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Characterization of *sine oculis, eyes absent* and *dachshund* Mutant Phenotypes and the Search for Novel Regulators

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Abstract: The complexity of the *Drosophila* compound eye is under the control of eight "master control" genes. These genes are known to interact genetically and biochemically. Documentation of various gene expressions in various eye gene mutants is reported here as well as preliminary results from a deficiency screen to find novel regulators of the eye gene *dachshund*. Interestingly, in addition to the varying effects of eye genes on one another, we report alternate functions for which *sine oculis, eyes absent* and *dachshund* are critical such as gut formation and nervous system development. We also propose slight modifications to the current model of genetic pathways patterning the eye to reflect the abilities of *sine oculis* and *eyes absent* to independently activate their downstream target *dachshund* and their independence of positive feedback from *dachshund* as well.

Introduction:

Recently, much attention has been paid to the processes by which the single cell of a fertilized embryo can form complex structures such as the compound eye. The compound eye of *Drosophila melanogaster* is highly organized into 750 repeating units called ommatidia. Each individual ommatidium is made up of a cornea, three pigment cells and eight photoreceptor cells called rhabdomeres that plug in at the base to a sensory nerve axon. The organization of the ommatidia arises most directly from the movement of the morphogenic furrow from the posterior of the eye imaginal disk to the anterior. This movement patterns behind it between 32 and 34 sequential rows of ommatidia. The movement of the morphogenic furrow and the organization of the subsequent eye structures, such as ommatidia and sensory bristles, have been well documented (Wolff, T. et al., 1993). However, the specification of the eye begins during embryogenesis and continues through the larval stages, but is much less well understood (Kumar, J. et al., 2001b).

The fly eye arises through a temporal and spatial network of eight "master control" genes. They are considered "master control" genes because of their ability to produce ectopic eyes (except for *sine oculis*) when misexpressed and because when gene function is lost, the eye is deleted (Kumar, J., 2001c). Signaling pathways first set up compartmental boundaries between the future eye and antennal imaginal disks, which are immediately adjacent to one another. EGFR (epidermal growth factor receptor) tends to promote an antennal fate while inhibiting eye development. The Notch signaling pathway promotes eye development while inhibiting antennal development (Kumar, J. et al., 2001c). Through this reciprocal inhibition and activation it is thought that a hierarchy of the eight "master genes" (*twin of eyeless (toy), eyeless (ey), eyes absent (eya), sine oculis (so), dachshund (dac), eye gone (eyg), twin of eye gone (toe) and optix (opt)*) is established within the eye field (Fig. 1).

The organization of this hierarchy has been genetically deduced. *toy* is upstream of the other six, controlling *ey*, which itself interacts with *eyg*. Directly downstream of *ey*, *eya* and *so* have been shown to biochemically interact and complex with one another *in vitro* (Pignoni, F. et al., 1997). The nature of the interaction of this complex with its downstream target, *dachshund (dac)*, is still unclear. However, it is thought that they interact genetically in some form of feedback loop. The functions of *eyg, toe* and *opt* are still unclear.

These "master control" genes are both necessary and sufficient for eye formation, but are expressed in areas of the embryo other than the eye. These genes are being used elsewhere to pattern other parts of the embryo unrelated to the eye. How this function is differentially regulated is unknown. In this study, it is shown that indeed several of these

master control genes, specifically *dac, so,* and *eya*, play key roles in other areas of

embryonic development. Beyond this, *dac* functions to help pattern the central and

peripheral nervous systems. It has also been shown to be transcriptionally active when its

two known upstream regulators are taken away. So, it has been hypothesized that other

regulators must exist outside of this eye patterning hierarchy. Preliminary data reported
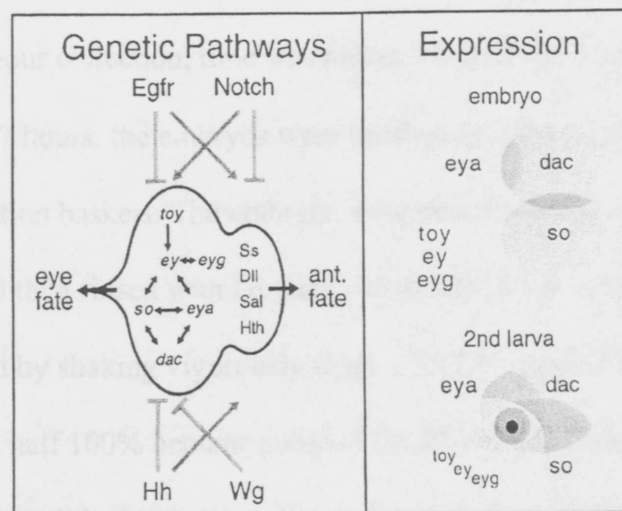
here appears to support this.

