Development 125, 2149-2158 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV7644

Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms

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Accepted 20 March; published on WWW 6 May 1998

SUMMARY

In newly hatched *Drosophila* larvae, quiescent cells reenter the cell cycle in response to dietary amino acids. To understand this process, we varied larval nutrition and monitored effects on cell cycle initiation and maintenance in the mitotic neuroblasts and imaginal disc cells, as well as the endoreplicating cells in other larval tissues. After cell cycle activation, mitotic and endoreplicating cells respond differently to the withdrawal of nutrition: mitotic cells continue to proliferate in a nutrition-independent manner, while most endoreplicating cells reenter a quiescent state. We also show that ectopic expression of *Drosophila* Cyclin E or the E2F transcription factor can drive quiescent endoreplicating cells, but not quiescent imaginal neuroblasts, into S-phase. Conversely, we demonstrate that quiescent imaginal neuroblasts, but not quiescent endoreplicating cells, can be induced to enter the cell cycle when co-cultured with larval fat body in vitro. These results demonstrate a fundamental difference in the control of cell cycle activation and maintenance in these two cell types, and imply the existence of a novel mitogen generated by the larval fat body in response to nutrition.

Key words: Cell cycle, Neuroblasts, Endoreplication, Nutrition, *Drosophila*

INTRODUCTION

The coordination of cell proliferation with other developmental processes is essential in forming any complex, multicellular organism. Much has been learned about the conserved basic cell cycle machinery from simple systems such as yeast and tissue culture cells, where studies have focused on the regulation of the conserved machinery by environmental cues such as growth factors, mating pheromones and nutrition. However, to fully understand the process of integrating cell proliferation with other developmental processes it must be considered within the context of a developing metazoan. *Drosophila melanogaster* has been used extensively to investigate the control of embryonic cell cycle regulation (for review see Edgar and Lehner, 1996), but the embryo is a contained system which is influenced only minimally by its environment. We have begun to investigate the regulation of cell cycle initiation in *Drosophila* larvae, where the cell cycle is influenced by environmental as well as developmental cues.

The larval stage of the *Drosophila* life cycle is specialized for eating and growth. Larvae increase their mass about 200 fold during the 3 days which they spend feeding prior to pupariation (Church and Robertson, 1966). Almost all of this growth is due to increases in cell size in larval-specific tissues. During embryonic and larval development, these larval-specific tissues progress through a modified cell cycle which consists of successive rounds of DNA synthesis without intervening mitoses (Smith and Orr-Weaver, 1991). This modified cell cycle, the endoreplication cycle, leads to increased DNA ploidy and is required for growth (Royzman et al., 1997; our unpublished results). The endoreplicative tissues (ERTs) in the growing larva include the gut, fat body, salivary glands, malpighian tubules, trachea, muscle and epidermis.

Little is known about the regulation of the basic cell cycle machinery during endoreplication, although it has been demonstrated that *Drosophila* Cyclin E is required for the embryonic endoreplication cycles (Knoblich et al., 1994). Endoreplication begins during mid-embryogenesis, and many of the larval-specific tissues undergo at least one round of endoreplication prior to hatching (Smith and Orr-Weaver, 1991). The embryonic endoreplication cycles occur in tissue-specific domains at specific developmental times (Smith and Orr-Weaver, 1991). In contrast, the endoreplication cycles which occur after larval hatching occur in smaller sub-domains of those observed during embryogenesis and lack strict developmental control, suggesting that environmental as well as developmental factors are controlling the cell cycle in ERTs (Smith and Orr-Weaver, 1991).

While increases in DNA ploidy and cell size occur in the ERTs, cells which have been set aside to generate adult-specific tissues proliferate using a mitotic cell cycle, leading to an increase in cell number rather than cell size. The mitotic tissues include the imaginal discs, larval neuroblasts, the germ cells and the abdominal histoblast nests. In this study the mitotic cells we have focused on are the larval neuroblasts, a population of stem cells in the central nervous system (CNS)

which divide asymmetrically to regenerate themselves and produce a pool of undifferentiated neural precursor cells which will be incorporated into the adult CNS during metamorphosis.

Neuroblasts proliferate during embryogenesis to give rise to the neurons and glia which compose the larval CNS (Hartenstein and Campos-Ortega, 1984), arrest their cell cycle after completing this embryonic program, and remain quiescent until after larval hatching (Prokop and Technau 1991). In the first instar larva, neuroblasts reenter the cell cycle in a characteristic spatiotemporal pattern (White and Kankel, 1978; Truman and Bate, 1988; Ito and Hotta, 1992) and perform a second round of neurogenesis. A special class of neuroblasts, the mushroom body neuroblasts (MBNbs), do not become quiescent during embryogenesis but instead proliferate continuously throughout embryonic and larval development (Prokop and Technau 1991; Ito and Hotta, 1992). MBNbs are one of the few cell types that are cycling at the time of larval hatching (White and Kankel, 1978; Truman and Bate, 1988; Smith and Orr-Weaver, 1991; Ito and Hotta, 1992).

The process of neuroblast cell cycle activation has been observed in detail in the ventral nerve cord (VNC) (Truman and Bate, 1988) and in the brain (Ito and Hotta, 1992). Larval neuroblasts reactivate in an anterior to posterior wave: neuroblasts in the brain reactivate first, followed by those of the thoracic VNC, and lastly those in the abdominal and terminal VNC (Fig. 1B, for review see Truman et al., 1994). The small quiescent neuroblasts undergo a characteristic enlargement which begins before the initiation of their first S-phase (Truman and Bate, 1988).

Genetic studies have identified two genes which act together to regulate the timing of neuroblast cell cycle activation, *ana* and *trol*. *ana* encodes a secreted glycoprotein which is made in glial cells located adjacent to larval neuroblasts. In *ana* mutant larvae, neuroblasts reenter the cell cycle precociously, indicating that *ana* encodes a repressor of S-phase initiation (Ebens et al., 1993). While *trol* has not yet been cloned, it seems to oppose the ANA repressor to allow S-phase initiation at the proper time. *trol* mutant neuroblasts fail to reenter the cell cycle and remain arrested in the quiescent state. *ana* is epistatic to *trol*, indicating that *trol* is not required for cell cycle activation in the absence of the ANA repressor (Datta, 1995). It is not yet known how *trol* activity is temporally regulated or how the *trol*/*ana* pathway impinges on the basic cell cycle machinery to regulate the transition from G_1 into S-phase.

