Expression of evolutionarily conserved eye specification genes during *Drosophila* embryogenesis

Justin P. Kumar and **Kevin Moses**

Department of Cell Biology, Emory University School of Medicine, 1648 Pierce Drive, Atlanta, GA 30322, USA

Abstract

Eye specification in *Drosophila* is thought be controlled by a set of seven nuclear factors that includes the Pax6 homolog, Eyeless. This group of genes is conserved throughout evolution and has been repeatedly recruited for eye specification. Several of these genes are expressed within the developing eyes of vertebrates and mutations in several mouse and human orthologs are the underlying causes of retinal disease syndromes. Ectopic expression in *Drosophila* of any one of these genes is capable of inducing retinal development, while loss-of-function mutations delete the developing eye. These nuclear factors comprise a complex regulatory network and it is thought that their combined activities are required for the formation of the eye. We examined the expression patterns of four eye specification genes, *eyeless* (*ey*), *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*) throughout all time points of embryogenesis and show that only *eyeless* is expressed within the embryonic eye anlagen. This is consistent with a recently proposed model in which the eye primordium acquires its competence to become retinal tissue over several time points of development. We also compare the expression of Ey with that of a putative antennal specifying gene Distal-less (Dll). The expression patterns described here are quite intriguing and raise the possibility that these genes have even earlier and wide ranging roles in establishing the head and visual field.

Keywords

Eye; Evolution; Embryogenesis; *Drosophila*

Introduction

The specification of the compound eye of the fruit fly, *Drosophila melanogaster*, has been shown to be under the concerted action of seven nuclear proteins. This group of proteins is comprised of the Pax6 homologs Eyeless (Ey) and Twin of Eyeless (Toy), the Pax2 homolog Eye Gone (Eyg), the Six homologs Sine Oculis (So) and Optix, and two novel genes Dachshund (Dac) and Eyes Absent (Eya, Bonini et al. 1993; Cheyette et al. 1994; Mardon et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994; Jun et al. 1998; Czerny et al. 1999; Seimiya and Gehring 2000). Evidence from genetic, molecular and biochemical studies show that these proteins do not lie in a linear biochemical pathway, but rather form a complicated and interwoven regulatory network. For instance, the transcription of *so* and *eya* is activated by the direct binding of Ey protein to upstream promoter sequences within these genes and ectopic expression of *eya* requires *ey* expression to induce ectopic eyes (Bonini et al. 1997; Halder et al. 1998; Niimi et al. 1999; Bui et al. 2000; Zimmerman et al. 2000). Additional protein-protein

interactions between So, Dac, and Eya have also been shown to occur in in vitro assays (Chen et al. 1997; Pignoni et al. 1997).

Homologs of the *Drosophila* eye specification genes have been found throughout the animal kingdom (Gehring and Ikeo 1999; Wawersik et al. 2000). Indeed several orthologs of *toy, ey, so, eya*, and *dac* are expressed in mouse and human retinas (Ton et al. 1991; Grindley et al. 1995; Oliver et al. 1995; Xu et al. 1997; Zimmerman et al. 1997; Hammond et al. 1998; Caubit et al. 1999; Davis et al. 1999; Leppert et al. 1999; Nishina et al. 1999; Terzic and Saraga-Babic 1999; Winchester et al. 1999; Davis et al. 2001). The expression patterns of these genes suggest that they have been used several times during evolution to specify eye development. Indeed, mutations in the mouse and human homologs of *toy, ey, so*, and *eya*, and possibly *dac*, underlie several severe retinal disease conditions (Hogan et al. 1986; Hill et al. 1991; Abdelhak et al. 1997a,b; Wallis et al. 1999; Azuma et al. 2000; Klesert et al. 2000; Pasquier et al. 2000; Sarkar et al. 2000; Davis et al. 2001).

The widespread use Eyeless/Pax6 in retinal development throughout the animal kingdom has led to the title of "master control gene" for eye specification (Halder et al. 1995b; Gehring 1996; Callaerts et al. 1997). In *Drosophila* this title has been justified by (1) the deletion of the adult compound eye in loss of function *ey* mutants, (2) the induction of ectopic eyes by the misexpression of *ey*, and (3) the ability of the mouse and human homologs of *ey* to induce retinal development in the fly despite the tremendous evolutionary distance between vertebrates and flies (Quiring et al. 1994; Halder et al. 1995a). Recently, the downstream targets and interacting components of *ey* have been identified and have been given the same title based on the above criteria (Bonini et al. 1997; Shen and Mardon 1997; Pan and Rubin 1998; Czerny et al. 1999; Seimiya and Gehring 2000).

