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Origin of Leg Musculature during *Drosophila* Metamorphosis

(imaginal disks/transplantation/arginine kinase/α-glycerolphosphate dehydrogenase/alcohol dehydrogenase)

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ABSTRACT **Pro- and mesothoracic leg imaginal disks of late third-instar larvae of genotypes affecting the electrophoretic mobilities of α-glycerolphosphate dehydrogenase (EC 1.1.1.8) and arginine kinase (EC 2.7.3.3) were transplanted into host larvae of different genotypes. The metamorphosed implants were analyzed microscopically for the presence of musculature, histochemically for the distribution of enzyme activity, and electrophoretically for determination of the phenotypes of the two muscle-marker enzymes. The results permit the conclusion that leg imaginal disks contain muscle stem-cells.**

The origin of skeletal muscle in *Drosophila* metamorphosis was not directly identified. *In vitro* culture of leg imaginal disks of late third-instar larvae (1) yields fully differentiated legs that are devoid of musculature. Lack of innervation (2) can hardly be held responsible for this failure of musculature to form, since the disks were cultured in the presence of and remained connected to the cerebral complex throughout the period of culture and differentiation (1). *In vivo* culture of leg disks (3), on the other hand, leads to the formation of differentiated (though inverted) legs that "often" (3) have musculature attached.

Do leg imaginal disks contain muscle stem-cells? The result of the *in vitro* observations reported above would argue that they don't, but perhaps the culture conditions were inadequate for muscle differentiation. The *in vivo* observations would argue that they do, but these stem-cells might have migrated from the host organism onto the developing disk during culture and/or metamorphosis.

In the present investigation, we used electrophoretic enzyme markers in combination with *in vivo* culture techniques to answer this question.

METHODS

General. Pro- and mesothoracic leg imaginal disks were dissected from mid-to-late third instar larvae (donors), and transplanted individually into mid-third instar larvae (hosts), by a standard technique (4). After metamorphosis, the hosts were aged for 1–3 weeks, and the implants were recovered by dissection and either examined histochemically, or homogenized and analyzed electrophoretically for enzyme phenotype. The sensitivity of the gel-electrophoresis assays was such that between 50 and 100 metamorphosed implants were needed for each assay.

αGPDH (EC 1.1.1.8.). The αGPDH of the donor strain, $\alpha Gpdh^s$ (5), migrates more slowly toward the anode than does that of the host strain, *Sevelen.* Electrophoresis of homogenates of metamorphosed implants and donor and host controls was performed in 1% Noble Agar gels [sodium citrate–boric acid–Tris·HCl (pH 8.6) (6)], for 40 min at 150 V. The gels were stained at 37° in 25 ml of 80 mM Tris·HCl (pH 7.5) containing in final concentration, 37 mM glycerol-1-phosphate, 1.68 mM NAD^+, 30 IU chicken aldolase A_4 (provided by Dr. H. Lebherz of this laboratory), 120 IU rabbit muscle triose phosphate isomerase, 80 μM NaCN, 18 mg of nitro-blue tetrazolium, and 0.5 mg of phenazine methosulfate. The same reaction mixture was also used for histochemical analysis of frozen sections.

ADH (EC 1.1.1.1.). Reciprocal transplantations were performed with a $w; Adh^{n1}$ strain (7) that completely lacks ADH activity, and the wild-type strain *Sevelen.* Freshly dissected metamorphosed implants were either processed for frozen-section histochemistry or stained as whole organs with the reaction mixture described in (8), except that 2-butanol was substituted for ethanol.

