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Stimulation of Midgut Stem Cell Proliferation by *Manduca sexta* α-Arylphorin

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Extracts of the green-colored perivisceral fat body of newly ecdysed *Manduca sexta* pupae stimulate mitosis in midgut stem cells of *Heliothis virescens* cultured in vitro. Using a combination of cation- and anion-exchange chromatography, we have isolated a protein from these fat body extracts that accounts for the observed stem cell proliferation. SDS-PAGE analysis of the protein results in a single band of 77 kDa. Sequences of tryptic peptides from this protein are identical to internal sequences of the storage hexamer α-arylphorin. The α-arylphorin isolated by our procedure represents a small fraction of the total arylphorin present in the fat body extract. However, it alone seems responsible for the stimulation of mitotic activity in *H. virescens* midgut stem cells. Arch. Insect Biochem. Physiol. 55:26–32, 2004. Published 2003 Wiley-Liss, Inc.[†]

INTRODUCTION

In both vertebrate and invertebrate animals, differentiated epithelial cells have short lifetimes. Once mature, they soon die, to be replaced by uni- or multi-potent stem cells that multiply and subsequently differentiate as they move from the place where they are sequestered into the existing epithelium. Repair of injury is accomplished by rapid multiplication and maturation of stem cells (Jones et al., 1995; Slack, 2000). In insects, midgut stem cells multiply rapidly just before each molt. They mature as they interdigitate into the existing midgut epithelium during the molt, increasing the size of the gut to fit the larger next stage larva (Baldwin et al., 1993). Multiplication and differentiation of vertebrate stem cells are finely controlled by a number of known growth factors and cytokines (Watt and Hogan, 2000). Although 4 peptides have been isolated that induce insect stem cells to differentiate to mature forms (Loeb et al., 1999; Loeb and Jaffe, 2002), little is known about the specific factors that control the multiplication of stem cells in insects.

In vitro, lepidopteran midgut follows the same pattern of development as midgut in vivo, in that only stem cells multiply and subsequently differentiate to mature cell types; mature cells undergo apoptosis, and are replaced by newly differentiated stem cells (Loeb and Hakim, 1996; Loeb et al., 2001). In order to induce the stem cells to multiply in vitro, fat body tissue (Sadrud-Din et al., 1994) or an aqueous extract of fat body tissue (Sadrud-Din et al., 1996) was added to the culture medium. The source of the fat body was the green abdominal tissue found in newly eclosed Lepidopteran pupae; fat body or fat body extract (FBX) from *Heliothis virescens, Manduca sexta*, or *Lymantria dispar* were equivalent in stimulating proliferation of midgut stem cells obtained from larvae of either *M. sexta*, *H. virescens* (Loeb and Hakim, 1996) or *L. dispar* (Dougherty et al., 2000).

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In the present study, we demonstrate that the factor present in *M. sexta* FBX responsible for midgut stem cell proliferation in vitro is α-arylphorin.

MATERIALS AND METHODS

Experimental Animals

Eggs of the tobacco budworm, *H. virescens* were provided by the Cotton Insects Laboratory, USDA (Phoenix, AZ), and the larvae were raised on artificial diet (Stonefly industries, Inc., Bryan, TX) at 30°C under LD 16:8 as described by Loeb and Hakim (1996). Approximately 24 h prior to molting, larvae become swollen due to the release of molting fluid into the intercutaneous space (the puffy stage). Close to the end of this period, and a few hours prior to the actual molt, the old head capsule becomes displaced forward, and exposes the neck membrane (slipped-head stage). It is this stage that is used for midgut cell culture, as described in Loeb and Hakim (1996).

M. sexta eggs were obtained from the rearing facility at the University of Arizona (Tucson) and larvae were reared as described by Hoffman et al. (1966). When the larvae entered the prepupal stage, they were removed to individual paper cups until pupation. Pupae were sacrificed for fat body dissection within hours of pupation, before the cuticle hardened and turned brown.