The evidence from genetic, molecular, and biochemical experiments suggests that these nuclear factors should act together as a transcriptional unit within the retinal primordium to specify the eye. This is also expected if this set of genes does in fact represent master control genes for eye specification. However, it is known that each of the eye specification genes is also expressed in other tissues that do not give rise to retinal tissue (Fasano et al. 1991; Cheyette et al. 1994; Mardon et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994; Bonini et al. 1998; Jones et al. 1998; Leiserson et al. 1998; Czerny et al. 1999; Seimaya and Gehring 2000; Kumar and Moses 2001). Furthermore, since these are without exception nuclear factors, these genes should be expected to receive instructions from upstream signal transduction cascades. We have recently shown that both the epidermal growth factor receptor (Egfr) and Notch signaling cascades lie upstream of the above-mentioned eye specification genes. Hyperactivation of Egfr or downregulation of Notch signaling results in the homeotic transformation of the eye into an antenna (Kumar and Moses 2001). In such cases, the transcription of the eye specification genes is downregulated and is accompanied by the upregulation of antennal specification genes (Kumar and Moses 2001). These two pathways join the Hedgehog and transforming growth factor-beta (TGFb) pathways in regulating the eye specification genes (Chen et al. 1999; Curtiss and Mlodzik 2000; Kumar and Moses 2001).

This dramatic effect on eye and antennal specification surprisingly does not take place during embryogenesis, as was expected based on previous interpretations, but rather it occurs during the latter half of the second larval instar (Younossi-Hartenstein et al. 1993; Kumar and Moses 2001). Concomitant with eye specification at this time point is the co-expression of the Notch receptor and of all eye specification genes within the eye imaginal disc (Kumar and Moses 2001). We therefore argued that the co-expression of all seven master control genes during the second larval instar is crucial for eye specification. This view of eye development is in conflict with established models in which eye specification is thought to take place during embryogenesis. For this to be correct the eye specification genes should be co-expressed within

the retinal primordium during its early development. However, at the latest phases of embryogenesis (time point 16) only *toy, ey*, and *eyg* have been localized to the embryonic eye imaginal disc primordium (Quiring et al. 1994; Jones et al. 1998; Czerny et al. 1999; Kumar and Moses 2001). They are joined later by the remaining eye specification genes in a spatially and temporally sequential manner (Bui et al. 2000; Kumar and Moses 2001).

The expression patterns of the eye specification genes during early embryonic development is less well described. We set out to describe in detail the expression pattern of *ey, so, eya*, and *dac* in relation to each other throughout embryogenesis. We find that all four of these genes are expressed in dynamic patterns during embryogenesis, but that only *ey* is expressed within the eye imaginal disc. However, the early expression patterns of *so, eya*, and *dac* suggest that they may be involved in the specification of the head and visual field. Such a role for these genes in evolution is supported by the head and visual system defects that are seen in mouse models and human patients (Abdelhak et al. 1997a,b; Wallis et al. 1999; Azuma et al. 2000; Klesert et al. 2000; Pasquier et al. 2000; Sarkar et al. 2000; Davis et al. 2001). We also find that contrary to the suggestion made by in vitro biochemical interactions, these three proteins have very distinct and in most cases non-overlapping expression patterns. In addition, a comparison of Ey expression with that of the antennal specifying gene Distal-less (Dll) indicates that, although Dll is expressed in several domains within the embryonic head, these do not appear to correspond to the future antennal segment. These results are consistent with a model in which eye and antennal specification occurs (1) during the second larval instar, (2) requires the Egfr, Notch, and TGFb signal transduction cascades, and (3) requires the co-expression of the eye and antennal specification genes in the appropriate spatial compartment.

Materials and methods

Drosophila

stocks and antibodies

The following fly strains were used in this study: *ey-lacZ* (Halder et al. 1998) and *so-lacZ* (Cheyette et al. 1994). Both lacZ lines are known to be faithful to the normal expression patterns of the *eyeless* and *sine oculis* genes and are used here to facilitate comparisons with other gene products in double labeling experiments within individual embryos. The following antibodies were used in this study: mouse anti-Dac 1:100 (Mardon et al. 1994), mouse anti-Eya 1:100 (Bonini et al. 1993), and rabbit anti Dll 1:100 (Dong et al. 2000).

Staging and staining of embryos

Embryos harboring an ey-lacZ or a so-lacZ insertion were collected for 1 h at 18°C. Embryos were then aged at 25°C to form a 1-h series starting at 1 h and continuing until 16 h (i.e., 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, 13 h, 14 h, 15 h, 16 h). Embryos at each time point were then stained to look for the following combinations: *ey-lacZ*/Dll, *ey-lacZ*/Dac, *ey-lacZ*/Eya, *so-lacZ*/Dac, and *so-lacZ*/Eya. Embryos were stained as described in Kumar and Moses (2001). We did not stain embryos for both Dac and Eya, since all available antibodies to these two proteins are made in mouse and cross-reactivity between antibodies will compromise the analysis of the staining patterns.

