Establishment of *Drosophila* imaginal precursor cells is controlled by the *Arrowhead* gene

Jennifer Curtiss and Joseph S. Heilig*
Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309, USA

*Author for correspondence

**SUMMARY**

Metamorphosis in *Drosophila melanogaster* requires synchronization of numerous developmental events that occur in isolated imaginal precursor tissues. The imaginal primordia are established during embryonic stages and are quiescent for much of larval life. The *Arrowhead* gene is necessary for establishment of proper numbers of cells within a subset of imaginal precursor tissues. Loss-of-function mutations in *Arrowhead* reduce the number of abdominal histoblasts and salivary gland imaginal ring cells before the proliferative stages of their development. The number of abdominal histoblasts in mutant animals is approximately half that of wild-type, as might result from failure of a single early division of these cells. A neomorphic *Arrowhead* allele results in the specific loss of the retinal precursors by the early third instar, before they have begun to differentiate. Since *Arrowhead* mutations affect only subsets of imaginal tissue, there must be distinctions in the developmental regulation of different imaginal precursors. *Arrowhead* may be part of a regulatory pathway responsible for establishing the proper number of abdominal histoblasts and salivary gland imaginal ring cells. The neomorphic *Arrowhead* allele, which may cause misexpression of the *Arrowhead* gene in the eye-antenna imaginal disc, interferes with the establishment or proliferation of retinal precursor cells.

Key words: imaginal precursors, *Drosophila melanogaster*, cell proliferation, abdominal histoblasts, salivary gland imaginal ring, compound eye

**INTRODUCTION**

Development of *Drosophila melanogaster* and other holometabolous insects culminates with metamorphosis, resulting in a complete transformation of the body of the animal. To effect this transformation essentially all juvenile tissues are histolyzed and adult (imago) tissues are generated. Metamorphosis requires the dramatic proliferation and differentiation of adult primordia that are sequestered during embryonic development, yet do not contribute to the embryonic or larval stages. These imaginal precursor cells proliferate during the larval stages but do not begin to differentiate until metamorphosis commences at the end of larval life. Establishment of the imaginal precursors and coordination of their proliferation and differentiation occurs with remarkable synchrony and precision.

Establishment of the imaginal precursor cells occurs during the embryonic stages (references include Poulson, 1965; Madhavan and Schneiderman, 1977; Bate and Martinez Arias, 1991; Cohen, 1993; Meise and Janning, 1993). The precise time at which imaginal precursors become committed has been difficult to determine, but cells capable of contributing to some imaginal structures appear to be present at, or immediately following, the cellular blastoderm stage (Garcia-Bellido and Merriam, 1969; Wieschaus and Gehring, 1976a,b; Cohen, 1993). Single-cell transplantation experiments demonstrated that although capable of forming imaginal precursors, cells of the early gastrula stage are not yet committed to a larval or imaginal fate (Meise and Janning, 1993). It has been suggested that imaginal cells are established no later than stage 13, following germ band retraction (Cohen, 1993), since by the beginning of embryonic stage 13 most, if not all, imaginal precursors express the *escargot* gene (Whitely et al., 1992).

Imaginal precursor cells share three characteristics that distinguish them from larval cells. (1) After cell proliferation ceases at embryonic stage 5, the cellular blastoderm stage, larval cells never divide again whereas the imaginal precursor cells reinitiate proliferation during the larval or pupal stages. (2) The nuclei of larval cells become polytene whereas imaginal precursors remain diploid. (3) Imaginal cells remain undifferentiated until the onset of metamorphosis.

Proper control of proliferation of imaginal precursors is important and regulation of the process is complex. A variety of experimental approaches has demonstrated that the imaginal tissues arise from very small numbers of embryonic founder cells (Wieschaus and Gehring, 1976a,b; Madhavan and Schneiderman, 1977; Meise and Janning, 1993). Both the timing of reinitiation of mitosis and the extent of proliferation vary significantly between different populations of imaginal precursors. The cells of the eye-antenna imaginal discs are the first to resume post-embryonic division, beginning in the middle of first instar development (Madhavan and Schneider-
man, 1977) and increasing approximately five hundred fold in number by the beginning of metamorphosis. Cells of the imaginal rings of the gut and salivary glands do not resume mitosis until the transition between second and third instars and increase only about ten fold in number (Madhavan and Schneiderman, 1977; Bryant and Levinson, 1985). The abdominal histoblasts, the cells that will form the imaginal abdominal epithelium, do not resume mitosis until the pupal stage (Guerra et al., 1973; Madhavan and Schneiderman, 1977) and then divide at approximately three times the rate of other imaginal cells (Roseland and Schneiderman, 1979). These striking differences in mitotic behavior indicate that imaginal precursor cells constitute a complex population of cells subject to a variety of regulatory mechanisms.