Figure 1. Proposed genetic pathway as it relates to expression patterns seen in the embryo. The Egfr and Notch signaling pathways work reciprocally to promote eye identity though the downstream hierarchy shown (Kumar et al., 2001c).

Experimental Procedures:

Drosophila Stocks

The following stocks were used in this study: cs, dac^4, eya^{D1} so^{3}lacZ, so^D, so^{3}eya^{D1}

(Kumar, 2003). All stocks were maintained at 25°C, or room temperature during the egg

collection period.

Embryo Collection

Flies were transferred from their stock bottles to plastic egg-lay chambers topped with an agar plate containing 1% ethyl acetate and 5% sucrose. A drop of yeast paste was put on the plate before topping the chamber. The flies were allowed to lay for 4-hour intervals, at which time the old plates were replaced with fresh ones. The flies were moved back into bottles overnight.

Collected embryos were incubated for seven hours measured from the midpoint of the time laid (ie: in a 4 hour collection, time was measured from the 2 hour point) at 25°C in a humidified box. At 7 hours, the embryos were brushed off of the agar plates and into plastic mesh dechorionation baskets. The embryos were dechorionated by soaking in 50% bleach for 3 minutes and then rinsed with DI water for at least 30 seconds. Dechorionated embryos were then fixed by shaking vigorously in an 1.5 ml Eppendorf tube containing half 10% formaldehyde, half 100% heptane solution for 20 minutes. After this, the phases were allowed to separate and the bottom aqueous layer was removed. An equal part of 100% methanol was added and the embryos were shaken vigorously again for 1 minute to remove the vitelline membrane. The top organic layer was removed and replaced with more 100% methanol. The embryos were then either stored at 4°C for later staining or were stained immediately by the following procedure.

Antibody Staining

The following primary antibodies were used in this study: mouse anti-Eya, mouse anti-Dac, rabbit anti-lacZ, mouse anti-22C10, rat anti-ELAV, mouse anti-AbdB, mouse anti-

Dfd, mouse anti-Pb, mouse anti-Lab, mouse anti-Scr, mouse anti-Ubx, mouse anti-Antp. (*Hox* gene antibodies are courtesy of the Kaufman lab.)

Embryos stored in 100% methanol were rehydrated by nutating in 50% PBT, 50% methanol solution for 10 minutes and then nutating in 100% PBT for another 10 minutes. The embryos were then sometimes blocked by nutating in 10% goat serum solution for 10 minutes. Sometimes this step was skipped and showed no significant difference in the overall staining. The 10% goat serum solution (if used) was then replaced with the primary antibody solution containing 10% goat serum and varying concentrations of the primary antibody. The embryos were allowed to incubate at room temperature overnight in this solution. The following day the primary antibody was removed and the embryos were rinsed for 5-10 minutes in wash buffer. Then a secondary antibody solution was added that contained 10% goat serum and either 2% or 4% secondary antibody. The embryos were allowed to incubate at room temperature in this solution for at least 3 hours. The secondary antibody was then removed, the embryos were rinsed for 5-10 minutes in wash buffer and the wash buffer was then removed. To develop the embryos they were then incubated at room temperature in development solution made according to the "Bio-Rad" HRP development kit. The embryos were developed until a dark and clear pattern could be discerned. The reaction was stopped by removing the development solution and rinsing the embryos in wash buffer for 2-3 minutes. The wash buffer removed and "Vectashield" mounting medium was added. The embryos were mounted on slides and stored at -20°C until ready to be viewed under the light microscope and digitally photographed, usually the same day.

Deficiency Screen

Deficiency kits for each arm of the three autosomes were ordered from the Bloomington

Stock Center. Stocks were maintained in bottles at room temperature for the duration of

embryo collection. Embryos were collected, fixed and stored in the same manner as

described under *Embryo Collection*. Once enough embryos from any given deficiency

were collected, they were stained against Dac protein in the manner described under

Antibody Staining. Stained embryos were then observed under bright field optics and

changes in *dac* expression, if any, were photographed and described.