Results

Clonal analysis and fate mapping data have suggested that the antenna and compound eyes are specified during embryogenesis (Postlethwait and Schneiderman 1971). Morphological analysis has further indicated that the antennal and eye disc primordia fuse to form a single complex near the end of embryogenesis (Younossi-Hartenstein et al. 1993). We analyzed the expression of an *ey-lacZ* transgene, which is known to faithfully reflect *ey* expression within

the developing imaginal disc, in batches of embryos that were separated by 1 h and compared this with the expression of Dll, an antennal specifying gene. We found that *Dll* precedes *ey-lacZ* expression and is first seen in anterior regions of the head at approximately 5 h AED (Fig. 1A, BM). *Dll* expression can be found in the maxillary and mandibular segments along with the leg precursors at approximately 6 h AED (Fig. 1C–H). *ey-lacZ* expression is first detectable at approximately 11 h AED in two symmetrical regions on the dorso-lateral surface of the embryo (Fig. 1K, L). At this time point the *ey-lacZ* domains are adjacent to two domains of *Dll* expression. Interestingly, *Dll* expression is not seen in the presumptive antennal discs. However, as development proceeds and the eye imaginal disc adopts a more dorso-medial position and the domains of *Dll* expression become further separated from that of *ey-lacZ* (Fig. 1M–P). This separation continues through to the end of embryonic development. While *ey* expression is seen throughout the eye-antennal disc complex at this time point, *Dll* expression is not detected in the eye-antennal imaginal disc complex until the second instar larval time point, and is not restricted to just the antennal portion of the disc until the beginning of the third larval instar (Kumar and Moses 2001).

Genetic experiments have established *ey* as residing near the top of the eye specification hierarchy and *dac* as the most-downstream member of this signaling cascade. Nevertheless, genetic and molecular epistasy experiments have shown a complex reciprocal interaction between the two genes. Both are able to induce the transcription of the other in ectopic expression experiments, and both require the function of the other for ectopic eye development (Shen and Mardon 1997). Although it is still unclear if these connections are though direct binding of these proteins to each other's promoters, it suggests that both genes should be expressed within the same cells during eye imaginal disc development. We expected *dac* expression to be induced in all places where *ey-lacZ* was visualized and visa versa. We compared the expression of *ey-lacZ* with that of *dac* and were surprised to find that *dac* is expressed in an *ey*-independent manner during embryogenesis. *dac* expression is first detected in two anterior-dorsal domains at approximately 4 h AED and increases in complexity throughout the embryonic head by approximately 5 h and 6 h AED (Fig. 2A–F). Subsets of cells within the brain hemispheres express *dac* beginning at approximately 7 h AED (Fig. 2G, H), while cells within each segment of the central nervous system (CNS) express *dac* at approximately 8 h AED (Fig. 2I, J). At approximately 11 h AED *ey-lacZ* expression, which demarcates the eye imaginal disc, is not co-localized with *dac*, which is predominant in the optic lobes, brain and CNS (Fig. 2O. P). As the imaginal disc continues to develop through embryogenesis, *dac* and *ey-lacZ* expression are never co-localized to the same groups of cells (Fig. 2Q–T). This is further surprising, since ectopic *dac* expression in imaginal discs is sufficient on its own to induce *ey* transcription, and thus we did expect to see regions of *ey-lacZ* and *dac* colocalization.

Ey directs the transcription of *eya* by binding to regulatory regions within the *eya* promoter (Halder et al. 1998; Bui et al. 2000; Zimmerman et al. 2000). Ectopic expression of eya was not however observed to induce eye transcription (Bonini et al. 1997). It is therefore possible to see cells that are Eya positive and Ey negative, but any cell that is Ey positive should also be Eya positive. To determine if this is indeed the case we compared the expression of *ey-lacZ* with that of *eya*, with the expectation that all cells (especially the eye imaginal disc primordia) that express *ey-lacZ* will also express *eya*. Surprisingly, *eya* expression begins even earlier than that of *dac* and is obviously also independent of *ey* regulation. Eya protein is first seen at approximately 2 h AED at which the embryo is still in the synctial blastoderm time point and can be seen as a band of cells that runs along the dorsal surface of the embryo (Fig. 3A–D). By approximately 4 h AED this band is transformed into a crown that extends more laterally (Fig. 3E–J). As is the case with *dac, eya* begins to be expressed in subsets of cells within the embryonic brain by approximately 7 h AED, but these cells are distinct from those that express *dac* (Fig. 3K, L). Unlike *dac, eya* is not expressed within the ventral nerve cord,

but rather is found in a small clustering of cells within the segmental grooves of the embryo (Fig. 3M–P). From the onset of *ey-lacZ* expression at approximately 11 h AED through the end of embryogenesis, *eya* is not expressed within the eye imaginal disc (Fig. 3Q–X). These results are consistent with those reported in Bonini et al. (1998). Eya protein is first detected in the eye imaginal disc during the first larval instar (Bui et al. 2000).