Imaginal precursor cells are established as discrete groups of cells localized to specific regions of the embryo. The precursors of the adult head structures, appendages and genitalia form from invaginations of the embryonic epithelium and make up the imaginal discs, groups of cells not directly associated with the larval integument. The precursors of the abdomen and the internal organs of the adult such as the gut, salivary glands and brain arise from nests or rings of cells intimately associated with larval structures. For example, the salivary gland imaginal rings are embedded in the larval salivary glands, the midgut imaginal rings are embedded in the larval midgut and the abdominal histoblast nests form among the cells of the larval abdomen. A distinction can be made between those imaginal precursor cells that develop separately from the larval cells and those that develop in close association with larval cells. In this paper we define as incorporate imaginal precursor cells those, including the abdominal histoblasts and salivary gland imaginal rings, that are embedded in larval tissue. During metamorphosis, incorporate imaginal cells replace the cognate larval organ in which the precursor cells are located. We define as excorporate imaginal precursor cells the imaginal discs, which develop separately from larval tissue. During metamorphosis, excorporate imaginal cells elaborate structures unique to the adult. We propose that differences in the larval environments in which incorporate and excorporate imaginal precursor cells develop impose important distinctions on the requirements for control of the establishment, maintenance and proliferation of different groups of imaginal cells.

Genetic analyses of imaginal precursor development have focused on excorporate precursors, particularly the thoracic imaginal discs (Shearn et al., 1971; Stewart et al., 1972; Shearn, 1974; Shearn and Garen, 1974; Sharma and Chopra, 1976; Shearn, 1978; Spencer et al., 1982; Cohen and Jürgens, 1989; Cohen, 1993). Few mutations affecting development of incorporate imaginal cells have been reported (Bryant and Levinson, 1985). Here we describe the effects of mutations in the Arrowhead (Awh) gene which is necessary for the development of the proper number of precursor cells required for the elaboration of the adult abdomen and salivary glands. Mutations in Awh result in a reduction in the number of precursor cells of at least these two incorporate imaginal tissues. We also describe a neomorphic allele of Awh that results in the specific elimination of cells that will form the adult retina. In animals homozygous for the neomorphic mutation, the retinal precursors are missing by the late second or early third larval instar, the time they are thought to become determined (Becker, 1957), and before they have begun to differentiate. The Awh gene product may participate in a regulatory mechanism controlling the establishment of the proper number of abdominal histoblasts and salivary gland imaginal ring cells. The neomorphic allele may interfere with a similar regulatory mechanism controlling the establishment or proliferation of the precursors of the compound eye. Taken together, these mutant phenotypes reveal a fundamental role for the Awh gene in imaginal cell establishment.

MATERIALS AND METHODS

Drosophila stocks

Stocks were maintained on standard cornmeal/molasses/yeast medium at 18˚C or 25˚C. Genetic nomenclature is that of Lindsley and Zimm (1992). The wild-type strain used was Oregon R. The esg<sup>63</sup> (Hayashi et al., 1993) and l(3)S634 enhancer detector stocks were provided by the Berkeley Drosophila Genome Project. Flies carrying the gl-lacZ construct, type C (Moses and Rubin, 1991) and dpp-lacZ (Blackman et al., 1991) were provided by K. Moses. Stocks used for generation of FLP induced mosaics were constructed from 80-NM (Xu and Rubin, 1993) and w<sup>hsFLP</sup>; TM3/TM6, which were obtained from the Bloomington Stock Center and T. Laverty. Deletions GN34 (63D,E-64B1,2) and GN19 (63E6,9-64B1,2) (Harrison et al., 1995), and the E12 and G14 alleles of l(3)S636a (Wohllwill and Bonner, 1991; Harrison et al., 1995) were provided by S. Harrison.

Isolation of Awh loss-of-function alleles

Awh<sup>18</sup> were isolated by reverse of the Awh<sup>1</sup> dominant phenotype: homozygous Awh<sup>1</sup> males were mutagenized with either 4 krad of X-irradiation (Awh<sup>1</sup>-Awh<sup>18</sup>), or 25 mM EMS (Lewis and Bacher, 1968) (Awh<sup>10</sup>-Awh<sup>18</sup>), and crossed en masse to homozygous Awh<sup>1</sup> e virgin females. The progeny of this cross (Awh<sup>1</sup> /hsflp<sup>1</sup> e<sup>1</sup>) were screened for flies with small eyes. Putative loss-of-function alleles, Awh<sup>1</sup> h (* denotes a mutation) were crossed to Awh<sup>1</sup> e/TM3 Sb e flies to establish an Awh<sup>1</sup> h/TM3 Sb e stock and to confirm that the reverting mutation segregated with the Awh<sup>1</sup> h chromosome. Awh<sup>19</sup>-Awh<sup>22</sup> were recognized as mutations failing to complement the pupal lethality of Awh<sup>1</sup>. Awh<sup>19</sup> was isolated by treating red e males with EMS as above and crossing individual males to TM3 Sb e/TM6B Hu Tb e virgin females. A single red e/TM6B Hu Tb e male was picked from the progeny of each cross and individually crossed to Awh<sup>1</sup> h/TM3 Sb Tb e stock and to confirm that the reverting mutation segregated with the Awh<sup>1</sup> h chromosome. Awh<sup>19</sup>-Awh<sup>22</sup> were isolated using hybrid dysgenesis. A P-element insertion at 63F4-6, l(3)S634, was mobilized using P[<i>y</i>, Δ2-3] (Robertson et al., 1988). Male l(3)S634/P[<i>y</i>, Δ2-3] progeny were individually crossed to TM3 Sb e/TM6B Hu Tb e virgin females. A single 4<sup>th</sup> l(3)S634/TM6B Hu Tb e male was picked from the progeny of each cross and individually crossed to Awh<sup>1</sup> h/TM6C Sb Tb e virgin females. These strains were screened for those in which red e/TM6B Hu Tb e male was picked from the progeny of each cross and individually crossed to Awh<sup>1</sup> h/TM6C Sb Tb e stock and to confirm that the reverting mutation segregated with the Awh<sup>1</sup> h chromosome. A single 4<sup>th</sup> l(3)S634/TM6B Hu Tb e male was picked from the progeny of each cross and individually crossed to Awh<sup>1</sup> h/TM6C Sb Tb e stock and to confirm that the reverting mutation segregated with the Awh<sup>1</sup> h chromosome.