Preparation of Midgut Cell Cultures

H. virescens larvae were surface sterilized in consecutive baths of 20% detergent (Septisol™, Vestal Laboratories, St. Louis, MO), 0.1% p-hydroxybenzoic acid methyl ester (Sigma, St. Louis, MO) and 0.1% sodium hypochlorite (Clorox™). Larvae were transferred in sterile water to a continuous flow hood (Aerosafe, Bengton Associates, King of Prussia, PA) and were dissected immediately in sterile modified Ringer solution (Barbosa, 1974) containing 0.5% gentamicin (Sigma), 0.01% antibiotic-antimycotic (Sigma), and Clorox™ (0.0003%). A dorsal cut down the abdomen exposed the midgut, which was then slit open and the contents removed; the midgut was detached at both ends and rinsed in Ringer solution. Midguts were pooled in a few milliliters of sterile medium (Loeb and Hakim, 1996) and left undisturbed for 1.5 h. At the end of this time, the preparation was sieved through a 70-μm mesh filter (Beckton Dickinson, Franklin Lakes, NJ). The tissue fragments caught in the sieve were discarded. Approximately 50–70% of the free cells in the seivate were stem cells that had migrated out of the gut. The cells in the sievate were pelleted by gentle centrifugation at 600*g* for 5 min. Further enrichment of preparations containing 80–90% stem cells was achieved by centrifuging a 1-ml suspension of these cells through 4 ml Ficoll-Paque (Pharmacia, Uppsala, Sweden) for 15 min at 400*g* and collecting the upper 1 ml. After 2 washes in Ringer solution to remove the Ficoll-Paque, the cells were resuspended in 1 ml of medium.

Preparation of Fat Body Extract

Green fat body was dissected from abdomens of 50 or more newly eclosed green pupae of *M. sexta* and pooled in modified Ringer solution. Tissue was later sieved (70 μm, Falcon, Franklin Lakes, NJ) to remove the Ringer containing hypochlorite, and removed to 50-ml tubes and covered with modified Ringer solution without hypochlorite. The preparations were sonicated (Heat Systems Inc. Farmingdale, NY) and centrifuged at 4°C (3,150*g*) for 30 min. The middle layers were removed and frozen at –20°C. After approximately 24 h, the combined middle layers were thawed, re-centrifuged as above, and middle layers were again removed and combined. The process was repeated another time, since 2 freeze-thaw cycles are necessary to break up gel-like material that interferes with further purification. The resulting FBX was filtered through a 0.45-μm Gelman acrodisc, followed by a 0.2-μm sterilizing Gelman filter (Pall Corp., Ann Arbor, MI). Aliquots of 500 μl were pipetted into sterile microfuge tubes and frozen at –20°C for future use. As non-FBX material tends to precipitate each time a tube is thawed, FBX was centrifuged for 1 min at 10,000*g* (Jouan, Winchester, VA); only the supernatant was used. Prior to use, each batch of FBX was bioassayed for its ability to

increase cell number; usually 20 µl/ml medium was sufficient to sustain cultures of insect midgut.

Fractionation of FBX

One milliliter of FBX supernatant was filtered for 140 min at 5,000*g* using a 30-kDa cutoff Centricon filter. Both filtrate and retentate were reconstituted to 1 ml with Ringer solution and then bioassayed.

Initial fractionation of FBX was performed on an HP 1100 high-pressure liquid chromatograph (Hewlett-Packard GmbH; Waldbronn, Germany) equipped with a diode array detector. A 250-µl aliquot of FBX was diluted to 4 ml with 20 mM sodium acetate buffer (pH 4.75) and loaded onto a Vydac 400VHP575 strong cation exchange column equilibrated with the same buffer. After the UV absorbance had stabilized, bound material was eluted with a gradient of 0–500 mM KCl in the sodium acetate buffer over a 50-min period at a flow rate of 0.5 ml/min. Fractions were collected at 1-min intervals and assayed. Larger quantities of FBX were subsequently fractionated by FPLC (Pharmacia; Uppsala, Sweden) using a 2.6 × 10 cm SP Sepharose FF column (Pharmacia; Uppsala, Sweden). Five milliliters of FBX diluted to 50 ml with 20 mM sodium acetate (pH 4.75) was loaded onto the column, which was equilibrated with the same buffer. After the UV absorbance of the effluent had stabilized, the column was eluted with a gradient of 0–500 mM KCl in the same buffer over a 25-min period at 4 ml/min. Fractions were collected at 1-min intervals.