Recently it has been shown that the patterning genes *hedgehog* (*hh*) and *decapentaplegic* (*dpp*) are required for the specification in the eye (Chen et al. 1999; Curtiss and Mlodzik 2000). In an interesting model it has been proposed that Hh signals to Eya which then in turn induces (directly or indirectly) the transcription of both *so* and *dac* (Curtiss and Mlodzik 2000). This would then suggest that during embryogenesis all three proteins should have overlapping expression patterns during the allocation of the eye disc. We first compared the expression of a *so-lacZ* transgene with that of *dac*. Interestingly, while the onset of expression of both genes are first detected at approximately 4 h AED, their expression patterns abut each other and are not overlapping. While *dac* is expressed in two clusters of dorsal medially located cells, *so-lacZ* expression is seen in a broad swathe of cells that extends from one lateral surface to another. Its expression appears to be delimited by the more-anterior domain of dac expression and the cephalic furrow (Fig. 4A, B). By approximately 5 h AED there is a cluster of cells along the lateral margins just anterior to the cephalic furrow in which both *so-lacZ* and *dac* are co-expressed (Fig. 4C–F). However, the vast majority of *so-lacZ* and *dac* expression is non-overlapping. Not unlike *eya, so-lacZ* is expressed in a subset of cells within the developing brain but is not expressed in the ventral nerve cord (Fig. 4G–T). There is considerable overlap between the *dac* and *so-lacZ* expression patterns within the developing brain lobes. In the segmental grooves *so-lacZ* expression can be seen much like that of *eya* (Fig. 4K–T). At approximately 11−14 h AED there is no expression of *so-lacZ* within the developing eye imaginal disc (Fig. 4M–T).

Eya can form a complex with So in vitro (Pignoni et al. 1997). Within the eye imaginal disc of second and third instar larvae, both genes are expressed in overlapping, although not completely exact, patterns (Cheyette et al 1994; Mardon et al. 1994; Serikaku and O'Tousa 1994; Kumar and Moses 2001). It has also been shown that *so* and *eya* act synergistically in promoting ectopic eye development (Bui et al. 2000). We compared the expression of *so-lacZ* with that of *eya*. Eya protein is first detected in the cellular blastoderm at approximately 2 h AED (Fig. 5A–D), while *so-lacZ* expression is not seen until approximately 4 h AED (Fig. 5E. F). The dorsal expression of both genes overlaps considerably (Fig. 5E–L). There is also considerable overlap in their expression patterns within the developing brain lobes (Fig. 5M–R). By the end of embryogenesis *so-lacZ* expression is severely reduced (Fig. 5S, T). Interestingly, the patterns of expression of *so-lacZ* and *eya* within the segmental grooves are not overlapping (Fig. 5N–Q). However, the degree of overlap between the patterns within the embryo and the late second and third eye imaginal discs furthers supports the in vitro biochemical evidence that these genes do interact.

Discussion

The dramatic differences in morphology and optical properties between invertebrate and vertebrate eyes have been used to justify the argument that the eye has a polyphyletic origin. In fact it has been argued that eye evolution has occurred independently at least 40 if not 65 times throughout history (Salvini-Plawen and Mayr 1997). It has been estimated that eyes can evolve in a relatively short period of time, furthering the polyphyletic argument (Nilsson and Pelger 1994). In addition to the morphological and optical differences, the differences in lens components and phototransduction machinery have been used to forcefully argue the polyphyletic origin of the eye (Land and Fernald 1992; Fernald 1997, 2000).

An alternate view of eye evolution came with the dramatic finding that the fruit fly *Drosophila* contained a homolog of the Pax6 gene (*eyeless*) and it was sufficient on its own to direct eye development (Quiring et al. 1994; Halder et al. 1995a). The demonstration that vertebrate Pax6 genes could direct ectopic eye development in flies identified a functional conservation that was unexpected given the tremendous differences in eye morphology (Halder et al. 1995a). Over the last few years, Pax6 genes have been identified throughout the animal kingdom and homologs from several different phyla have been shown to function in flies. These findings have led to the proposal that the eye does in fact have a monophyletic origin (Gehring 1996; Gehring and Ikeo 1999).

A number of additional genes have been identified in *Drosophila* that are essential for the specification of the eye and are capable of inducing ectopic retinal development (Treisman 1999; Heberlein and Treisman 2000). They have a similar ability to induce ectopic eyes and have been also considered master control genes for eye specification. The simplest explanation for the complex genetic, molecular, and biochemical interactions among these genes is that they work in concert within the same cells to direct the fate of the eye. We have previously reported that all seven of these eye specification genes are co-expressed during the second larval instar, a point in time that is surprisingly later than expected considering that the fate of the eye was thought to be determined during embryogenesis (Kumar and Moses 2001). In fact only three eye specification genes (*toy, ey*, and *eyg*) are expressed in the embryonic eye imaginal disc (Quiring et al. 1994; Jones et al 1998; Czerny et al. 1999). If these are the only eye specification genes to be expressed in the eye primordium then it is unlikely that eye specification could occur during embryogenesis in light of the results of Kumar and Moses (2001).

In this report we provide a detailed description of the *ey, so, eya*, and *dac* expression patterns during all time points of embryogenesis. We found that (1) *so, eya*, and *dac* are expressed in several regions that are not fated to become eyes, (2) *so, eya*, and *dac* expression begins prior to *ey* expression, (3) *ey* does not induce the transcription of *so, eya*, or *dac* in the eye imaginal disc during embryogenesis, and (4) the patterns of *so, eya*, and *dac* are dynamic and in most instances non-overlapping.