Histology

To identify imaginal ring nuclei, salivary glands were dissected from larvae in PBS, fixed for 10 minutes in 1% glutaraldehyde in PBS, and washed in PBS. They were then incubated in 0.5 µg/ml Hoechst 33258 (Sigma) in PBS for 10 minutes, washed in PBS, and examined immediately by epifluorescence. β-galactosidase activity was detected in tissues fixed in 1% glutaraldehyde in PBS for 15 minutes, washed twice in PBS, and incubated in staining solution (Simon et al., 1985) for 2 hours to overnight. After washing in PBS, stained tissues were mounted in 80% glycerol.

Inspection of pharate adults

Pupal cases containing pharate adults were collected by applying a small amount of water to dissolve glue. The entire pupa was fixed by
immersion in boiling water for 2 minutes. The pupal case was gently dissected in PBS, and the pharate adult teased out.

**Scanning electron microscopy**

Wild-type, *Awh*1/+ , and *Awh*1/*Awh*1 flies were dehydrated in an ethanol series (25%, 50%, 75% and 2·100%) for 12 hours at each concentration. Following dehydration and critical point drying, heads were removed and mounted on SEM stubs with double-stick tape, sputter coated with gold, and examined on an ISI-30 scanning electron microscope.

**Immunohistochemistry**

Fixation and treatment of eye-antenna imaginal discs was performed as described by Tomlinson and Ready (1987) with the following modifications. Discs were dissected in fixative without removing the peripodial membrane and 0.1% Triton X-100 was substituted for 0.1% saponin. β-galactosidase was detected with a monoclonal antibody (Promega) used at 1:1000 dilution. The monoclonal anti-hairy antibody (gift from N. Brown) was diluted 1:5 and the monoclonal anti-scabrous antibody (gift from N. Baker) was diluted 1:2. The secondary antibody was HRP-conjugated goat anti-mouse (BioRad) used at a 1:1000 dilution.

**Awh1 mosaic analysis**

Mosaic analysis was performed as described by Xu and Rubin (1993). Mosaic animals were generated by crossing w; *Awh*1 FRT males to w hsFLP1; 80-NM females at 25˚C. Eggs were collected for 12 hours and then aged to the appropriate stage before recombination was induced by placing vials in a 37˚C water bath for 60 minutes. Recombination was induced in embryos and larvae between 0-96 hours after egg laying (AEL). Eyes of adult flies were inspected for clones 3-4 days after eclosion. Appropriate chromosomes were constructed to allow identification of *Awh*1 homozygous clones, in separate experiments, as those expressing either zero or two copies of P[w*].

**RESULTS**

**Recovery of mutations in Awh**

The original *Arrowhead* mutation, *Awh*1 , is a gain-of-function mutation that arose spontaneously and that eliminates the eyes when homozygous; it is described in detail below. To determine the role of *Awh* in normal development we generated loss-of-function mutations in the *Awh* locus. The screen was based on the observation that flies hemizygous for *Awh*1 have small eyes. Thus, loss-of-function *Awh* mutations could be identified by screening for reversion of the homozygous *Awh*1 phenotype (see Materials and Methods). Seventeen alleles were recovered in this screen. Of these, 8 (*Awh*2-*Awh*9 ) were induced by X-irradiation (of 18,343 chromosomes screened) and 9 (*Awh*10-*Awh*18 ) were induced by EMS (of 29,925 chromosomes screened). All 17 alleles are recessive lethal mutations and fail to complement one another in all combinations. In order to confirm that we could recover alleles independent of the original *Awh*1 mutation, we also screened for mutations that fail to complement *Awh*1 (see Materials and Methods). Four alleles were recovered, one (*Awh*19 ) induced by EMS (of 5,922 chromosomes screened), and three (*Awh*20, *Awh*22 ) induced by hybrid dysgenesis (Tower et al., 1993) (of 6,436 screened). All four alleles recovered by noncomplementation of *Awh*1 also fail to complement *Awh*2-*Awh*22 .

All of the x-ray induced alleles have cytologically visible aberrations in polytene chromosome region 63E. Although several of these mutations result in complex rearrangements,
three are clearly interpretable deletions or inversions. Awh2, Awh4, and Awh6 result from a deletion of 63B10.11 through 63E6.9, an inversion of 63E4 to 66B, and an inversion of 63E4 to 64B, respectively. Analysis of independently isolated deletions in the region confirm the location of Awh. The deletion l(3)GN34 removes the region 63D,E through 64B, and fails to complement all mutant phenotypes (described below) of all of the loss-of-function alleles. A deletion of the region 63E6.9 through 64B1.2, l(3)GN19, complements all mutant phenotypes of all alleles of Awh. From this analysis we conclude that Awh is located in polytene chromosome region 63E4.9. This location is consistent with our initial meiotic mapping of Awh1 to 11 map units on chromosome arm 3L.