The active cation-exchange fractions were further purified by anion exchange chromatography. Fractions were diluted 1:10 with 20 mM Tris-HCl (pH 8.6) and loaded onto a BioChrom Hydrocell NP10 DEAE column (7.8 × 75 mm). The bound material was then eluted with a gradient of 0–250 mM KCl in the same buffer at 0.5 ml per min.

Analysis of HPLC Fractions

Fractions generated by HPLC were bioassayed as described below and analyzed by SDS-PAGE using 10% gels as described by Laemmli (1970). Gels were stained with PhastGel Blue R (Pharmacia; Uppsala, Sweden) according to the manufacturer's instructions.

A tryptic digest of the active peak was performed, and the digest fractionated by reverse phase chromatography. Two peaks were chosen for analysis by automated Edman degradation on an Applied Biosystems 477A Sequencer.

Bioassay

The number of cells in a 5-µl aliquot of a 1-ml suspension of stem cells were counted, enabling accurate dispersion of 500 cells to each well of a 24-well plate (Falcon, Becton Dickinson Corp.). Aliquots of partially purified polypeptides or column fractions were added to the wells in duplicate. Duplicate controls containing the same volume of Ringer solution as the protein aliquots were included in each bioassay. The plates were incubated at 26°C for 3 to 4 days. After incubation, the number of cells in each well was counted.

RESULTS

Fractionation of FBX

All of the bioactivity in the FBX separated using Centricon size exclusion filters appeared in the fraction that was equal to or greater than 30 kDa. Twenty microliters of the greater than 30-kDa fraction induced doubling of cell number in a midgut stem cell preparation in 4 days, from a mean of 500 cells to a mean of 1,003 cells. However, 20 µl of the <30-kDa fraction only induced an increase from 500 to 700 cells in 4 days, which was statistically the same increase observed in the control cultures ($P = 0.156$). Subsequent fractionation of FBX proceeded under the assumption that the proliferation factor was a protein.

Cation exchange fractionation of FBX by cation exchange HPLC resulted in a single major peak that eluted between 15 and 20 min (Fig. 1A). The UV absorbance spectrum indicated that the peak contained protein with substantial aromatic amino acid content (not shown). Bioassay of pooled fractions indicated that stem cell proliferation was

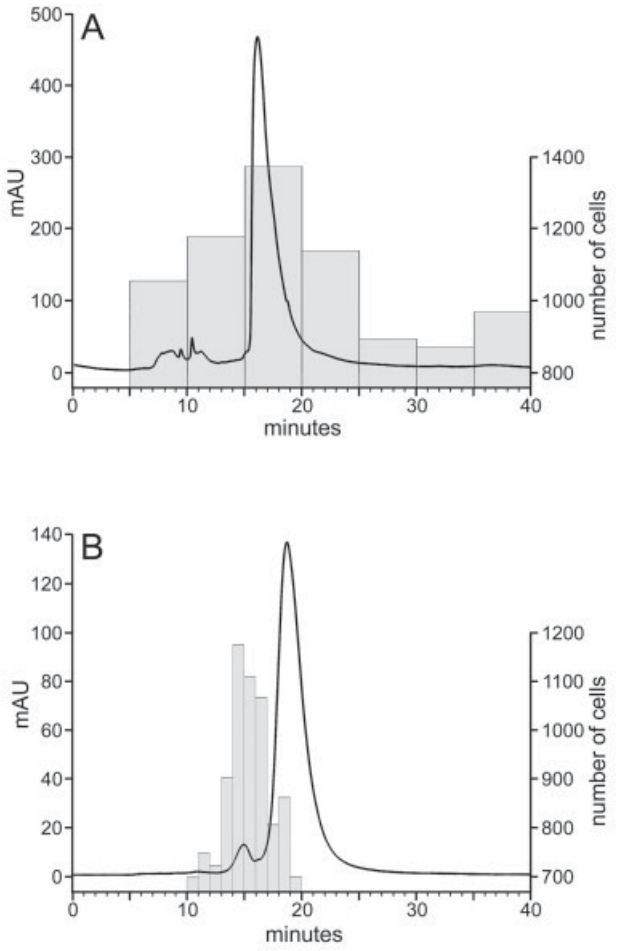

Fig. 1. **A:** HPLC cation-exchange fractionation of 250 µl FBX. The superimposed histogram represents the effect of pooled fractions on stem cell numbers. **B:** HPLC anion-exchange chromatography of pooled active fractions from A. The histogram represents the effect of individual chromatographic fractions on stem cell numbers; most activity was associated with the smaller peak.

maximally stimulated by fractions from 15–19 min, coinciding with the observed peak of UV absorbance (Fig. 1A).