Here we show that cell cycle initiation in *Drosophila* larvae is regulated at the organismal level by a nutritional cue. We demonstrate that endoreplicative and mitotic cell cycles respond differently to changes in nutrition and seem to be regulated by distinct signals. We also show that these two types of cell cycles respond differently to overexpression of G_1/S cell cycle regulators. Finally, we identify the larval fat body as the source of a mitogenic factor which acts on quiescent larval neuroblasts in vitro.

MATERIALS AND METHODS

Fly strains

The wild-type stock Sevelen was used for all experiments except as noted. The mutant allele *ana*^{9} was used (Ebens et al., 1993). The lines used for ectopic expression are as described: hs-dE2F hs-dDP (Duronio et al., 1996), hs-Cyclin E (Richardson et al., 1995), and UAS-dE2F UAS-dDP (Neufeld, de la Cruz, Johnston and Edgar, unpublished). The UAS-GFP act>CD2>GAL4 line was generated by recombination of UAS-GFP (Neufeld, de la Cruz, Johnston and Edgar, unpublished) and act>CD2>GAL4 (Pignoni and Zipursky, 1997).

Nutritional regimens

'Fed' larvae were provided a standard *Drosophila* diet of corn meal, molasses and agar supplemented with live yeast (fly food). Larvae referred to as nutrient-deprived or starved were cultured from hatching on a medium of 20% sucrose in PBS. Embryos were collected, dechorionated, and transferred to the sucrose solution where they floated on the surface. Food withdrawal experiments were conducted by removing larvae from fly food and transferring them to sucrose solution at the indicated time. Larvae were transferred to fresh sucrose every 24 hours after food withdrawal until they no longer fouled the solution. Complete synthetic medium and drop out media are according to Sang (1978). Axenic cultures of live larvae were carried out on these agar-based media. Cycloheximide was used at a concentration of 500 μg/ml. Larvae which were used for fat body donors in the culture experiments were raised on fly food but were not provided a live yeast supplement in order to minimize the potential for contamination of cultures.

Histology

5-Bromodeoxyuridine (BrdU) was fed to larvae at a concentration of 100 μg/ml. Larvae were dissected in PBS and the tissues fixed in 8% formaldehyde/PBS. Immunohistochemistry was performed using standard techniques (Patel, 1994) except that tissues were handled in polyester transwell baskets (Corning Costar) in 24-well tissue culture plates. The primary antibody was a mouse anti-BrdU (Becton-Dickenson) used at a 1:100 dilution. The secondary antibody was a HRP-coupled goat anti-mouse (Jackson ImmunoResearch) used at a 1:350 dilution. Tissues were transferred to watch glasses for the HRP color development reaction.

Toluidine blue staining was performed as described by Truman and Bate (1988).

In vitro organ culture

In vitro organ culture was performed using a method based on that of Awad and Truman (1997). Larvae were cultured under sterile conditions on either standard medium or 20% sucrose/PBS. Larvae were surface sterilized in a solution of 70% ethanol for 5-7 minutes, rinsed in sterile water, and dissected in D-22 insect cell culture medium (Sigma) using sterile tools. Tissues were transferred to a Petri dish containing a 20 μl drop of D-22 medium with 7.5% fetal bovine serum and 1% of an antibiotic/antimycotic solution containing 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B (Sigma). A Spemann pipet or dissecting needle was used to transfer the tissues in a minimal volume of medium. In co-culture experiments, the entire fat bodies from ten feeding mid-third instar larvae were added to the cultures. Cultures were incubated at 25°C under a 5% CO_2 atmosphere in humidity chambers in a standard tissue culture incubator. BrdU was diluted in D-22 and added to the cultures to a final concentration of 100 μg/ml. Cultured tissues were fixed and stained as described above. D-22 medium was buffered with 20 mM HEPES to facilitate the diffusion of O_2.

Mutant analysis

For analysis of *ana*^{9}, embryos were collected at 23°C and transferred to sucrose. BrdU was added to the sucrose solution to a final concentration of 100 μg/ml immediately after larval hatching. After approximately 24 hours of labeling, larvae were dissected, fixed and immunochemically processed as above. The parental genotype was *ana*^{9}/*In (3LR) Gla Bc Elp*. In this experiment, 33 mutant larvae were examined.

Ectopic expression

The hs-dE2F, hs-dDP and hs-DmcycE constructs used were under the control of the *hsp-70* heat shock promoter. Embryos were collected at 23°C and transferred to sucrose. After hatching, larvae were heat shocked for 1 hour at 37°C to induce expression. To simultaneously express *dE2F* and *dDP* under the control of the UAS in multiple tissue types, we used the flp-out GAL-4 technique (Pignoni and Zipursky, 1997). Embryos of the genotype hs-flp1; UAS-dE2F UAS-dDP / UAS-GFP Act>CD2>GAL4 were allowed to hatch on sucrose, and then heat shocked for 2 hours at 37°C to induce Flp expression. GFP expression was monitored to ensure that a 2 hour heat shock was sufficient to induce UAS-directed transcription in all larval tissue types. In both types of overexpression experiment, BrdU was added to the sucrose solution to a final concentration of 100 μg/ml immediately after heat shock. Larvae were dissected, fixed and immunochemically processed after at least 24 hours of labeling.

RESULTS

Nutrition activates both mitotic and endoreplicative cell cycles in the larva

At larval hatching only a few cell types are cycling and incorporate BrdU. These include endoreplicating cells in the hindgut and midgut (Smith and Orr-Weaver, 1991), the mitotic MBNbs and lateral Nbs in the CNS and their progeny (Truman and Bate, 1988; Ito and Hotta, 1992), and the mitotic germ cells in the developing gonad (data not shown but see Fig. 1D). All other larval cells are quiescent. As larvae feed, cells in other tissues reenter the cell cycle and begin to incorporate BrdU. By the second instar, BrdU incorporation is observed in all of the larval endoreplicating tissues (ERTs), activated neuroblasts, and the imaginal discs (Fig. 1A,B).