Previous analyses of this region of the genome have revealed that several essential genes are located in this region (Wohlwill and Bonner, 1991; Harrison et al., 1995). We obtained representatives of four lethal complementation groups and tested them for the ability to complement Awh loss-of-function alleles. The two available members of the pupal lethal complementation group l(3)63Ea (Wohlwill and Bonner, 1991) fail to complement all mutant phenotypes of Awh mutations. We conclude that l(3)63Ea alleles affect the Awh locus.

Animals transheterozygous for any two Awh alleles die during metamorphosis. The phenotypic analysis described below was performed in transheterozygous combinations of three loss-of-function alleles, Awh4, Awh10, and Awh12. All phenotypes described were seen in all combinations of these alleles as well as all other allelic combinations. We will subsequently refer to transheterozygous combinations of these alleles as Awh-.

Awh- pupae fail to develop abdominal epithelium

Each adult abdominal segment forms from four pairs of histoblast nests: the anterior and posterior dorsal pairs which produce the tergites, the ventral pair which produce the sternites and pleurites, and the spiracular pair which form the spiracle and the surrounding pleurite tissues (Madhavan and Schneiderman, 1977; Madhavan and Madhavan, 1980). Mosaic and histological studies including our own have shown that each anterior dorsal and ventral histoblast nest is composed of approximately 16 cells, each posterior dorsal histoblast nest of approximately five cells (Guerra et al., 1973; Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980; Figs 1E, 2), and each spiracular histoblast nest of approximately three cells (Madhavan and Madhavan, 1980). The abdominal histoblasts do not divide during the larval stages, but begin to divide within the first 3 hours after pupariation (Guerra et al., 1973; Madhavan and Schneiderman, 1977; Madhavan and Madhavan, 1980). They continue to divide until approximately 15 hours of pupal development without displacing the larval cells (Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980). At about 15 hours of pupal life, the abdominal histoblast cells begin to migrate and displace the larval cells, which are histolyzed (Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980). Following proliferation and migration, cells of adjacent segments fuse at dorsal/ventral and segmental boundaries (Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980). During the terminal stages of abdominal development the cells differentiate to produce epidermal tissues including the microchaetae and macrochaetae, and secrete the adult cuticle (Guerra et al., 1973; Madhavan and Schneiderman, 1977; Roseland and Schneiderman, 1979; Madhavan and Madhavan, 1980; Fristrom and Fristrom, 1993).

The result of normal abdominal development is shown in

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**Fig. 2.** Average number of abdominal histoblasts is reduced in Awh- larvae. Abdominal histoblasts were identified by expression of esgP3 and counted in (AD) anterior dorsal, (PD) posterior dorsal and (V) ventral nests of wild-type and Awh4/Awh10 third instar larvae (for example, Fig.1E,F). The average numbers of cells detected in each histoblast nest in segments 1-7 is indicated. Error bars denote the standard error of the mean. The ratio of the average number of histoblasts present in wild type to the average number present in Awh- is indicated above each pair of bars. Note that the ratio is approximately 2 for most nests. Significance of the difference between wild-type and Awh- was determined by student’s t-test. At 95% confidence, values for P ranged between 1.1×10^-3 and 1.35×10^-10.
Role of Awh in imaginal development

Fig. 3. Awh− larvae lack salivary gland imaginal rings. (A) Wild-type and (B) Awh10/Awh12 third instar larval salivary glands stained with Hoechst 33258. Large nuclei in both panels are the polytene nuclei of the larval salivary gland cells. Small nuclei, arrow in A, are the non-polytene nuclei of the salivary gland imaginal ring. The imaginal ring cells are absent in the Awh− gland shown in B. Scale bar in A (for A and B) 100 μm.

Fig. 1A,C, which depict a wild-type pharate adult. In Awh− pupae of the same stage little or no development of abdominal epithelium occurs. In the Awh− pharate adult shown in Fig. 1B,D, a single row of bristles developed in the anterior-most segment. No other development of the abdominal epithelium occurred, as evidenced by the absence of bristles and cuticle. In the absence of abdominal epithelium, histolyzed larval tissue can be seen through the pupal membrane. As shown in Fig. 1B,D, when partial development of abdominal epithelium occurs in Awh− pupae, the cuticle and bristles appear normal. Because Awh− abdominal histoblasts that do develop appear to differentiate normally we conclude that Awh does not affect differentiation of the cells, but does affect the establishment or proliferation of the precursors. Development of excorporate imaginal tissues is completely normal in Awh− animals and, as can be seen in Fig. 1B,D, development of the head, eyes, wings, legs and genitalia is indistinguishable from wild type.

To determine whether the abdominal histoblasts appear normal before they resume proliferation during the pupal stages, we examined abdominal histoblast nests from Awh− third instar larvae. Expression of the escargot (esg) gene is first detected in imaginal cells at embryonic stage 13 and continues throughout larval and early pupal development (Whitely et al., 1992; Fuse et al., 1994). In the enhancer detector strain esgP3, lacZ expression is controlled by the esg gene, allowing the detection of abdominal histoblasts and other imaginal precursor cells from stage 13 onward (Hayashi et al., 1993). Examination of abdominal segments from wild-type and Awh− third instar larvae carrying esgP3 (Fig. 1E,F) shows that Awh− larvae have significantly fewer cells in each histoblast nest. By counting the number of cells in each histoblast nest we found that Awh− larvae have approximately one-half the number of abdominal histoblasts found in wild-type larvae (Fig. 2). We also inspected abdominal histoblasts in wild-type and Awh− stage 14 embryos carrying esgP3. Although precise enumeration of the cells is difficult at this stage, the number of abdominal histoblasts in Awh− embryos appears to be approximately half that of wild-type (data not shown). This suggests that Awh may be necessary to generate the proper number of abdominal histoblasts in the embryo.