These results have several implications for our current thinking on how the *Drosophila* eye is specified. It has been shown that the ectopic expression of each of the eye specification genes (with the exception of *so*) is sufficient to induce the formation of ectopic eyes. What prevents the induction of retinal tissue throughout the embryo? Based on these and our previous findings we would like to argue that expression of all eye specification genes are required for eye determination. Within the embryo we do not find a region in which all these factors are present. It is not until the second larval instar that all genes are expressed within the same tissue (Kumar and Moses 2001). Within the embryo, positive or repressive mechanisms must be in place to prevent the eye specification genes from being co-expressed. For example, *ey* is capable of directly inducing the transcription of both *so* and *eya* within the mature eye imaginal disc, but within the eye anlagen these genes are not expressed, although Ey protein is present. The nature of this regulatory mechanism is unknown, but its existence is supported by the finding that the Notch receptor and thus Notch signaling is highly upregulated within the eye imaginal disc at the time at which all eye specification genes are co-expressed. At earlier time points Notch is seen even throughout the eye and antennal primordium (Kumar and Moses 2001).

It is still unclear as to why only three eye specification genes (*toy, ey*, and *eyg*) are expressed within the embryonic eye primordium. Is the expression of these three genes within the eye primodium a priming step for the eventual specification of the eye or is it simply a step that distinguishes one disc from another? Since Ey protein has been shown to directly bind to the *so* and *eya* promoters, there must be an inhibitory signal within the eye disc that prevents the

transcription of these genes from being induced. This repression is first released for *eya* transcription as it is localized to the first instar eye disc. The inhibition upon the remaining genes is released during the second larval instar. Unraveling this mystery will certainly require extensive molecular and biochemical analysis on embryonic and early larval eye discs.

Another lingering question focuses on the fates of the cells that are derived from the initial expression of *so, eya*, and *dac*. All three of these genes are expressed very early; for instance *eya* is expressed in a cluster of cells at the cellular blastoderm time point. Do these cells contribute to the formation of the visual field? Are these three proteins committing cells to adopt an eye imaginal disc fate, an event that will occur much later in embryogenesis? Such questions can only be addressed by precise single cell fate mapping experiments. Only by labeling a single cell and tracing its progeny will we be able to know if the earliest cells that express *so, eya*, and *dac* will later become cells of the eye imaginal disc. How the expression patterns described here correlate with the genetic, molecular, and biochemical interactions of the eye specification genes is an interesting problem that will undoubtedly require the identification of additional instructive and inhibitory signals.

Finally, are the earliest expression patterns of these eye specification genes homologous between vertebrates and invertebrates? This is certainly a much more-difficult question to answer. A decade ago this question would be easily answered with a resounding "no". Now as more molecular and physiological similarities between the visual systems of vertebrates and invertebrates are being discovered the answer to this developmental question may not be as easily or as negatively answered. It would be truly remarkable if a common developmental history underlies the use of identical molecules to create the different types of eyes seen throughout the animal kingdom. The key to such questions may lie in the precise fate mapping of individual cells that express each of the genes responsible for eye specification.