Anion exchange fractionation of the active peak obtained by cation exchange chromatography (Fig. 1B) resulted in small peak eluting between 14 and 17 min (Peak 1) and a much larger peak of UV absorbance eluting between 17 and 23 min (Peak 2). Bioassay of individual fractions from 11–20 min indicated a peak of stem cell proliferation stimulating activity that coincided with Peak 1 (Fig. 1B).

Analysis of the Peaks 1 and 2 shown in Figure 1B by SDS-PAGE (10% acrylamide) revealed that Peak 2 produced two bands of equivalent intensity that had apparent molecular weights of 77 and 72 kDa, while Peak 1 produced a single band of 77 kDa (Fig. 2).

Automated Edman degradation of two peaks from a reverse phase fractionation of a tryptic di-

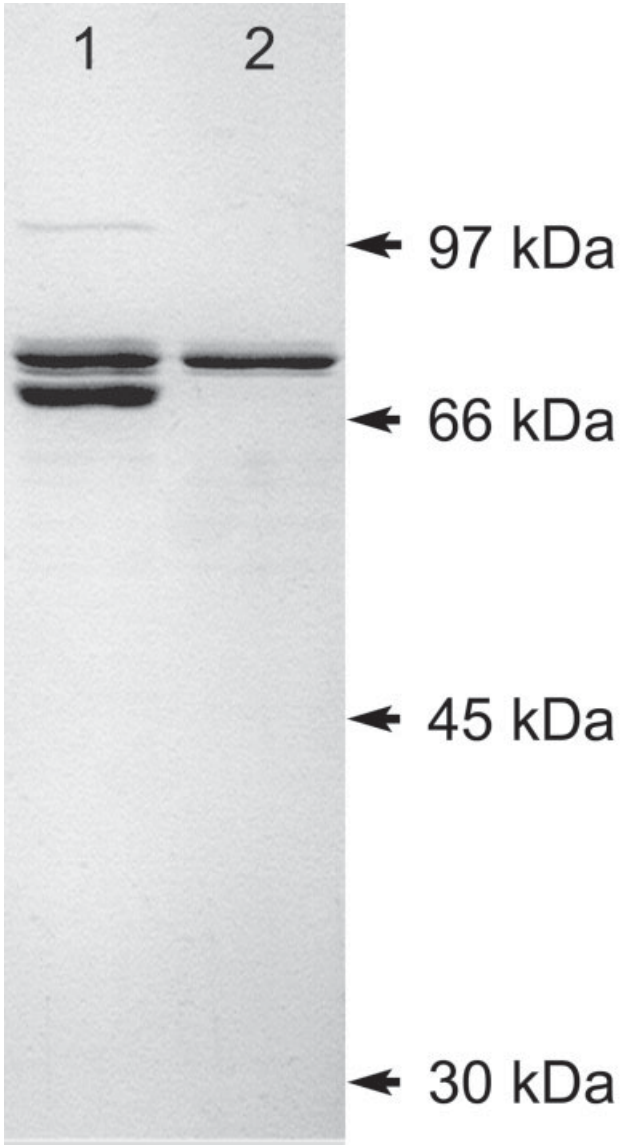

Fig. 2. SDS-PAGE analysis of peaks from anion-exchange chromatography (10% acrylamide). **Lanes 1** and **2** represent, respectively, 2 µg and 1 µg of protein from the major and minor peaks of UV absorbance depicted in Figure 1B. The molecular weights of the major bands were calculated to be 77 and 72 kDa.

gest of the active 77-kDa protein resulted in one major and one minor peptide sequence for each peak. The sequences of the four peptides were found to be identical to internal sequences of *M. sexta* α-arylphorin (Willott et al., 1989). Sequences of the tryptic fragments, aligned with the sequence of α-arylphorin, are shown in Figure 3. None of the tryptic fragments were consistent with β-arylphorin.