Results:

Alternate functions for the eye genes *eya*, *so* and *dac*

To discern other possible functions we looked at the axonal (PNS) and nuclear (CNS)

nerve development in *eya*, *so* and *dac* mutant embryos. In *so* null mutants the peripheral

nervous system is severely disturbed. The embryo is misshapen and the regular parallel

and segmental arrangement of the axons seen in a wild type embryo are missing or

irregular. The central nervous system is entirely absent and no nuclear staining of nervous

tissue could be detected.

In *dac* mutants the PNS is intact and shows minimal disturbance, while the CNS

is extremely disrupted. Staining along the ventral nerve cord and in the presumptive brain

is reduced and, in some places, absent.

eya mutants show correct CNS and PNS development; however, they did display

an unexpected phenotype. The gut of *eya* mutants fails to constrict and remains a large

ball. In failing to constrict properly it distorts some of the surrounding tissue that would

otherwise appear normal (Fig. 2D-E).

Genetic interaction within the eye patterning hierarchy

Removing *so* function or *eya* function did not significantly affect *dac* gene expression.

While the morphology of the head was changed, the number of *dac*-expressing cells and

the intensity of their expression remained comparable to wild type. Interestingly, the

dominant *so* allele caused a more severe phenotype than the null allele. When the So

protein was present, but not functional, there was a significant decrease in *dac* gene

expression (Fig. 2C).

When *so* gene function was removed, Eya protein remained in a pattern

comparable to wild type expression. Expression of *so* gene in the other mutants could not

be assayed at this time because of the lack of effective antibodies against the So protein

(Fig. 2B).

dac **regulation outside of the eye patterning hierarchy**

When so³*eya*^DI double mutants were stained with antibodies directed against the Dac

protein it was hypothesized that, because of *dachshund*'s downstream location in the

hierarchy, that it would not be active due to the absence of both its upstream regulators

(Fig. 1). However, the stained embryos showed small clusters of cells still expressing the

dac gene. The number of cells expressing and their level of expression was variable. The

CNS and PNS of the double mutant showed phenotypes most similar to that of *so*³ single

mutants, and the additional lack of *eya* gene function did not appear to increase the

severity of the mutant phenotype (Fig. 2D-E, Fig. 3). Because of this as well as other evidence, it was hypothesized that regulators of the *dac* gene outside of the eye patterning hierarchy must exist. The following experiments were in response to this question. As a first possible candidate for outside regulators, we surveyed seven of the eight *Drosophila* Hox genes for changes in expression in eye gene mutants. Lack of neither *eya*, *so*, nor *dac* gene function changed *Hox* gene expression. (Results not shown)

Preliminary results from the deficiency screen for *dac* regulators

In light of the above-mentioned results concerning the mysterious activation of *dac* in the head, a deficiency screen has been undertaken. By looking at sequential deletions of the *Drosophila* genome, and screening for changes in *dac* gene expression, we will be able locate areas of the genome that contain genes critical for proper expression. Through screening progressively more refined deficiencies, single genes will eventually be isolated. At the time of this writing, 60 deficiencies spanning both arms of the second chromosome have been evaluated for novel regulators of *dac*. Of those 60, 18 of them have yielded changes in *dac* gene expression. All of the changes have been either decreased or absent expression patterns. No deficiency causing an increase in *dac* gene expression has been found yet (Fig. 4).

As a control, all deficiencies causing a complete lack of *dac* gene expression were checked for genes known to be within the deficiency. The two deficiencies overlapping the *dachshund* gene were located this way, thus confirming the effectiveness of the screen.