Acknowledgements

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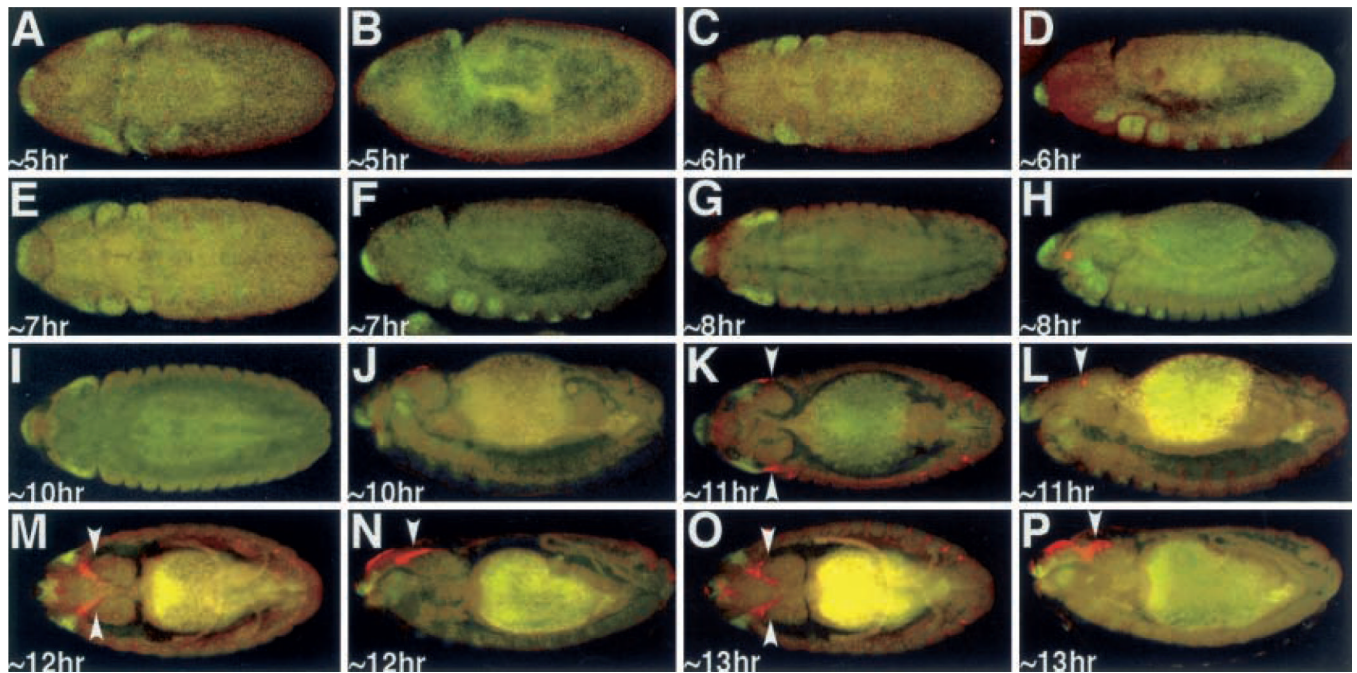
Fig. 1A–P.
Expression of Dll and *ey-lacZ*. **A, C, E, G, I, K, M, O** Dorsal view. **B, D, F, H, J, L, N, P**
Lateral view. *Arrowheads* denote the eye imaginal discs. Age of embryos listed at *bottom