Awh− larvae fail to develop salivary gland imaginal rings

The adult salivary glands develop from imaginal rings located at the anterior end of each larval salivary gland (Bodenstein, 1950). The imaginal ring cells resume mitosis at the molt from second to third instar (Madhavan and Schneiderman, 1977). The larval salivary gland degenerates during the early pupal stages until it is completely dissolved by approximately 24 hours of pupal development (Robertson, 1936; Bodenstein, 1950). At approximately 10 hours of pupal development the imaginal ring cells begin to grow out anteriorly and posteriorly to form the adult salivary gland (Bodenstein, 1950).

Although the number of cells established in the embryo as salivary gland imaginal ring cells has not been precisely determined, each ring contains approximately 150 cells in mature third instar larvae (Bryant and Levinson, 1985; J.C. and J.S.H., unpublished data). We observe approximately 10 cells in each ring of wild-type late second instar larvae and presume that this approximates the number of embryonic founder cells. In salivary glands of wild-type third instar larvae stained with Hoechst 33258 the non-polytene nuclei of the imaginal ring cells are clearly distinguishable from the nuclei of the larval cells as small nuclei located at the proximal region of the gland (Fig. 3A). In Awh− larvae these nuclei are often completely absent (Fig. 3B). In some Awh− larvae the imaginal rings are

Fig. 4. Awh specifically eliminates the eye in a dosage-dependent manner. Scanning electron micrographs of dorsal view of heads from (A) wild-type, (B) Awh heterozygote (also representative of Awh/Df and Awh/Dp), and (C) Awh homozygote. Adult structures other than the retina are not affected by Awh.
alleles, and these trans-heterozygotes exhibit only the Awh- allele with one copy of + (Fig. 4B) (data not shown). All other tissues arise from the eye-antenna imaginal discs, including the ocelli, develop normally (data not shown). All other structures arising from the eye-ommatidia when examined in 1 of the compound eyes. The ommatidia that form in the Awh- flies are viable and exhibit no defects other than the reduction in size of wild-type eyes (Fig. 5). Heterozygous and homozygous Awh- flies are intermediate in size (data not shown). The absence of the compound eye and of any apparent photoreceptor differentiation in eye-antenna imaginal disc cells is the same in all panels. Anterior is left.

**The gain-of-function allele affects compound eye development**

We conclude that Awh- is a neomorphic (Muller, 1932) allele. Awh- is viable in combination with all loss-of-function Awh alleles, and these trans-heterozygotes exhibit only the Awh- heterozygous phenotype. Awh- is, therefore, appears to retain all normal Awh function. Retinal development is never initiated in Awh- homozygotes

Because the adult retina never forms, we wanted to determine at which stage eye development is disrupted by the Awh- mutation. The adult compound eyes form from cells of the posterior region of the eye-antenna imaginal discs (Haynie and Bryant, 1986). The cells of the eye-antenna imaginal disc, like those of other imaginal tissues, are established during embryogenesis and are the first imaginal cells to resume division; they do so by the middle of the first larval instar and continue to divide throughout the larval stages (Madhavan and Schneiderman, 1977). During the late third instar, photoreceptor differentiation begins (reviews include Tomlinson, 1988; Ready, 1989; Banerjee and Zipursky, 1990; Rubin, 1991; Zipursky and Rubin, 1994). We examined Awh- eye-antenna imaginal discs during the second larval instar, when the eye-antenna imaginal disc cells are proliferating, and during the late third instar, when photoreceptor differentiation has already begun, to determine at which stage retinal development is interrupted by Awh-

We compared expression in wild-type and Awh- homzygous Awh- flies are intermediate in size (data not shown). The absence of the compound eye and of any apparent photoreceptor differentiation in eye-antenna imaginal discs cells is the same in all panels. Anterior is left.

**Relative to the size of the antennal region, the presumptive eye region of Awh- eye-antenna discs is significantly smaller than that of wild-type eye-antenna discs (Fig. 5). Awh- heterozygous eye-antenna discs are intermediate in size (data not shown). The absence of the compound eye and of any apparent photoreceptor differentiation in eye-antenna imaginal discs cells is the same in all panels. Anterior is left.**

**Fig. 5. Eye-antenna imaginal discs from Awh- third instar larvae do not express markers of retinal differentiation.** (A-D) Wild-type eye-antenna imaginal discs, (E-H), Awh- homozygous eye-antenna imaginal discs. A and E, expression of hairy protein; B and F, expression of dpp as indicated by dpp-lacZ. C and G, expression of scabrous protein; D and H, expression of gl as indicated by gl-lacZ. Expression of hairy and scabrous proteins was determined immunohistochemically; expression of gl-lacZ and dpp-lacZ was determined by β-galactosidase activity (see Materials and Methods). a, presumptive antennal region of disc; e, presumptive eye region of disc; lon, larval optic nerve. Scale bar in A 100 μm, magnification is the same in all panels. Anterior is left.

Using Hoechst 33258 we inspected other incorporate imaginal tissues in Awh- larvae, including the fore- and hindgut imaginal rings and midgut histoblast nests. Although these cells are present in Awh- individuals, the tissues do not appear normal and fewer cells may be present.