In contrast to the BrdU incorporation patterns observed in fed larvae, larvae that were provided only a sucrose diet from hatching (nutrient-deprived larvae) showed incorporation only in the MBNbs, the progeny of the MBNbs, and the gonads (Fig. 1D,E). Those gut cells that were endoreplicating at the time of larval hatching ceased incorporating BrdU within the first 12 hours on a sucrose diet (data not shown). The starved larvae survived for at least 8 days on the sucrose diet (Table 1), and the MBNbs continued to incorporate BrdU throughout this period. During the extended period of culture on sucrose, larvae remained arrested as small first instars. This developmental arrest was completely reversible by restoring the larvae to a normal diet.

Nutritional control of larval neuroblast activation is not mediated by the *trol*/*ana* pathway

The *trol*/*ana* pathway regulates the timing of neuroblast activation (Datta, 1995; Ebens et al. 1993). To determine whether nutritional regulation of neuroblast activation is mediated by the *trol*/*ana* pathway as well, we investigated the effects of starvation on neuroblast enlargement. During normal development, quiescent neuroblasts initiate a characteristic enlargement before the onset of S-phase. In *trol* mutant larvae neuroblasts fail to enter S-phase, although they do enlarge (Datta, 1995). We examined the larval neuroblasts in nutrient-deprived larvae to see if cell cycle activation was blocked before or after neuroblast enlargement. Toluidine blue preferentially stains the cytoplasm of enlarged neuroblasts (Altman and Bell, 1973). The CNS from a normally fed second instar larva shows staining of many enlarged neuroblasts in the brain region (Fig. 1C). When central nervous systems from nutrient-deprived larvae were treated with toluidine blue only the four MBNbs were stained (arrowheads, Fig. 1F), indicating that nutrition is required for neuroblast enlargement as well as S-phase initiation. This result suggests that nutritional control affects the process of neuroblast activation upstream of *trol*.

We also examined the phenotype of *ana* mutant larvae under

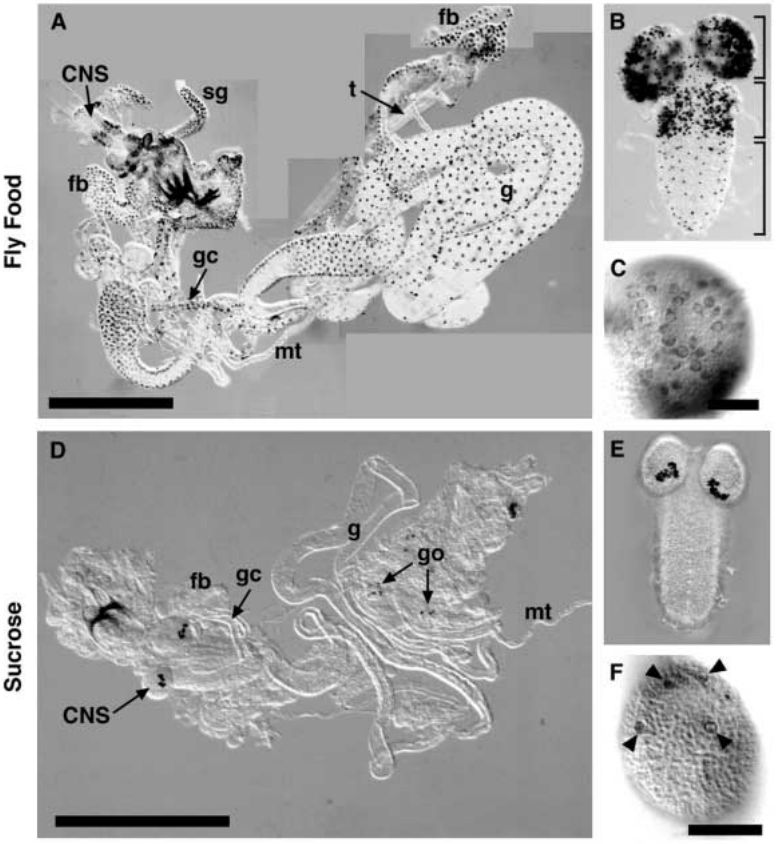

Fig. 1. Nutrition activates the cell cycle in quiescent larval tissues. (A) A second instar larva fed BrdU for 24 hours prior to dissection. Anterior is to the left. (B) A ventral view of the isolated CNS from a second instar larva that was fed BrdU for 24 hours prior to dissection. From top to bottom, brackets denote the brain region, the thoracic region of the VNC, and the abdominal/terminal region of the VNC. Anterior is up. (C) A single brain lobe of a second instar larva stained with toluidine blue to label the many enlarged neuroblasts. (D) A first instar larva that was fed only sucrose from the time of larval hatching. BrdU was added 24 hours after larval hatching, and larvae were dissected at 29 hours after larval hatching. (E) A dorsal view of the isolated CNS from a first instar larva that was fed on sucrose from the time of hatching. BrdU was added approximately 48 hours after larval hatching, and larvae were dissected at approximately 72 hours after larval hatching. (F) A single brain lobe of a starved first instar larva stained with toluidine blue to show the four labeled MBNbs (arrowheads). CNS, central nervous system: go, germ cells of gonad: fb, fat body; gc, gastric cecae; g, gut; mt, malpighian tubule; sg, salivary gland; t, trachea. Scale bars (A,D) 300 μm; (C,F) 30 μm.

Table 1. The effects of modified diets on DNA replication

Media	MBNb replication (M)	Gonad (M)	Nb and Disc Replication (M)	ERT replication (ER)	Survival/ Rescuability
Complete	Yes	Yes	Yes	Yes	NA
−inosine & uridine	Yes	Yes	Yes	Yes	10 days
−choline & cholesterol	Yes	Yes	Yes	Yes	9 days
−vitamins	Yes	Yes	Yes	Yes	6-8 days
−amino acids	Yes	Yes	No	No	10 days
−amino acids+one amino acid	Yes	Yes	No	No	10 days
complete+cycloheximide	No	No	No	No	>12 days
20% sucrose/PBS	Yes	Yes	No	No	8 days

Larvae were hatched on either complete synthetic medium, medium lacking various classes of essential dietary components, or complete synthetic medium containing 500 μg/ml cycloheximide, and monitored for growth and survival for up to 12 days. BrdU incorporation patterns were examined after a 24 hour feeding pulse from ~48-72 hours after transfer. Rescuability was determined by the ability of larvae to produce adult flies when transferred from synthetic media to normal fly food. For all samples, *n*>20 larvae. M, mitotic cell type; ER, endoreplicating cell type.

nutrient-deprived conditions. The *ana* gene encodes a repressor of S-phase initiation (Ebens et al., 1993), and in fed *ana* mutant larvae S-phase initiation occurs precociously. We reasoned that ANA might be responsible for maintaining the quiescent state of larval neuroblasts under nutrient-deprived conditions. However, we observed no neuroblast activation in nutrient-deprived *ana* mutant larvae (data not shown). This further confirms that the *trol*/*ana* pathway is not involved in nutritional regulation of cell cycle activation.