Wild Type	so^3 Mutant (null)	so^D Mutant	eya^{D1} Mutant	dac^4 Mutant

A

NA NA NA NA
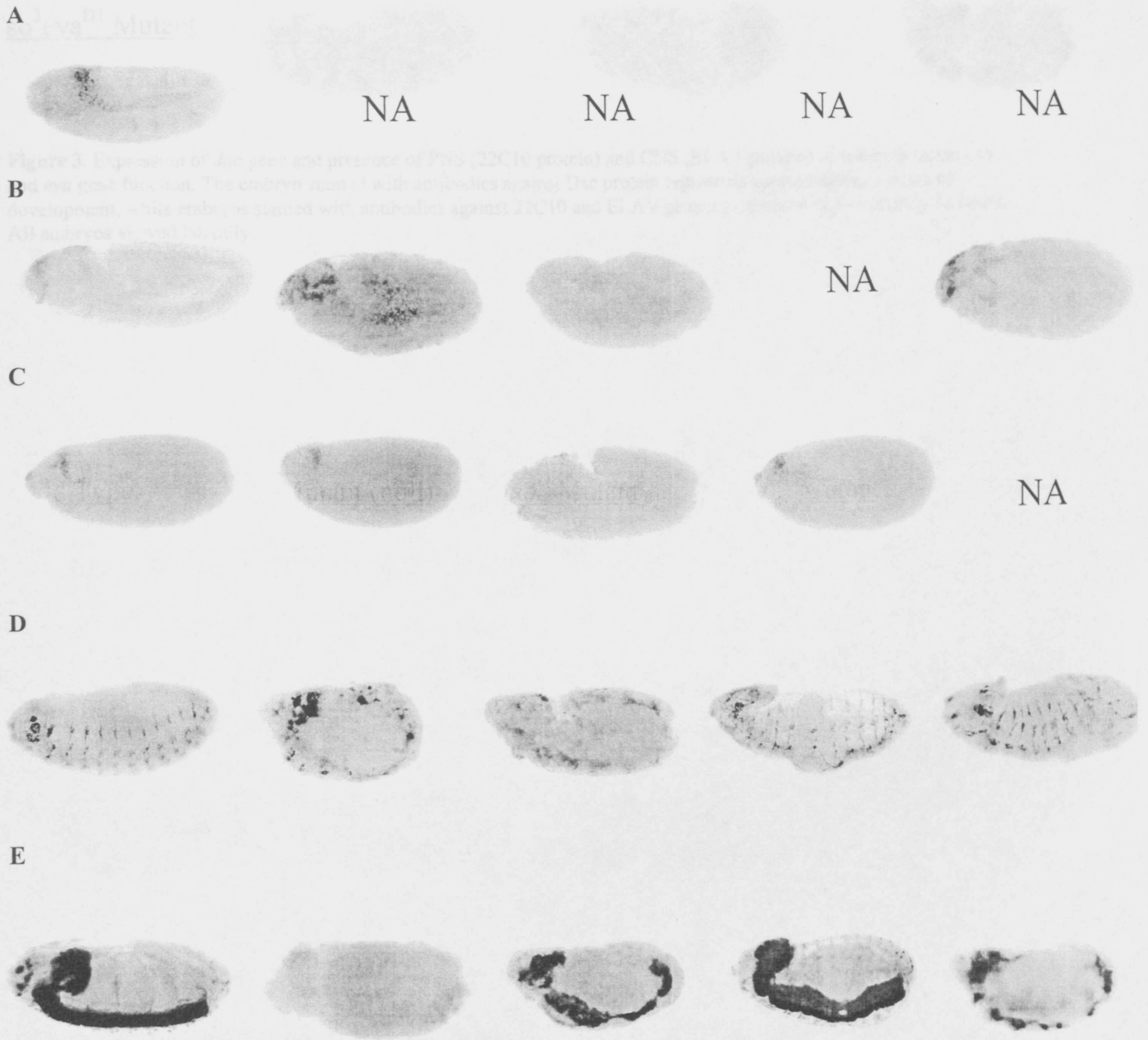
B

NA

C

NA

D

E

Figure 2. Expression of various eye genes, and nervous system phenotypes of various eye mutants. (A) so-lacZ wild type expression (B) Eya in wild type, so^3 mutant, so^D mutant and dac^4 mutant embryos (C) Dac in wild type, so^3 mutant, so^D mutant, and eya^{D1} mutant embryos. (D) 22C10 stain against nerve axons in wild type, so^3 mutant, so^D mutant, eya^{D1} mutant and dac^4 mutant embryos (E) ELAV stain against nerve nuclei in wild type, so^3 mutant, so^D mutant, eya^{D1} mutant and dac^4 mutant embryos. Figures A-C show embryos at approximately 7 hours of development, while D-E show embryos at approximately 12 hours. All embryos viewed laterally.
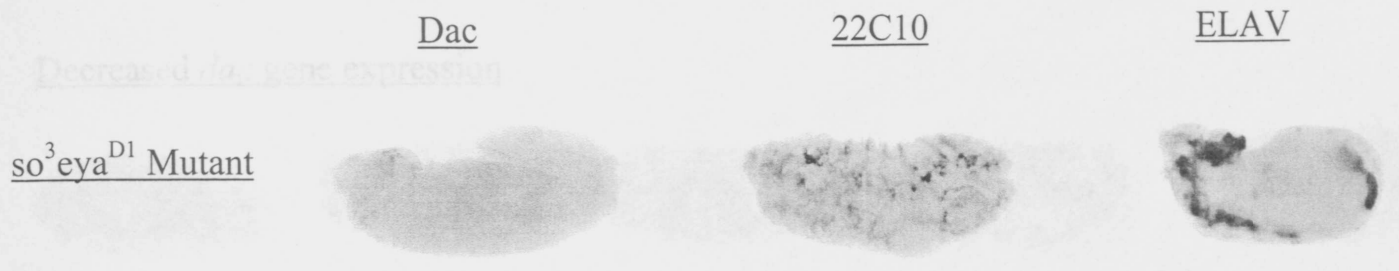
Dac   **22C10**   **ELAV**