Dose-response experiments performed with the purified α-arylphorin indicated that maximal stimulation of stem cell multiplication occurred at a concentration of approximately 125 ng per ml (Fig. 4). At this titer, multiple cell divisions resulted in the formation of clusters, or nidi, of stem cells.

DISCUSSION

We have determined that the component of *M. sexta* FBX that causes rapid multiplication of cultured midgut stem cells from *H. virescens* is the α-subunit of arylphorin. Arylphorins are a class of insect storage proteins that have an unusually high content of the aromatic amino acids phenylalanine and tyrosine (Telfer et al, 1983) and belong to the larger class of storage proteins known as the hexamerins. The arylphorins are high molecular weight complexes composed of six similar (or identical) subunits. They are related to the arthropod hemocyanins, and more distantly to the phenyloxidases (Burmester, 2002). Ryan et al. (1985) cited a number of studies suggesting diverse roles for the arylphorins, such as serving as a reservoir of tyrosine for cuticle sclerotization, a protein component of the cuticle, and a carrier for ecdysteroids and xenobiotics.

Kramer et al. (1980) first isolated and characterized *M. sexta* arylphorin (originally termed Manducin) from hemolymph of 5th instar larvae. In this study, it was determined that *M. sexta* arylphorin was a hexameric protein rich in aromatic amino acids with a molecular weight of approximately 450,000, and consisting of two distinct subunits. These authors also reported that *M. sexta*

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                                                        NVNQLDYEAE
MKTVVILAGLVALALSSAVPPPKYQHHYKTSPVDAIFVEKQKKVFSLFKNVNQLDYEAE

YYK[1]
YYKIGKDYDVEANIDNYSNKKVVEDFLLLYRTGFMPKGFEFSIFYERMREEAIALFELF

YYAKDFETFYKTASFARVHVNEGMFLYAYYIAVIQRMDTNGLVLPAPYEVYPQYFTNME

VLFKVDRIKMQDGFLNKDLAAYYGMYHENDNYVFYANYSNSLSYPNEEERIAYFYEDIG

LNSYYYYFHMHLPFWWNSEKYGPFKERRGEIYYYFYQQLIARYYLERLTNGLGEIPEFS

WYSPVKTGYYPMLYGSYYPFAQRPNYYDIHNDKNYEQIRFLDMFEMTFLQYLQKGHFKA

            XNFVGNYXQANAXLYNEE[2]
FDKEINFHDVKAVNFVGNYWQANADLYNEEVTKLYQRSYEINARHVLGAAPKPFNKYSF

IPSALDFYQTSLRDPVFYQLYDRIINYINEFKQYLQPYNQNDLHFVGVKISDVKVDKLA

TYFEYYDFDVSNSVFVSKKDIKNFPYGYKVRQPRLNHKPFSVSIGVKSDVAVDAVFKIF

LGPKYDSNGFPIPLAKNWNKFYELDWFVHKVMPGQNHIVRQSSDFLFFKEDSLPMSEIY

 LLDEGKIPSDMSXSSDTLPQ[1]                                    XIVP
KLLDEGKIPSDMSNSSDTLPQRLMLPRGTKDGYPFQLFVFVYPYQAVPKEMEPFKSIVP

DSKPFGYPFDRPVHPE[2]
DSKPFGYPFDRPVHPEYFKQPNMHFEDVHVYHEGEQFPYKFNVPFYVPQKVEV
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Fig. 3. Sequences of tryptic fragments of the lesser peak of UV absorbance in Figure 1B aligned with the sequence of *M. sexta* α-arylphorin. X: Residues that could not be determined. Superscripts following fragments identify which sequences were determined simultaneously; italicized fragments represent the minor sequences.