Once initiated, mitotic proliferation becomes nutrition independent

To better understand the nutritional requirements for cell cycle activation and maintenance in the larval neuroblasts, we studied the effects of nutrient withdrawal after a feeding pulse. Larvae were fed for either 1 or 2 days and then transferred to a sucrose diet on which they were maintained for up to 7 days. BrdU was added to the culture on the final day before dissection and fixation. Neuroblasts reenter the cell cycle in an anterior to posterior wave which initiates in the brain region, then progresses to the thoracic VNC, and finally reaches the abdominal and terminal VNC (see brackets in Fig. 1C). At the time of food withdrawal, larvae which were fed for 1 day showed neuroblast proliferation in the brain and thoracic region of the VNC (Fig. 2A), while those fed for 2 days showed proliferation in the abdominal and terminal regions of the VNC as well (Fig. 2D). Interestingly, we observed that neuroblasts which had reentered the mitotic cell cycle during the feeding period continued to cycle after nutrient withdrawal. Larvae which were fed for 1 day before transfer to the sucrose diet continued to incorporate BrdU into the neuroblasts in the brain and thoracic region of the VNC for up to 7 days (Fig. 2B,C). Those larvae that were fed for 2 days before transfer to the sucrose diet showed persistent BrdU incorporation in the abdominal and terminal regions of the VNC as well as the brain and thoracic VNC (Fig. 2E,F). In contrast to the behavior of proliferating neuroblasts, neuroblasts which had not yet activated at the time of food withdrawal remained quiescent after food withdrawal (compare abdominal and terminal neuroblasts in Fig 2A-C to those in Fig. 2D-F). A reduction in the number of proliferating neuroblasts was observed over time after food withdrawal, probably due to a subset of the activated neuroblasts completing their programs of division during the extended period of culture on sucrose (see Discussion). BrdU incorporation patterns were also examined in imaginal discs after food withdrawal. We observed that like neuroblasts, disc cells continued to incorporate BrdU up to 7 days after food withdrawal (data not shown). These results demonstrate that nutrition is required for activation but not for maintenance of mitotic proliferation. Once activated, neuroblasts and disc cells cycle in a nutrition-independent manner like the MBNbs.

ERTs require continuous nutrition for maintenance of cell cycle progression

To compare the nutritional regulation of the ERTs to that of neuroblasts, we performed a similar food withdrawal experiment. Larvae were fed for 1 day and then transferred to a sucrose diet for up to 5 days. BrdU was fed to the larvae during the final 24 hours of culture prior to dissection and fixation (Figs 3B-C,E-F). As a control, larvae were fed BrdU

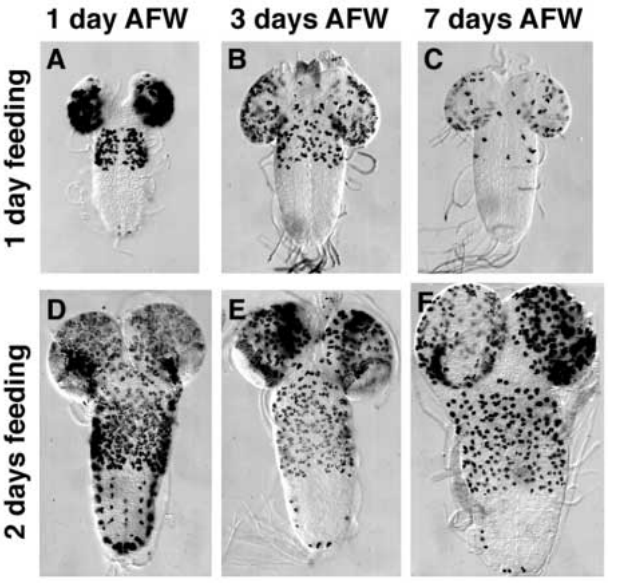

Fig. 2. The maintenance of larval neuroblast proliferation is nutrition-independent. BrdU incorporation patterns in the larval CNS after food withdrawal (AFW). Larvae were fed fly food for 1 (A-C) or 2 (D-F) days before transfer to sucrose. BrdU was added either immediately following food withdrawal (A,D), 48 hours after food withdrawal (B,E) or 144 hours after food withdrawal (C,F). Larvae were dissected approximately 24 hours after addition of BrdU.

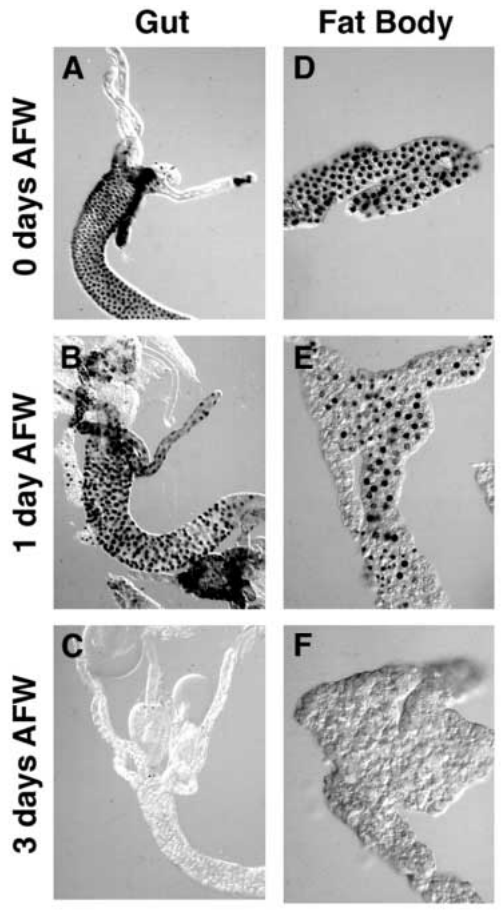

Fig. 3. The endoreplication cycles require continuous nutrition. BrdU incorporation patterns in larval gut (A-C) and fat body (D-F) after food withdrawal. Larvae were fed fly food for one day before transfer to sucrose. BrdU was added either during the one day of feeding (A,D), immediately following food withdrawal (B,E), or two days after food withdrawal (C,F). Larvae were dissected approximately 24h after addition of BrdU.