so^{3}eya^{D1} Mutant

Figure 3. Expression of *dac* gene and presence of PNS (22C10 protein) and CNS (ELAV protein) in embryos lacking *so* and *eya* gene function. The embryo stained with antibodies against Dac protein represents approximately 7 hours of development, while embryos stained with antibodies against 22C10 and ELAV proteins represent approximately 12 hours. All embryos viewed laterally.
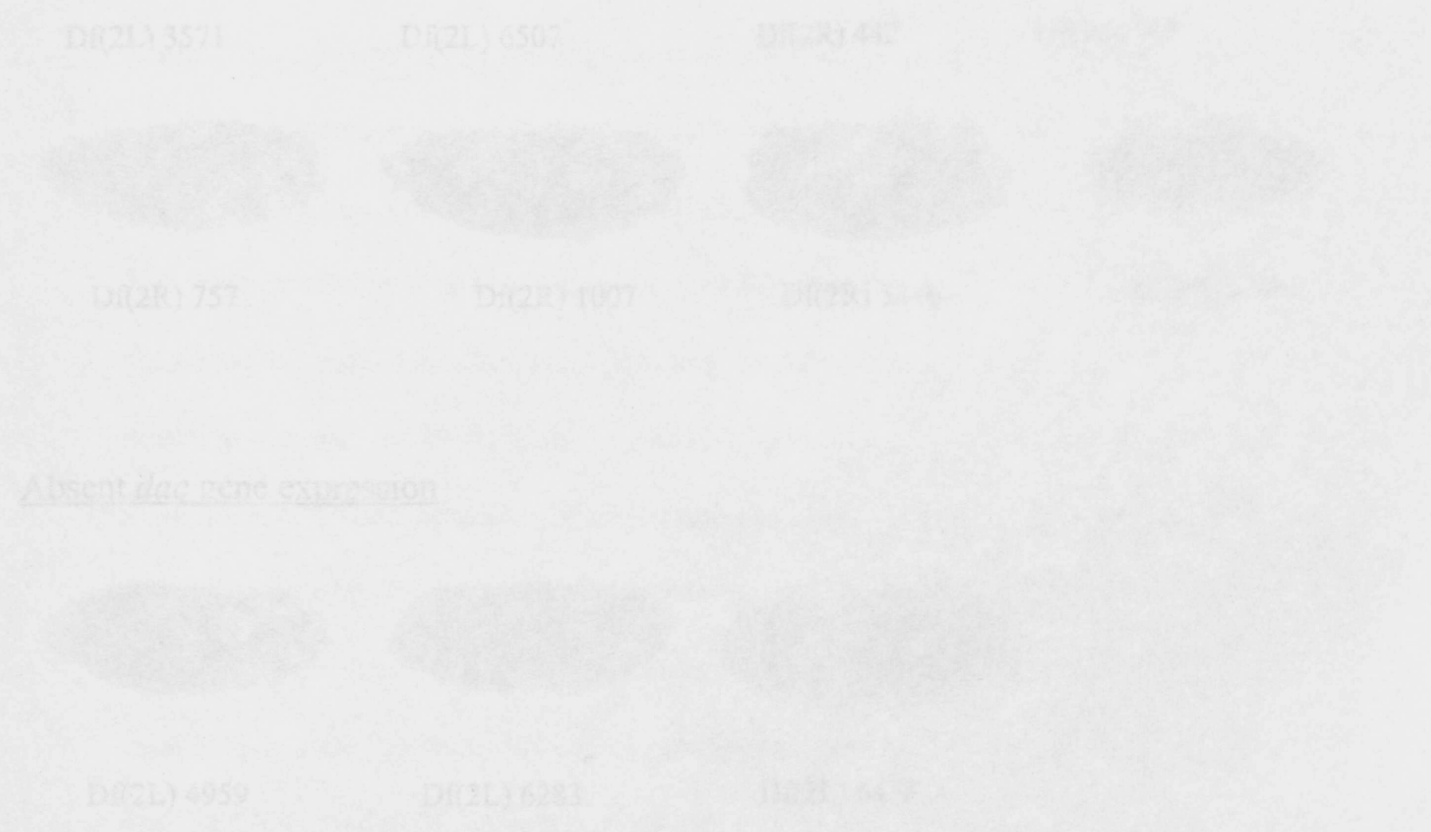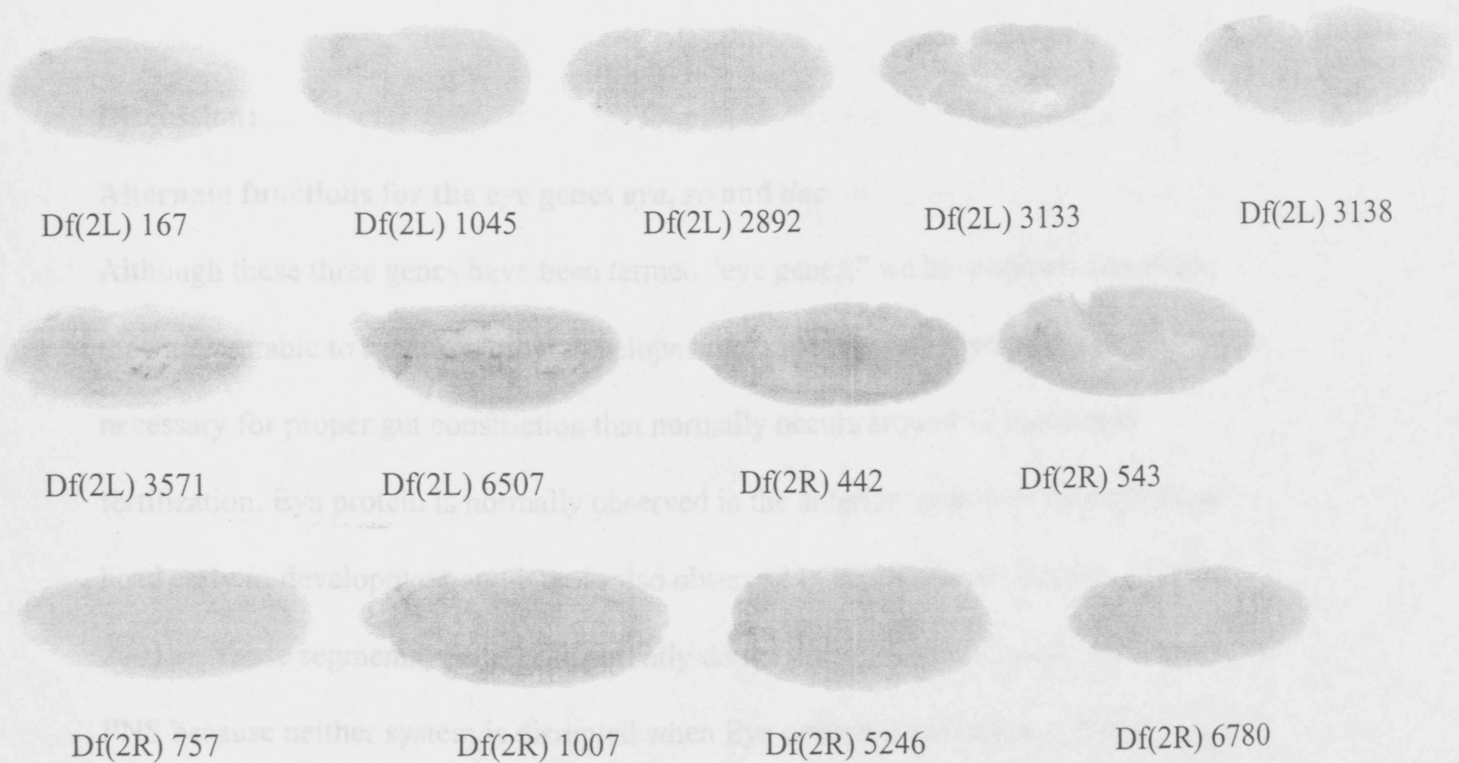
Decreased *dac* gene expression