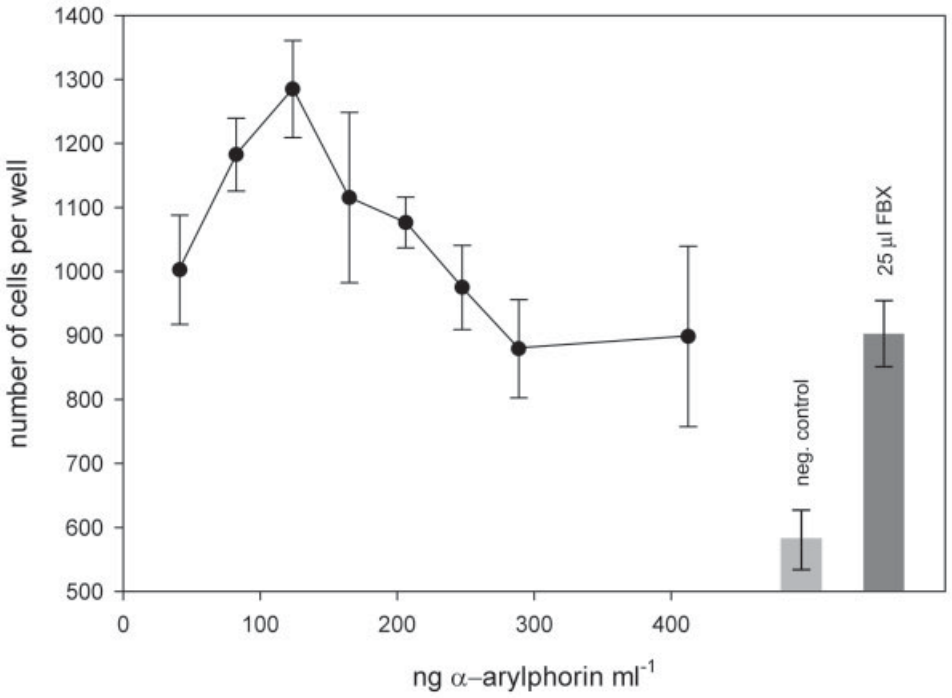

Fig. 4. Stem cell numbers in response to α-arylphorin concentration (solid circles). Each point represents the mean of 3–4 replicates (± SEM). Mean stem cell numbers (± SEM) in response to control (no α-arylphorin) and 25 µl FBX incubations are depicted by the indicated bars.

arylphorin isolated from pupal fat body was chromatographically and electrophoretically indistinguishable from that isolated from hemolymph. Arylphorin concentrations in the hemolymph of 3rd through 5th instar larvae were shown to peak late in each instar and fall during the molt; in the 5th instar, concentrations reached levels of 30–40 mg per ml, making it the major protein constituent of the hemolymph.

Webb and Riddiford (1988b) found that *M. sexta* arylphorin is expressed primarily by the fat body of late instar larvae, with lesser levels of expression occurring in salivary gland, muscle, and epidermis. This study also demonstrated that 20-hydroxyecdysone and cessation of feeding reduced expression of arylphorin by fat body.

In holometabolous insects, a dramatic decline in hemolymph arylphorin concentration is observed during pupal and adult development; this decline is accompanied by accumulation of arylphorin in the fat body (Telfer and Kunkel, 1991). Haunerland and Shirk (1995) showed that in *Helicoverpa zea*, arylphorin was sequestered only by the colored, perivisceral fat body. Only extracts of the green perivisceral fat body surrounding the midguts of newly eclosed lepidopteran pupae could support the growth of midgut cells in vitro, and caused increased mitosis in isolated midgut stem cells from *H. virescens* or *M. sexta* (Loeb and Hakim, 1996). Other uncolored fat body from the thorax or abdomen was not effective in vitro (Sadrud-Din et al., 1994; Loeb and Hakim, 1996), providing supporting evidence that the active factor in FBX was one of the storage proteins. Its proximity to the midgut may be related to its importance to the regulation of midgut tissue.

In 20 mM sodium acetate buffer at pH 4.75, *M. sexta* arylphorin can be purified to near homogeneity from FBX in a single step by cation exchange chromatography. In the present study, small amounts of pure α-arylphorin were subsequently separated from the cation exchange purified material by HPLC anion exchange chromatography using a non-porous matrix that is ideally suited to resolving large proteins. It appears that the purified α-arylphorin consists of free subunits, as it passes freely through a 100-kDa cutoff ultrafiltration membrane.

The free α-arylphorin stimulates the multiplication of midgut stem cells at very low concentrations; peak stimulation occurred at concentrations of 125 ng per ml. This concentration would seem

to be too low to be explained by a nutritional effect on the stem cells. The bulk of the arylphorin that we isolated from FBX, which is composed of both α and β subunits had little or no measurable activity on midgut stem cell proliferation. Further work will be required to determine whether free α-arylphorin is present in hemolymph, and if its occurrence there can be correlated with periods of stem cell proliferation in vivo.

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