during the 1 day of normal feeding (Fig. 3A,D). When larvae were labeled with BrdU for a 24 hour period while feeding normally, virtually every endoreplicating cell in the larval gut and fat body incorporated BrdU (Fig. 3A,D), as well as most cells in the salivary glands, epidermis and trachea (data not shown). However, when BrdU incorporation was performed during the 24 hour period immediately following food withdrawal a subset of the endoreplicating cells of the gut, fat body, epidermis, trachea and salivary glands did not label (compare Fig. 3B,E with Fig. 3A,D and data not shown). Between 48 and 72 hours after food withdrawal, none of the endoreplicating cells in the fat body incorporated BrdU (Fig. 3F) and in the gut only a few endoreplicating cells in the midgut labeled (Fig. 3C, data not shown). Occasional epidermal, tracheal and salivary gland nuclei were observed to incorporate BrdU as well (data not shown). By the fifth day after food withdrawal, no epidermal cells incorporated BrdU, but occasional midgut, salivary gland, and tracheal cells did label. These results show that unlike the neuroblasts, ERTs require nutrition to maintain the cycling state. The larval fat body shows the most rapid response to nutrient withdrawal, followed by the majority of the gut and epidermal cells. In addition to shutting down the cell cycle, the fat body also shows dramatic changes in texture and opacity in response to starvation (compare Fig. 3F with 3D).

The crucial dietary component for cell cycle activation is amino acids

To understand what dietary component(s) is critical for cell cycle initiation we utilized a synthetic complete diet defined by Sang (1978). Larvae were hatched on either complete medium or medium lacking various classes of dietary components (Table 1). Growth and survival were monitored for up to 12 days, and BrdU incorporation was examined after a 24 hour pulse on the third day of culture. Larvae cultured on synthetic complete medium grew normally and pupariated after 4 to 5 days. BrdU incorporation was observed in all tissue types in these larvae. Larvae cultured on media which lacked either nucleotide precursors, lipids, or vitamins were able to grow to the late second or early third instar before dying. All tissue types incorporated BrdU after culture on these media as well. In contrast, larvae cultured on medium lacking amino acids did not grow, and the only cells which incorporated BrdU were the MBNbs and germ cells. To determine whether one specific amino acid was required as a signal for cell cycle activation, each of the eleven essential amino acids was added back individually to the amino acid drop-out media. No single amino acid was sufficient to rescue larval growth or cell cycle activation, suggesting that protein synthesis is required for cell cycle activation and growth.

Inclusion of the protein synthesis inhibitor cycloheximide in the complete synthetic medium blocked larval growth and cell cycle activation as well as BrdU incorporation into all cells including the MBNbs. Cycloheximide had no effect on viability and the block to growth and development was reversible for more than 12 days (Table 1). The ability of the MBNbs to cycle in the absence of nutrition but not in the presence of cycloheximide suggests that starved larvae do possess enough stores of amino acids to allow some protein synthesis, however higher levels of protein synthesis must be required for the activation of quiescent cells.

Amino acids are not sufficient for the initiation of DNA replication in cultured larval tissues

To determine whether the requirement for amino acids was tissue-autonomous or if amino acids were required at the organismal level, we incubated tissues dissected from starved larvae in D-22 insect cell culture medium. Since D-22 is rich in all the essential amino acids, if the requirement for amino acids is at the cell or tissue level it should be provided by the culture medium. The quiescent larval CNS was dissected from larvae which had been starved for 3 days from hatching, and then cultured for 4 days. BrdU was added either immediately or for the final day of culture. BrdU incorporation was only observed in the MBNbs (Fig. 5A). When the quiescent larval gut or fat body was cultured in the presence of BrdU for up to 4 days, no incorporation was observed (data not shown). As a positive control, central nervous systems were dissected from fed third instar larvae and cultured. BrdU was added for the final day of culture. The larval neuroblasts incorporated BrdU even after 4 days in culture, demonstrating that our culture system was capable of supporting survival and cell cycle

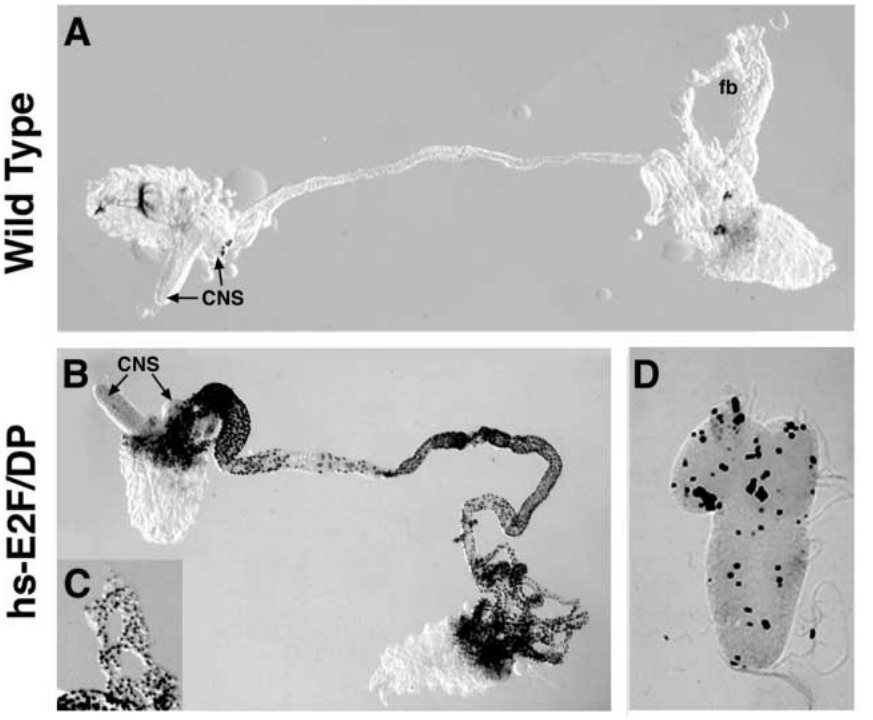

Fig. 4. Ectopic expression of G_1/S regulators drives ERTs but not neuroblasts into S-phase. Wild-type larvae (A) or larvae of the genotype P[w+, hs-dE2F] P[w+, hs-dDP] (B-D) were fed only sucrose for three days after larval hatching, then heat shocked for one hour at 37°C followed by the addition of BrdU. Larvae were dissected approximately 24 hours after heat shock. (C) An isolated fragment of the larval fat body showing BrdU incorporation after heat shock. (D) An isolated larval CNS showing scattered BrdU incorporation in non-neuroblast cells after heat shock. Abbreviations are as in Fig. 1.

progression. Thus the requirement for amino acids is not tissue-autonomous, suggesting that there must be some intermediary activating signal which is produced in vivo in response to dietary amino acids and which is not present in D-22.