left* of each panel. *Red ey-lacZ, green* Dll. Note the red spot in 1H is a microscope artifact and
does not reflect *ey-lacZ* signal. Note: each panel has a low level of background autofluoresence,
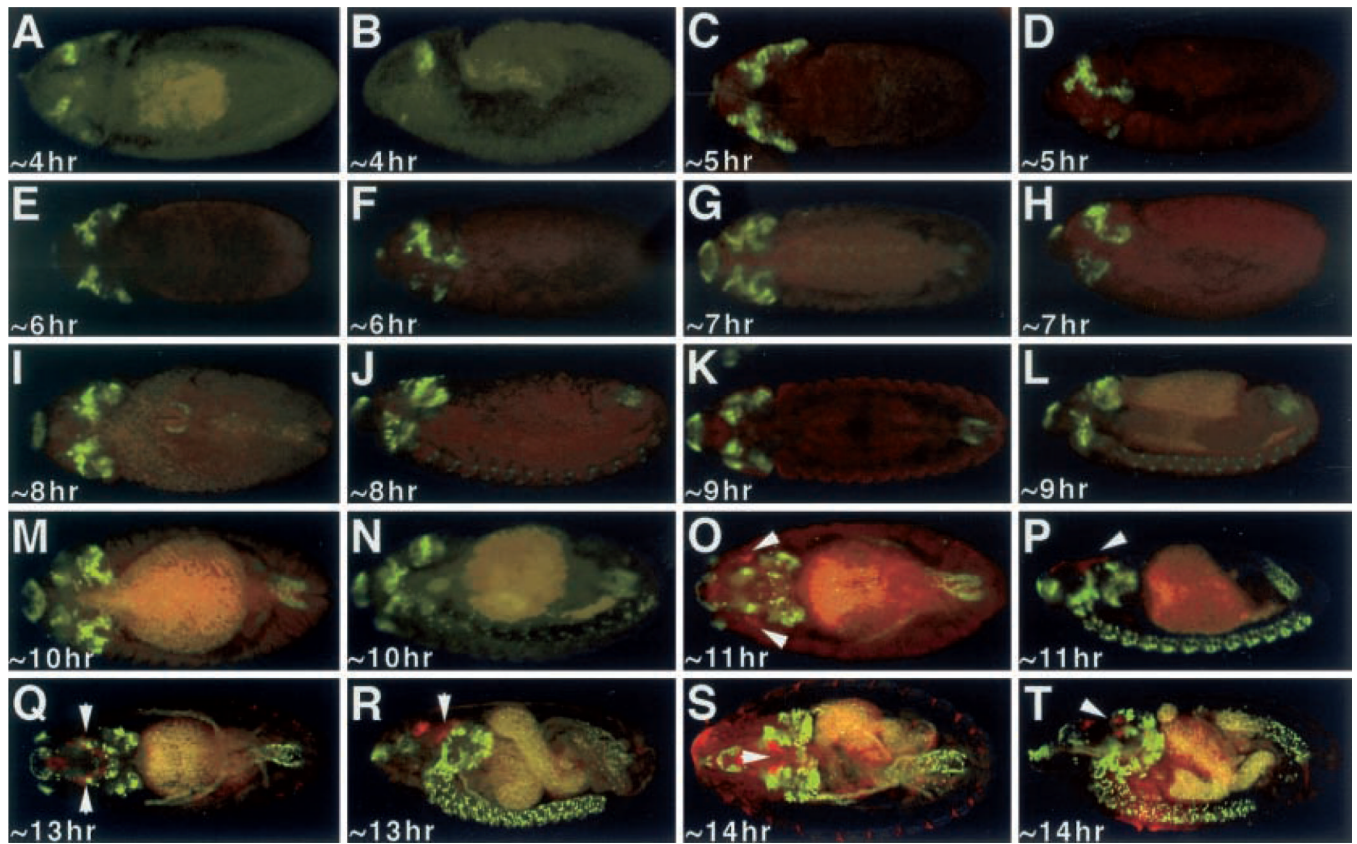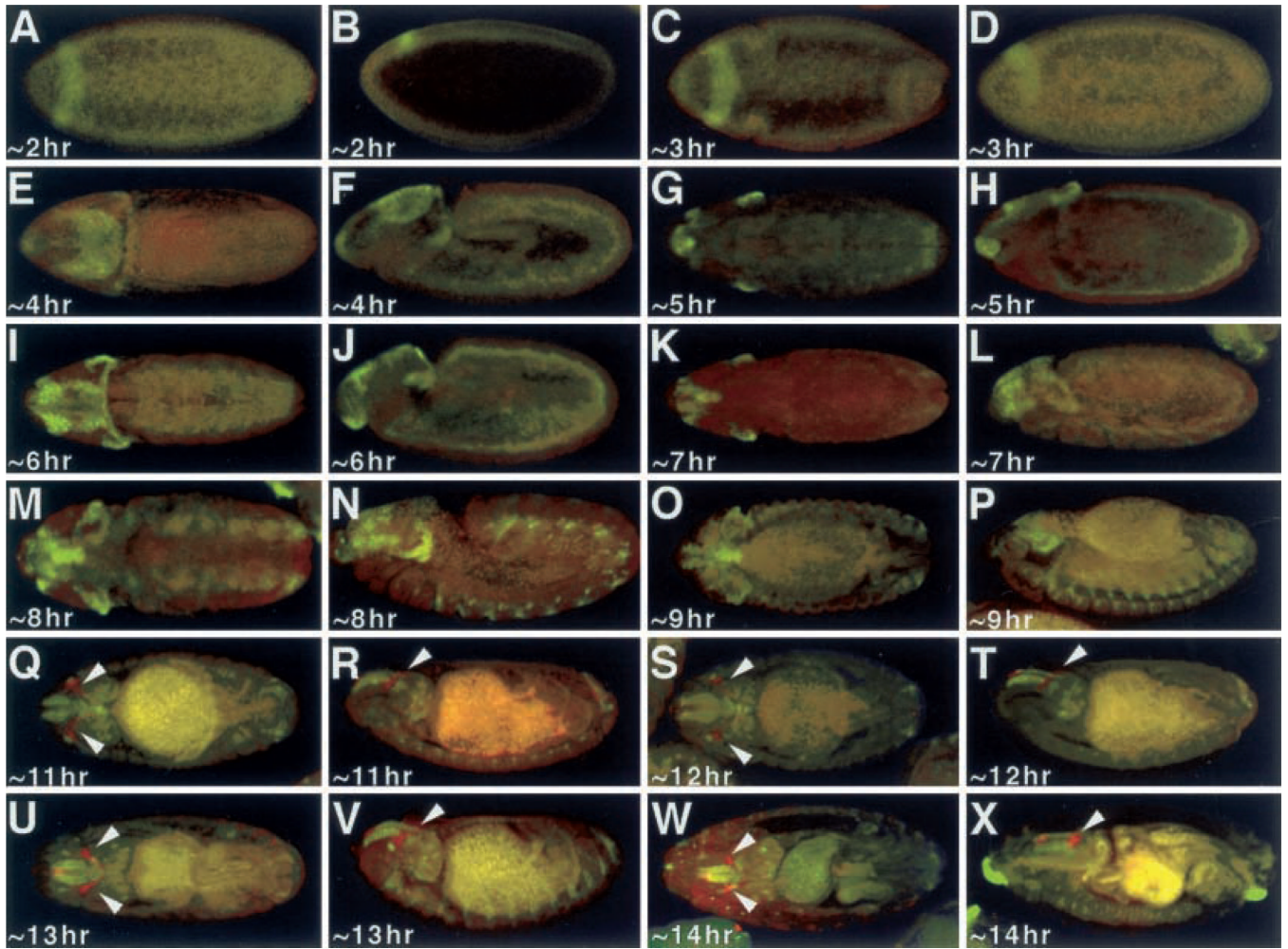while older embryos have high levels of background staining in the presumptive gut region,.