**The neomorphic Awh- allele affects compound eye development**

The gain-of-function allele Awh- arose spontaneously, and was identified in a fly with compound eyes approximately 50% the size of wild-type eyes (Fig. 4A,B). When homozygous, Awh- causes the complete elimination of the compound eye (Fig. 4C). Heterozygous and homozygous Awh- flies are viable and fertile and exhibit no defects other than the reduction in size of the compound eyes. The ommatidia that form in Awh- heterozygous flies are indistinguishable from wild-type ommatidia when examined in 1 μm serial sections of the eyes (data not shown). All other structures arising from the eye-antenna imaginal discs, including the ocelli, develop normally as do the larval photo-receptor organs. To determine the nature of the Awh- mutation, we crossed Awh- individuals to flies carrying deletions or duplications of polytene chromosome region 63E. The phenotype of one copy of Awh- heterozygous with either a deletion, Df(3)63C6,63E, or a duplication, Dp(3;3)62A,64C, of Awh* is indistinguishable from Awh- heterozygous with one copy of Awh* (Fig. 4B) (data not shown).

We conclude that Awh- is a neomorphic (Muller, 1932) allele. Awh- is viable in combination with all loss-of-function Awh alleles, and these trans-heterozygotes exhibit only the Awh- heterozygous phenotype. Awh- is, therefore, appears to retain all normal Awh function.

**Materials and Methods**

**Photoreceptor differentiation**

Using Hoechst 33258 we inspected other incorporate imaginal tissues in Awh- larvae, including the fore- and hindgut imaginal rings and midgut histoblast nests. Although these cells are present in Awh- individuals, the tissues do not appear normal and fewer cells may be present.

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We compared expression in wild-type and Awh- homzygous Awh- larvae of four genes normally expressed in cells undergoing photoreceptor differentiation in eye-antenna imaginal discs. Both hairy (h) (Brown et al., 1991) and decapentaplegic (dpp) (Blackman et al., 1991) are expressed ahead of the morphogenetic furrow, prior to obvious indicators of photoreceptor differentiation; scabrous (sca) is expressed very early in photoreceptor differentiation (Baker et al., 1990; Mlodzik et al., 1990); and glass (gl) is expressed in all photoreceptor cells throughout their development (Moses et al., 1989). In Awh- eye-antenna discs, none of these genes is expressed in patterns corresponding to compound eye development. However, all are expressed normally in other regions of the eye-antenna disc that are not related to compound eye development (Fig. 5).

Relative to the size of the antennal region, the presumptive eye region of Awh- eye-antenna discs is significantly smaller than that of wild-type eye-antenna discs (Fig. 5). Awh- heterozygous eye-antenna discs are intermediate in size (data not shown). The absence of the compound eye and of any apparent photoreceptor differentiation in eye-antenna imaginal discs cells is the same in all panels. Anterior is left.
initiation of photoreceptor development in Awh\(^{1}\) homozygotes suggests that the missing cells are exclusively those that would normally give rise to the eye.

We examined eye-antenna discs from larvae at the transition between second and third instar to determine if the eye precursor cells are missing from Awh\(^{1}\) eye-antenna discs before retinal differentiation is initiated. Based on size and morphological criteria, it appears that the eye precursors are missing from Awh\(^{1}\) eye-antenna discs at least as early as the early third larval instar (Fig. 6), approximately 36 hours before differentiation begins. Therefore, the eye precursors are already missing during the proliferative stages of eye-antenna disc development.

Absence of the retinal precursors is not the result of increased cell death in Awh\(^{1}\) homozygous eye-antenna discs. Acridine orange staining of Awh\(^{1}\) eye-antenna discs from late second instar through third instar larvae revealed neither the pattern of cell death associated with normal development (Wolff and Ready, 1991) nor any indication of abnormal cell death (data not shown). Therefore, the Awh\(^{1}\) phenotype must result from the death of eye precursors at an earlier stage of development, or from the failure of eye precursors to be established or proliferate.

**Awh\(^{1}\) prevents development of retinal precursors**

The fact that Awh\(^{1}\) eye-antenna discs are smaller than wild-type discs before differentiation begins indicates that Awh\(^{1}\) must affect either the establishment or proliferative stages of eye precursor development. In order to determine the critical time at which Awh\(^{1}\) affects eye development, we generated clones of Awh\(^{1}\) homozygous retinal tissue in an Awh\(^{1}\) heterozygous background. Clones were generated using the inducible FLP recombinase technique (Golic and Lindquist, 1989; Xu and Rubin, 1993). The P[white\(^{+}\)] marker used to identify the clones allowed us to distinguish regions of the eye composed of cells having zero, one or two copies of Awh\(^{1}\). Eggs were collected from flies of the appropriate genotypes (see Materials and Methods and legend to Fig. 7) for 12-hour periods and then aged to different developmental stages before being subjected to heat shock induction of the FLP recombinase. FRT specific recombination was induced in embryos or larvae ranging in age from 0-96 hours AEL. No clones of Awh\(^{1}\) homozygous tissue were recovered in flies derived from embryos or larvae heat-shocked before 60 hours AEL. Clones of Awh\(^{+}\) tissue were recovered in these flies indicating FRT specific recombination was occurring (Fig. 7A). In flies derived from larvae heat-shocked between 60 and 96 hours AEL clones of apparently normal retinal tissue were recovered that were homozygous for either Awh\(^{1}\) or Awh\(^{+}\) (Fig. 7B).