Ectopic expression of G_1/S regulators can drive endoreplicating but not mitotic cells into S phase

To determine whether the block to cell cycle activation in nutritionally deprived larvae could be bypassed by expressing G_1/S regulators, we ectopically expressed either *DmcycE* or *dE2F* and its binding partner *dDP* in starved larvae using a heat-shock inducible promoter. We also expressed *dE2F* and *dDP* using the flp-out Gal4 method (see Materials and Methods). *DmcycE* encodes the *Drosophila* Cyclin E, a G_1 cyclin which is required for entry into S-phase in both mitotic and endoreplicating cells (Knoblich et al. 1994). *dE2F* and *dDP* encode the two subunits which compose the *Drosophila* E2F transcription factor (Dynlacht et al., 1994; Ohtani and Nevins, 1994) which is required for the transcription of genes important for the mechanics of S-phase (Duronio and O'Farrell, 1994). In wild-type larvae, heat shock had no effect on BrdU incorporation in either ERTs or neuroblasts (compare Figs 1D, 4A). In contrast, hs-dE2F/dDP (Fig. 4B-D) or flp-out Gal4/UAS-dE2F UAS-dDP (data not shown) was sufficient to drive most endoreplicating cells into S-phase as assayed by BrdU incorporation. hs-DmcycE also drove endoreplicating cells into S-phase (data not shown), but not as efficiently as hs-dE2F/dDP. However, the larval neuroblasts did not incorporate BrdU after ectopic expression of either *dE2F/dDP* (Fig. 4D) or *DmcycE* or both *dE2F/dDP* and *DmcycE* (data not shown), suggesting that there is an additional requirement for activation in these cells. We did observe some cells in the CNS incorporating BrdU after induction of these G_1/S regulators (Fig. 4D), but these labeled cells never divided even if the larvae were transferred to fly food and allowed to feed for several days in the absence of additional BrdU (data not shown). This suggests that these cells are endoreplicating cells, not neuroblasts. In addition, the pattern of BrdU incorporation in the CNS did not resemble the spatial pattern of neuroblasts (compare Fig. 2D with 4D, especially the VNC) and the labeled nuclei appeared larger than those observed in the mitotic neuroblasts. Because quiescent endoreplicating cells can be forced to enter the cell cycle in response to ectopic expression of G_1/S regulators, these cells must be capable of supporting gene expression and cell cycle progression in the absence of external nutrition. This suggests that cell cycle initiation in the endoreplicating tissues is controlled by a regulatory event rather than as a cell autonomous response to nutrient availability. These results demonstrate that there is an intrinsic difference in regulation of the cell cycle machinery in quiescent mitotic and endoreplicating cell types, where the E2F transcription factor is limiting for S-phase initiation in the ERTs but not the larval neuroblasts. Due to the small size of the imaginal disc primordia in the first instar, we were unable to determine whether ectopic expression of Cyclin E or E2F was sufficient to drive these quiescent mitotic cells into S-phase.

The fat body generates a mitogenic factor that activates the cell cycle in larval neuroblasts

The larval and adult fat body show dramatic responses to starvation (Fig. 3D-F; Dean et al., 1985; Bownes et al., 1988). Additionally, imaginal discs cultured in vitro grow optimally when the medium is conditioned by larval fat body (Davis and Shearn, 1977). To dissect the regulatory mechanism of the nutritional response we performed co-culture experiments using the larval fat body. Quiescent central nervous systems from starved larvae were cultured for 4 days in insect cell culture medium with or without fat bodies from ten feeding,

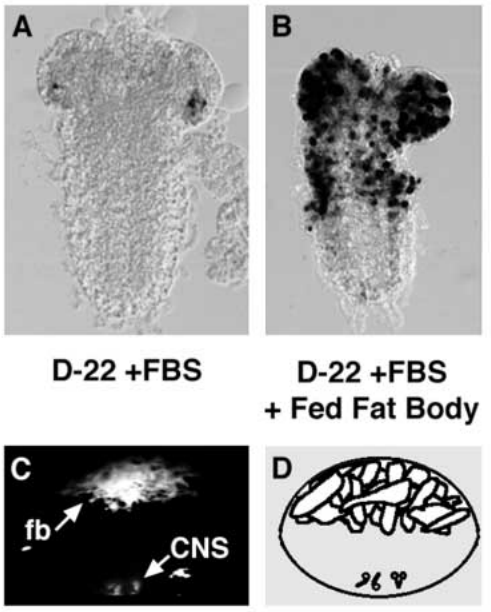

Fig. 5. The larval fat body produces a neuroblast mitogen. Central nervous systems were dissected from larvae which were fed only sucrose for 3 days after larval hatching, and then cultured for 4 days in D-22 insect cell culture media supplemented with fetal bovine serum (FBS) (see Materials and Methods) either without (A) or with (B) fat bodies collected from ten feeding mid-third instar larvae. In this example, BrdU was added after 72 hours in culture. Tissues were fixed approximately 24 hours after addition of BrdU. (C) A photograph of our co-culture system showing the separation of the two tissue types in a 20 μl drop of culture medium. (D) A cartoon representation of the photograph in C. Abbreviations are as in Fig. 1.