Arginine kinase (EC 2.7.3.3.). It was necessary to use the drosophilid *Zaprionus vittiger* as the donor organism, since a previous survey had revealed no electrophoretic arginine kinase variant among more than 80 different wild-type stocks of *D. melanogaster* and a series of other *Drosophila* species. The *Zaprionus* enzyme moves distinctly more anodally than does that of the host strain, *Sevelen.* This was determined by vertical electrophoresis in 14% starch gels (9). Arginine kinase activity was visualized by a modification of an overlay technique (10). Final concentrations of the overlay gels were 1% agar; 0.1 M glycine-NaOH (pH 8.6); 100 mM KCl; 10 mM Mg acetate; 5 mM ATP; 1.5 mM phosphoenol pyruvate; 1.4 mM NADH; 7 IU/ml of pig-heart lactate dehydrogenase (Boehringer); 3 IU/ml of rabbit muscle pyruvate kinase (Boehringer); and 30 mM L-arginine. After electrophoresis, the overlay gel was placed on the sliced starch gel for 10 min at 4°, and the gels were incubated for 40–60 min at 37°. Fluorescence of NADH was then excited with a UV lamp at 366 nm; disappearing NADH became apparent as dark spots and was recorded photographically by placing the overlay gel, supported by a glass plate, onto Agfa-LUPEX-LHI positive paper, by use of the same UV light source.

RESULTS

αGPDH

Histochemical analysis of frozen sections of untreated wild-type adult flies showed enzyme activity in musculature and fat-body. The muscle tissue that surrounds the chitinous

Abbreviations: αGPDH, α-glycerolphosphate dehydrogenase; ADH, alcohol dehydrogenase.

parts of metamorphosed implants also stained deep purple; fat-body cells that are attached to the implants, particularly if the hosts are not aged for more than 2 weeks after eclosion, also showed enzyme activity.

Electrophoretic analysis of the homogenized implants showed two zones of αGPDH activity, one corresponding to that of the donor's genotype (*αGpdh*^{s}), and the other corresponding to the host's genotype. The histochemical observations made us suspect that the fat-body cells attached to the implants accounted for the host-enzyme on the zymograms; i.e., we assumed that these fat cells are derived from the host. That this is indeed the case was shown by the next experiment.

ADH

Wild-type fat-body contains massive ADH activity (11). Flies of the *Adh*^{n1} genotype contain no such activity (7). The results shown in the Table prove that the fat cells attached to metamorphosed implants are derived from the host.

It seemed justified, therefore, to assign the donor-type αGPDH described above to the *musculature* of the implants. This was confirmed in the following experiment that used arginine kinase, an enzyme that is predominant in muscle and does not occur in fat-body.

Arginine kinase

Histological analysis of the implants (using αGPDH-staining) again revealed the presence of musculature in *Zaprionus* implants in *Drosophila* hosts. It should be noted that the *Zaprionus* legs that developed were markedly smaller than comparable *Drosophila* transplants. Nevertheless, the implants contained sufficient muscle to permit electrophoretic analysis. The zymogram clearly showed only one type of arginine kinase in the implants; its electrophoretic migration corresponded to that of the donor, *Zaprionus*.

DISCUSSION

In adult *Drosophila*, αGPDH is known to occur in fat-body and in musculature. Our present experiments with ADH markers show clearly that the fat-body cells that invariably accompany the metamorphosed implants are derived from the host. The only other tissue in the implants to reveal αGPDH activity is the musculature. Thus, it is reasonable to interpret the donor-specific αGPDH zone on our zymograms as derived from muscle. That this interpretation is correct is supported by the observation of donor-specific arginine kinase. We conclude, therefore, that leg imaginal disks contain stem-cells that, in the course of metamorphosis, differentiate into musculature.

We have no direct evidence for the localization of these stem-cells in the disks, but the observation (3) that the so-called adepithelial cells line up into linear arrays during metamorphosis and form muscle, coupled with the present findings, is strong indirect evidence that these adepithelial cells are indeed one source of adult skeletal muscle.

TABLE 1. *Alcohol dehydrogenase activity in fat cells and host fat-body*

Donor	Host	ADH activity in: Fat-cells, attached to implants	ADH activity in: Host fat-body
Sevelen	w; Adh^{n1}	−	−
w; Adh^{n1}	*Sevelen*	+	+
Sevelen	*Sevelen*	+	+
w; Adh^{n1}	w; Adh^{n1}	−	−

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