The results of this clonal analysis indicate that cells expressing two copies of Awh\(^{1}\) prior to 60 hours AEL are incapable of developing into retinal cells. The observation that cells that become homozygous for Awh\(^{1}\) after 60 hours of development are capable of forming apparently normal retinal tissue indicates that Awh\(^{1}\) interferes with a step of retinogenesis occurring prior to the onset of retinal differentiation.

**DISCUSSION**

A functional Awh gene is required for the generation of proper numbers of precursor cells of at least two incorporate imaginal tissues: the abdominal histoblasts and the salivary gland imaginal rings. Before the proliferative stages of imaginal precursor development, we find approximately half the normal number of abdominal histoblasts in Awh\(^{+}\) larvae. Similarly, the cells of the salivary gland imaginal rings are reduced in number in Awh\(^{+}\) larvae. In some larvae the abdominal histoblasts and salivary gland imaginal rings are completely absent. These phenotypes are consistent with the failure of an event necessary for the establishment of the founder cells of these tissues. The neomorphic allele Awh\(^{1}\) causes the specific loss of the precursor cells of the compound eye. The fact that eye-antenna...
imaginal discs of homozygous Awh

third instar larvae are smaller than wild-type and do not exhibit retinal differentiation, coupled with the results of the mosaic analysis suggest that Awh

affects eye precursor development long before retinal differentiation normally begins. We conclude that Awh

interferes with early events of establishment or proliferation of the retinal precursor cells. Loss-of-function and gain-of-function alleles of Awh affect early events in the development of subsets of imaginal precursor cells. Phenotypes of both types of alleles are consistent with the failure of an event necessary for the establishment or proliferation of the founder cells of these tissues.

**Awh is required to generate founder cells of a subset of incorporate imaginal tissues**

Awh loss-of-function mutations affect abdominal histoblast nests and salivary gland imaginal rings and other incorporate imaginal tissues, but do not appear to affect any excorporate imaginal tissues. Therefore, the Awh gene may normally be involved in the establishment of only incorporate imaginal tissues, suggesting there are differences in the regulation of the establishment of incorporate and excorporate imaginal tissues. These differences may be necessary since incorporate and excorporate imaginal precursors develop in distinctly different larval environments. During embryogenesis excorporate imaginal precursor cells are sequestered as imaginal discs, where they will develop separately from the larval tissues. In contrast, incorporate imaginal precursor cells are positioned within the larval tissues that they will displace during metamorphosis. During embryonic development, incorporate and excorporate imaginal cells must acquire characteristics enabling them to develop properly. Since incorporate imaginal cells develop in intimate association with larval tissues, errors in their positioning or proliferation could result in inappropriate invasion and disruption of larval tissues by imaginal tissues. A similar error occurring in excorporate imaginal cells may have less severe consequences for the larva. Therefore, more stringent control of proliferative capacity may be imposed on incorporate precursor cells.

The Awh gene product may participate in designating cells of the cellular blastoderm stage embryo to become precursor cells of the abdominal histoblasts and salivary gland imaginal rings. Alternatively, Awh may be involved in the proper localization of the abdominal histoblasts to the appropriate region of each larval segment and the cells of the salivary gland imaginal rings to the apical tip of the larval salivary gland. Or, Awh may be involved in establishing the relationship between imaginal and larval cells. Incorporate imaginal precursor cells may interact with and receive signals from larval tissues as one means of controlling their development. Evidence that the larval cells may be important for later stages of development of the abdominal histoblasts comes from the work of Roseland and Schneiderman (1979). They suggest that the cells of the larval abdomen provide positional cues to the abdominal histoblast cells as they migrate to cover the abdomen during metamorphosis. Although Awh appears to affect a much earlier stage of development, a series of signals may be exchanged between larval cells and the incorporate imaginal precursors they contact.

It is striking that in Awh

larvae the number of abdominal histoblasts in most nests is almost exactly half that found in wild-type (Figs 1E,F, 2). A simple interpretation of this observation is that the abdominal histoblasts fail to undergo a single mitotic division. Our studies of Awh

embryos expressing esg

indicate that the number of abdominal histoblasts does not change between embryonic stage 14 and the beginning of pupariation. Experiments of others have suggested that imaginal cells in general may divide once during the late embryonic stages, after larval cells have completed their last division (Madhavan and Schneiderman, 1977; Bate and Martinez Arias, 1991). It is possible that Awh is necessary for this last round of embryonic division in the abdominal histoblasts and salivary gland imaginal rings. Alternatively, Awh could be necessary to allow an even earlier division to occur, failure of which reduces the number of founder cells that ultimately are positioned in larval tissues. This series of establishment events that occurs in the embryo involves small numbers of cells. Small perturbations in any one of these events could result in large differences in the final numbers of cells available for development of the adult tissue during metamorphosis.

Although loss-of-function Awh mutations affect only incorporate imaginal tissues, it is possible that the reduction in cell number seen early in the development of abdominal histoblasts and the salivary gland imaginal ring in Awh

flies extends to excorporate imaginal tissues. If Awh

mutations do affect the numbers of all imaginal precursor cells established in the embryo, the observation that excorporate tissues develop normally may reflect a capacity of excorporate imaginal precursors to regulate the number of divisions they undergo during larval proliferation. Incorporate imaginal precursors may not possess the same degree of flexibility and may be tightly restricted in the number of divisions they can undergo when proliferation resumes. If Awh

mutations result in reduction of the number of all imaginal precursor cells, incorporate imaginal precursors are apparently unable to accommodate the reductions in size of the founder population.