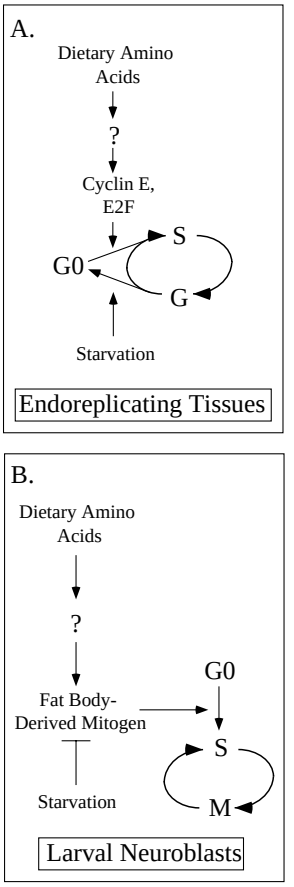

Fig. 6. Nutritional regulation of cell cycle initiation in larval tissues. A schematic diagram depicting the interactions between dietary amino acids and cell cycle activation in different tissue types. (A) Nutrition stimulates the quiescent cells of the larval ERTs to enter S-phase. The requirement for dietary amino acids can be bypassed by ectopic expression of Cyclin E or the E2F transcription factor. Nutrition is continuously required for these tissues to remain in the cycling state. (B) The larval neuroblasts only require nutritional input at the time of cell cycle activation. We propose that dietary amino acids stimulate the production or secretion of a mitogen from the fat body which acts on the quiescent larval neuroblasts. Starvation may regulate the production or secretion of the fat body-derived factor.

mid-third instar larvae. BrdU was added on the final day of culture. Co-culture of quiescent central nervous systems with fat body resulted in activation of neuroblast proliferation in the VNC and/or brain in 75.4% of the co-cultured central nervous systems (n=69 in nine independent cultures) (Fig. 5B), a result never observed when the fat body was omitted from the culture (n=71) (Fig. 5A). This fat body-dependent activation occurred in a relatively normal spatiotemporal pattern: neuroblasts in the larval brain activated on the third day of co-culture while neuroblasts in the thoracic VNC activated on the fourth day of co-culture. We also cultured quiescent central nervous systems in the presence of gut or CNS dissected from feeding third instar larvae, but observed no cell cycle activation under these conditions. These results suggest that the ability to activate the neuroblast cell cycle is specific to the larval fat body. The configuration of the cultured tissues in our system suggests that the mitogenic signal from the fat body must be able to diffuse, since the fat bodies float on the surface of the medium while the central nervous systems sink to the bottom (Fig. 5C,D). Quiescent larval guts were also co-cultured with fed larval fat bodies for up to 4 days in the presence of BrdU. We included quiescent central nervous systems in these cocultures as an internal control for cell cycle activation. Gut cells did not reenter the cell cycle in the presence of fat bodies (n=15 in two independent cultures) even though the quiescent central nervous systems in the same culture did show activation (66.7%, n=15) (data not shown). This result suggests that cell cycle activation in the ERTs is regulated by a factor which is distinct from the fat body mitogen.

DISCUSSION

Our results suggest that multiple pathways are involved in regulating the onset of cell proliferation in different tissue types in response to the global nutritional cue (Fig. 6). We show that mitotic and endoreplicating cell cycles are regulated differently in response to the nutritional state: the endoreplicating tissues (ERTs) require continuous nutrition to cycle, whereas the mitotic cells cycle in a nutrition-independent manner once activated. In addition, the mechanism of cell cycle arrest in the two types of quiescent cells is different: quiescent ERTs can be driven into S-phase by ectopic expression of either of the G_1/S regulators E2F or Cyclin E, while neither of these

regulators can induce quiescent neuroblasts to enter S-phase. Conversely, quiescent neuroblasts but not quiescent ERTs are induced to reenter the cell cycle in response to a mitogen produced by the larval fat body.

Differential regulation of mitotic and endoreplicative cell cycles

The differential responses of the mitotic and endoreplicative cell cycles to nutrient withdrawal (Fig. 6) may provide an important mechanism for survival of the organism and reproduction in the face of food shortages in the wild. When nutrients become limiting, available resources can be dedicated to maintaining growth and proliferation in the mitotic tissues which are required to form the reproductive adult. Indeed, larvae are capable of pupating at a much smaller size than they normally do. Bakker (1959) and Robertson (1963) defined a 'critical size' at which larvae are able to pupariate without further feeding. The small pupae which are formed by these larvae produce normal, fertile, but small adult flies.

Embryonic neuroblasts have an intrinsic program of cell proliferation (for review see Doe and Smouse, 1990; Doe and Technau, 1993). Each type of neuroblast has a specific identity, expresses unique and dynamic combinations of sublineage genes, and will give rise to a precise number and type of progeny before exiting the cell cycle. Interestingly, temporal control of sublineage gene expression in embryonic neuroblasts can be independent of cell cycle progression (Cui and Doe, 1995). Thus arresting a proliferating neuroblast in mid-lineage could lead to the desynchronization of sublineage gene expression and the loss of certain types of progeny, a result which could have disastrous consequences for the developing CNS.

In our food withdrawal experiment we observed that many activated neuroblasts continued to proliferate for up to 7 days after food withdrawal, however a subset of them did not. This observation was most striking in the abdominal region of the VNC (see Fig. 2D-F). The abdominal neuroblast lineages are much shorter than those of the majority of brain and thoracic neuroblasts, with a single abdominal neuroblast producing as few as four neurons during its postembryonic period of proliferation (Truman and Bate, 1988). Since the abdominal neuroblasts generally complete their entire larval program of proliferation in less than 2 days (see Truman et al., 1994), it is not surprising that after 7 days of culture on sucrose the majority of these neuroblasts have exited the cell cycle. We suspect that the reduction in labeled neuroblasts observed in all regions of the CNS over the course of this experiment is due to a subset of neuroblasts completing their intrinsic program of proliferation and exiting the cell cycle.

The role of the fat body in nutritional control

The insect fat body is the source of the majority of hemolymph proteins, including lipid binding proteins, juvenile hormone binding proteins and esterases, peptides which mediate the insect immune response, and vitellogenins involved in oocyte maturation in the adult female (for review see Dean et al., 1985; Keeley, 1985). The fat body is also responsible for synthesizing the stores of protein, lipid and glycogen which sustain the animal throughout metamorphosis. Ultrastructurally, the fat body shows a dramatic response to starvation. In *Calpodes* larvae, starvation leads to a rapid reorganization of the fat body including loss of mitochondria and rough endoplasmic reticulum (RER) by autophagy and depletion of stored metabolites. Refeeding induces mitochondrial divisions and increases in RER content as well as the eventual replenishment of depleted stores (in Dean et al., 1985). We have observed dramatic changes in the larval fat body in the course of our starvation experiments, including a loss of tissue cohesion and changes in opacity. These changes probably reflect the alteration in composition the fat body cells undergo as stores of metabolites are mobilized to support proliferating mitotic tissues during starvation.