Df(2L) 167   Df(2L) 1045   Df(2L) 2892   Df(2L) 3133   Df(2L) 3138

Df(2L) 3571   Df(2L) 6507   Df(2R) 442   Df(2R) 543

Df(2R) 757   Df(2R) 1007   Df(2R) 5246   Df(2R) 6780

Absent *dac* gene expression
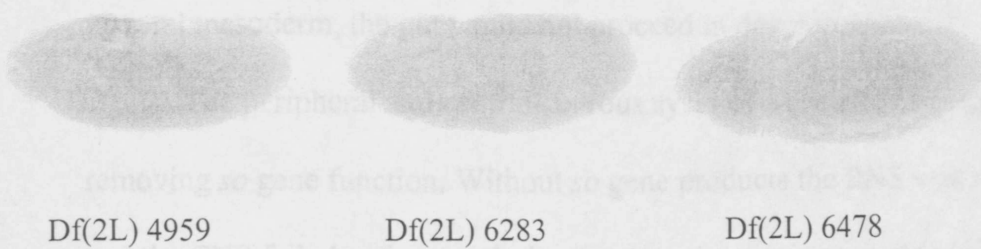
Df(2L) 4959   Df(2L) 6283   Df(2L) 6478

Figure 4. Deficiencies from the left and right arms of the second chromosome that have yielded changes in *dachshund* gene expression. Deficiencies that resulted in early embryonic death are not shown. Deficiencies that give an increase in *dac* gene expression are not shown because none have been found yet. Embryos all represent approximately 7 hours of development and are viewed laterally.

Discussion:

Alternate functions for the eye genes *eya*, *so* and *dac*

Although these three genes have been termed "eye genes," we have shown that when

they are not able to function, other developmental processes are disrupted. *eya* function is

necessary for proper gut constriction that normally occurs around 12 hours after

fertilization. Eya protein is normally observed in the anterior portion of the embryonic

head early in development and later is also observed in each segment (Kumar, J. et al.,

2001b). These segmental patterns apparently do not correspond with either the CNS or

PNS because neither system is disrupted when Eya protein is not present. It has been

suggested that gut constriction is, in part, regulated by processes of the visceral

mesoderm (Skaer, H., 1993). If this is indeed where the *eya* gene is being expressed, then

it would be consistent with the phenotype observed because without this factor within the

visceral mesoderm, the gut would not proceed in development.

The peripheral and central nervous systems were affected significantly by

removing *so* gene function. Without *so* gene products the PNS was reduced and irregular

and the CNS failed to form entirely. Previous expression patterns, as well as those given

here (Fig. 2A) have shown *so* to be present in each segmental groove. This is consistent

with the PNS irregularities. However, it has not been previously reported, nor found here,

that *so* is expressed in the ventral nerve cord. So, it is unclear as to why a lack of *so*

function would result in a complete loss of CNS development. It is known that So protein

interacts biochemically with Eya protein, so it may be that the So protein is also

interacting with other proteins critical for CNS development (Pignoni, F. et al., 1997).

Dac protein appears to be more critical for CNS development than for PNS

development as its mutant phenotype within the CNS is much more severe than in the

PNS (Fig. 2D-E). Dac is known to be expressed in the presumptive brain and along the

ventral nerve cord in later stage embryos, and not along the segmental grooves associated

with the PNS (Kumar, J. et al., 2001b). This observation supports the idea that the *dac*

gene plays a role in CNS development.

Genetic interaction within the eye patterning hierarchy

The results reported here support the current model for eye patterning. (Fig. 1, Kumar et

al., 2001c) However, the dependence of genes upstream of *dac* (*so* and *eya*) on positive

feedback from their counterparts appears less than perhaps first thought. When *dac*

function was removed, Eya protein expressions remained wild type, with minor

deviations attributable to the overall changes in head morphology (Fig. 2B). *eya* gene

expression appears almost entirely independent of *dac* gene products downstream. It is

currently unknown whether *so* is also independent of *dac*, due to a lack of quality

antibodies against the So protein.