**Awh

affects very early events in retinal precursor development**

Since the compound eyes develop normally in Awh

pharate adults, we do not believe Awh normally has a role in retinal development. The phenotype of the neomorphic Awh

mutation is likely the result of altered expression of Awh that interferes with retinal development. Nevertheless, an analysis of when and how Awh

interferes with normal retinal development provides useful clues about the early development of retinal precursors.

Awh

causes the specific loss of the precursor cells of the compound eye. The eye-antenna imaginal discs of homozygous Awh

third instar larvae are smaller than wild-type and do not exhibit retinal differentiation. Reduction in size of the presumptive eye region of the disc is apparent in very early third instar larvae suggesting that eye precursor cells are already absent during proliferative stages of eye-antenna disc development. There is no evidence that cell death is responsible for elimination of these cells, unless it occurs before the middle of the second instar. Mosaic analysis indicates that cells that are homozygous for Awh

prior to 60 hours AEL, the middle of second larval instar, are incapable of forming retinal tissue. After the mid-second instar, however, Awh

has no apparent effect on retinal development. These results indicate
that Awh\(^1\) affects early stages of retinal precursor development, but not retinal differentiation. A distinct change in the shape of the eye-antenna disc occurs at the molt between second and third instar. The tissue in the posterior region of the disc begins to extend ventrally. The larval optic nerve provides a useful reference for this growth (Figs 5D,H, 6). Eye-antenna discs isolated from wild-type and Awh\(^1\) larvae at mid-second instar are morphologically very similar whereas by early third instar, they are quite easily distinguished from one another (Fig. 6). The most striking difference is reduction in size of the posterior, ventral region of Awh\(^1\) discs. Similar morphological differences are seen in the third instar eye-antenna discs of larvae carrying mutations in sine oculis (Cheyette et al., 1994) and eyes absent (Bonini et al., 1993), both of which also eliminate the eyes. The posterior, ventral region of the late third instar eye-antenna imaginal disc normally develops into a substantial portion of the retina (Haynie and Bryant, 1986). Expansion of the posterior ventral region of the eye-antenna disc that occurs in late second instar larvae may reflect proliferation of precursor cells of the retina. Mosaic analysis performed by Becker (1957) indicated that a population of cells becomes restricted to a retinal fate around the time of transition between second and third instar. This result is consistent with the idea that specific proliferation of the retinal precursor pool occurs at this stage. Expansion of the posterior, ventral region of the eye-antenna disc does not occur in Awh\(^1\) homozygous larvae. Therefore, Awh\(^1\) may specifically prevent establishment or proliferation of the small number of founder cells of the eye.

**Awh may be involved in a pathway regulating establishment of subsets of imaginal precursors**

Loss-of-function and gain-of-function alleles of Awh affect early events in imaginal precursor cell development. Whereas the elimination of Awh gene product prevents development of incorporate imaginal precursors, retinal precursors are eliminated when Awh is misexpressed, as we presume results from the Awh\(^1\) allele. Similar tissue-specific differences are evident in loss-of-function mutations in the expanded gene (Boedigheimer and Laughon, 1993), which cause excessive proliferation of wing imaginal discs and reduced proliferation of eye-antenna imaginal discs. Thus, a signaling pathway may have opposite effects in different imaginal tissues.

The semi-dominant effect of Awh\(^1\), (i.e. increasing copies of Awh\(^1\) increases the severity of the phenotype) as well as the fact that occasionally a small number of abdominal histoblasts and salivary gland imaginal ring cells are present in Awh\(^-\) larvae, suggest that the response of an individual cell to variations in Awh expression is influenced by other intracellular factors. Thus, phenotypes of both loss-of-function and gain-of-function Awh alleles suggest that Awh participates in a regulatory pathway whose ultimate consequence is the initial identification of a cell as an incorporate imaginal precursor, or the decision of a cell to divide. Such developmental decisions may be based on the relative concentrations of several regulatory molecules, of which Awh is one. Regulation of apoptosis in mammals provides one example of a mechanism by which cell fate is determined by the relative concentrations of several regulatory molecules. Intracellular monitoring of the relative concentrations of Bcl-2 and Bax determine whether individual cells are committed to apoptotic death (Boise et al., 1993; Olsvai et al., 1993; Korsmeyer, 1995). A possible role of Awh is to modulate a signaling pathway whose endpoint is the control of cell proliferation or the establishment of cell identity. Small changes in Awh activity could result in activation or inactivation of the pathway. Misexpression of Awh, as may occur in Awh\(^1\) individuals, may interfere with a similar pathway in another tissue.

The results of our analysis suggest that Awh is important in the establishment of the abdominal histoblasts and salivary gland imaginal ring cells. The neomorphic Awh\(^1\) allele disrupts the establishment of the retinal primordia, indicating that the Awh\(^1\) gene product can radically affect developmental decisions in other imaginal tissues as well. Continued analysis of Awh will reveal the common element necessary for the development of these different imaginal tissues. Analysis of Awh will provide insight into early events in Drosophila imaginal tissue development which may, in turn, have implications for more general understanding of tissue specification and proliferation in other organisms.

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