Previous studies have demonstrated that the adult female fat body is able to regulate yolk gene transcription in response to the nutritional environment (Bownes et al., 1983; Bownes and Blair, 1986; Bownes et al., 1988; Bownes and Reid 1990; Sondergaard et al. 1995). Interestingly, there is evidence that a component of the adult female abdomen is also capable of supporting the proliferation of larval tissues in a nutrition-dependent manner. Schubiger (1972) demonstrated that the proliferation of imaginal disc fragments transplanted into the abdominal cavity of adult female hosts is dependent on nutrition. We have found that when quiescent central nervous systems from starved larvae are transplanted into the abdomens of fed adult female hosts, larval neuroblasts reenter the cell cycle in what appears to be a normal spatiotemporal pattern (J. S. B. and G. Schubiger, unpublished). An appealing hypothesis is that production of the neuroblast mitogen in the fat body is regulated at the transcriptional level under the control of nutritional enhancers similar to those identified in the regions upstream of yolk protein genes (Sondergaard et al., 1995). The ability of something in the adult female abdomen to activate proliferation in quiescent neuroblasts suggests that similar fat body-derived mitogens are produced in the larval and adult female fat bodies. This adult mitogen could have a role in controlling proliferation in the adult, perhaps functioning to regulate some oogenic process in response to the nutritional state. Indeed, oogenesis is inhibited in adult females fed on sucrose (David et al., 1973).

The dramatic response of the fat body to starvation, the demonstration that there is a mechanism for nutritional control of transcription in adult female fat body, and the similar abilities of the adult female abdomen and the larval fat body to support nutrition-dependent cell cycle activation lend support to our proposal that the fat body is responsible for mediating the nutritional response in larval neuroblasts. The results of our co-culture experiment demonstrate that the fat body supplies a diffusible factor which stimulates larval neuroblasts to enter the cell cycle (Fig. 6B).

We have tested a number of gain-of-function and loss-of-function mutations in known signaling pathways for a role in cell cycle activation in either neuroblasts or ERTs, including components of the Notch, Hedgehog, DPP, EGF, and FGF pathways as well as *ras* and *hopscotch* (the *Drosophila* JAK). We have also tested 20-hydroxyecdysone and bovine insulin for the ability to activate the cell cycle in quiescent larval tissues cultured in vitro. None of the candidate signaling components we tested influenced the process of cell cycle activation (data not shown). Our tests have eliminated a number of candidate growth factors, hormones, and signaling molecules, suggesting that the fat body-derived mitogen may be a novel *Drosophila* growth factor. Alternatively, the

nutritional regulation of larval neuroblast activation could be mediated by a combination of known signals or by a known signal which we have not yet rigorously tested such as juvenile hormone or Wg.

Nutritional control and neuroblast activation

We have demonstrated that nutritional control of neuroblast activation occurs independently of the *trol*/*ana* pathway. Nutrition is required for neuroblast enlargement as well as S-phase initiation, while the *trol/ana* pathway acts downstream of neuroblast enlargement. The requirement for multiple independent signals to activate cell proliferation is not unique to larval neuroblasts. For instance, the initial steps in the activation of T-cell proliferation depend on the stimulation of two independent receptors – the T-cell receptor complex (TCR) and the CD28 receptor (for review see Berridge, 1997).

Quiescent neuroblasts undergo a characteristic enlargement reminiscent of the growth requirement in early G_0/G_1 tissue culture cells (for review see Pardee, 1989). Imaginal disc cells also increase in mass prior to the onset of cell cycle activation (Madhavan and Schneiderman, 1977). Caldwell and Datta (personal communication) have demonstrated that enlarged larval neuroblasts which fail to activate in *trol* mutant larvae can be driven into S-phase by ectopic expression of Cyclin E. In contrast, quiescent neuroblasts in nutrient-deprived larvae cannot be driven into S-phase by ectopic expression of either Cyclin E or the E2F transcription factor. This suggests that nutrition is required for events which enable these G_1/S regulators to drive cells into S-phase. One possibility is that nutrition is required for the synthesis of basic components of the cell cycle machinery such as cyclin-dependent kinases (cdks). Another possibility is that nutrition is required for the downregulation of a cdk inhibitor.

Cell cycle regulation at the organismal level

We have begun to elucidate the interactions between different larval tissues in regulating cell cycle initiation in *Drosophila* larvae. Our results suggest that the animal contains a sensor capable of monitoring the nutritional environment and sending a signal, either directly or indirectly, to target tissues. Our analysis thus far suggests that the fat body sends a mitogenic signal to larval neuroblasts. We do not know if the fat body is directly sensing amino acid levels itself or if it is relaying a signal from some other tissue. Like neuroblasts, the endoreplicating tissues do not reenter the cell cycle as a cell-autonomous response to nutrients. This suggests that endoreplicating tissues also require a mitogenic signal generated elsewhere in the larva. We do not yet know the source of this second mitogen.

The function of growth factor homologs in *Drosophila* has been the subject of many studies. The known factors act within a tissue, generally only over a limited number of cell diameters (for reviews see Peifer and Bejsovec, 1992; Siegfried and Perrimon, 1994; Edgar and Lehner, 1996; Burke and Basler, 1997; Perrimon and Perkins, 1997). We have identified a regulatory pathway in *Drosophila* which seems to utilize at least one circulating mitogen. The identification and characterization of this factor and its effects will allow some insight into the role of endocrine growth factors in development.

We would like to thank Drs. Gerold Schubiger, James Truman, Allan Shearn, Sumana Datta and Michele Garfinkel for helpful discussions, as well as the many generous *Drosophila* researchers who made their mutants and constructs available to us for analysis. We are especially grateful to Craig Caldwell and Sumana Datta for allowing us to cite their unpublished work. We would also like to thank Dr. Gerold Schubiger for technical assistance in the transplantation experiments mentioned here and David Prober for constructing the recombinant UAS-GFP act>CD2>GAL4 chromosome. We are grateful to Drs. Tarif Awad and Paul Olivier as well as the members of the Edgar lab for useful comments on the manuscript. Technical support was provided by Tracy Balzer. J. S. B. is a Howard Hughes Medical Institute Pre-doctoral Fellow. B. A. E. is a Lucille P. Markey and a Rita Allen Scholar. This work was supported by NIH GM51186 to B. A. E.

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