The interaction of So and Eya proteins might be less critical in the eye patterning

hierarchy than first thought. When *so* gene function is lacking, functioning Eya protein is

sufficient to drive *dac* gene expression (Fig. 2C). Although So and Eya have been shown

to interact biochemically, *eya* gene function appears to be sufficient to activate *dac*

independent of *so*. It is unknown at this time whether *so* gene expression remains normal

when *eya* gene function is removed. The development of working antibodies against the So protein will allow further investigation. It is also unclear whether the So-Eya complex functions in the eye in *vivo*, or whether this complex serves a function elsewhere. Currently, this question is being examined biochemically in our lab.

With these observations revisions to the current model would be necessary. The interaction between So and Eya is lessened in the eye field and the flow of information from *so* and *eya* to *dac* would proceed predominantly downstream (Fig. 5).
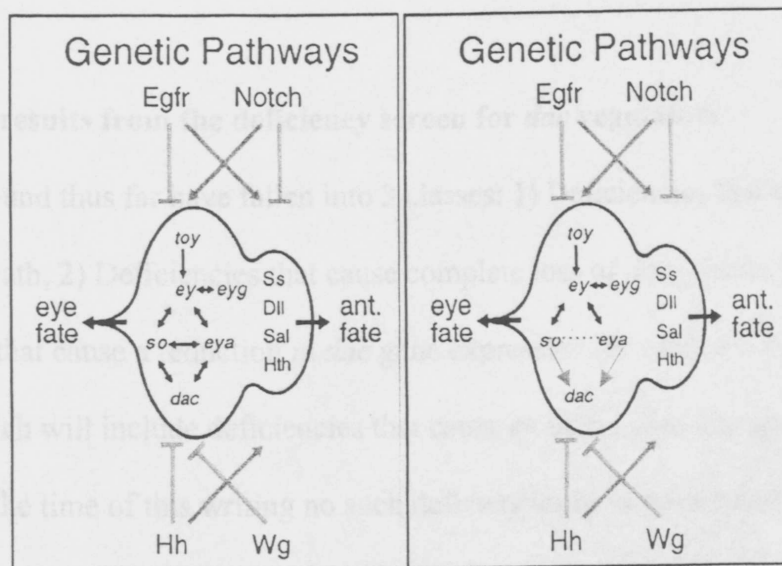
Figure 5. Left panel: current model of eye patterning (Kumar, J. et al., 2001c). Right panel: proposed changes to the genetic pathways of eye patterning. The interaction between So and Eya protein is made less significant and the positive feedback from *dac* to *so* and *eya* is eliminated.

dac regulation outside of the eye patterning hierarchy

It has been shown that Dac protein is present at different times and places throughout the embryo, and not just in the head. (Kumar, J., 2001b) It was significant then to find *dac* activation without the presence of its two known activators (*so* and *eya*). We have hypothesized that there must exist regulators of *dac* other than those known in the eye

patterning hierarchy. In light of this, and other evidence, a search was started for these unknown regulators. As a first pass, seven of the eight *Hox* genes were examined in various eye mutants. Because no changes in *Hox* gene expression were detected, it would suggest there is no direct interaction between the two systems. The reverse experiment (testing eye gene expression in *Hox* mutants) will further test this idea. Other classes of genes, such as antero-posterior, dorsal-ventral and gap, will also need to be tested for possible regulators. The deficiency screen, however, has yielded many possible leads as discussed in the following section.

Preliminary results from the deficiency screen for *dac* regulators

The results found thus far have fallen into 3 classes: 1) Deficiencies that cause very early embryonic death, 2) Deficiencies that cause complete loss of *dac* gene expression, 3) Deficiencies that cause a reduction in *dac* gene expression. Presumably there is a fourth category, which will include deficiencies that cause an increase in *dac* gene expression. However, at the time of this writing no such deficiencies have been found. The first class of mutants has been discarded because they die too early to observe any possible *dac* gene expression.

Interestingly, one deficiency on the right arm of the second chromosome produced a very dramatic and specific phenotype. The dorsal patches of *dac*-expressing cells were completely gone and only two cells expressed the Dac protein: one located on the anterior left and one on the anterior right side of the embryonic head. Apparently, these two cells are regulated differently than all the other *dac*-expressing cells in the head

because when this portion of the genome was removed only those cells successfully

expressed the Dac protein. It is surprising in its extreme specificity.

Each deficiency that has thus far yielded a change in *dac* expression and,

therefore, presumably a change in its regulation as well, will be further examined by

taking sequentially smaller deletions within the original deficiency. This will be

continued until a region containing a single gene regulating *dac* is located.

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