Fig. 2A–T.
Expression of Dac and *ey-lacZ*. **A, C, E, G, I, K, M, O, Q, S** Dorsal view. **B, D, F, H, J, L, N, P, R, T** Lateral view. *Arrowheads* denote the eye imaginal discs. Age of embryos listed at *bottom left* of each panel. *Red ey-lacZ, green* Dac. Note that Dac is not co-expressed with *ey-lacZ* at any time point in development. Note: each panel has a low level of background autofluoresence, while older embryos have high levels of background staining in the presumptive gut region

Fig. 3A–X.
Expression of Eya and *ey-lacZ*. **A, C, E, G, I, K, M, O, Q, S, U, W** Dorsal view. **B, D, F, H, J, L, N, P, R, T, V, X** Lateral view. *Arrowheads* denote the eye imaginal discs. Age of embryos listed at *bottom left* of each panel. *Red ey-lacZ, green* Eya. Note that Eya is not co-expressed with *ey-lacZ* at any time point in development. Note: each panel has a low level of background autofluoresence, while older embryos have high levels of background staining in the presumptive gut region
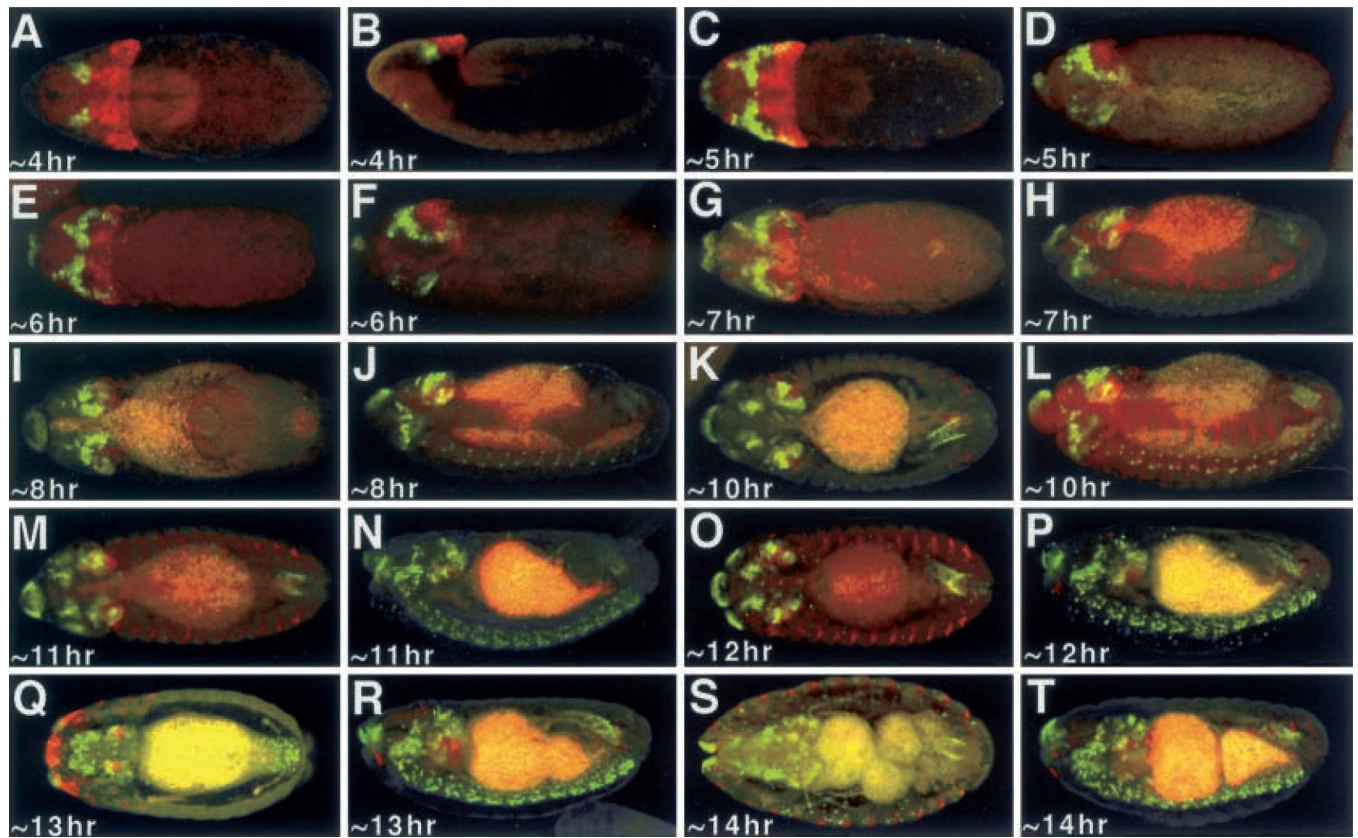
Fig. 4A–T.
Expression of Dac and *so-lacZ*. **A, C, E, G, I, K, M, O, Q, S** Dorsal view. **B, D, F, H, J, L, N, P, R, T** Lateral view. *Arrowheads* denote the eye imaginal discs. Age of embryos listed at bottom left of each panel. *Red so-lacZ, green* Dac. Note that Dac and *so-lacZ* is not found within the embryonic eye imaginal disc. Note: each panel has a low level of background autofluoresence, while older embryos have high levels of background staining in the presumptive gut region
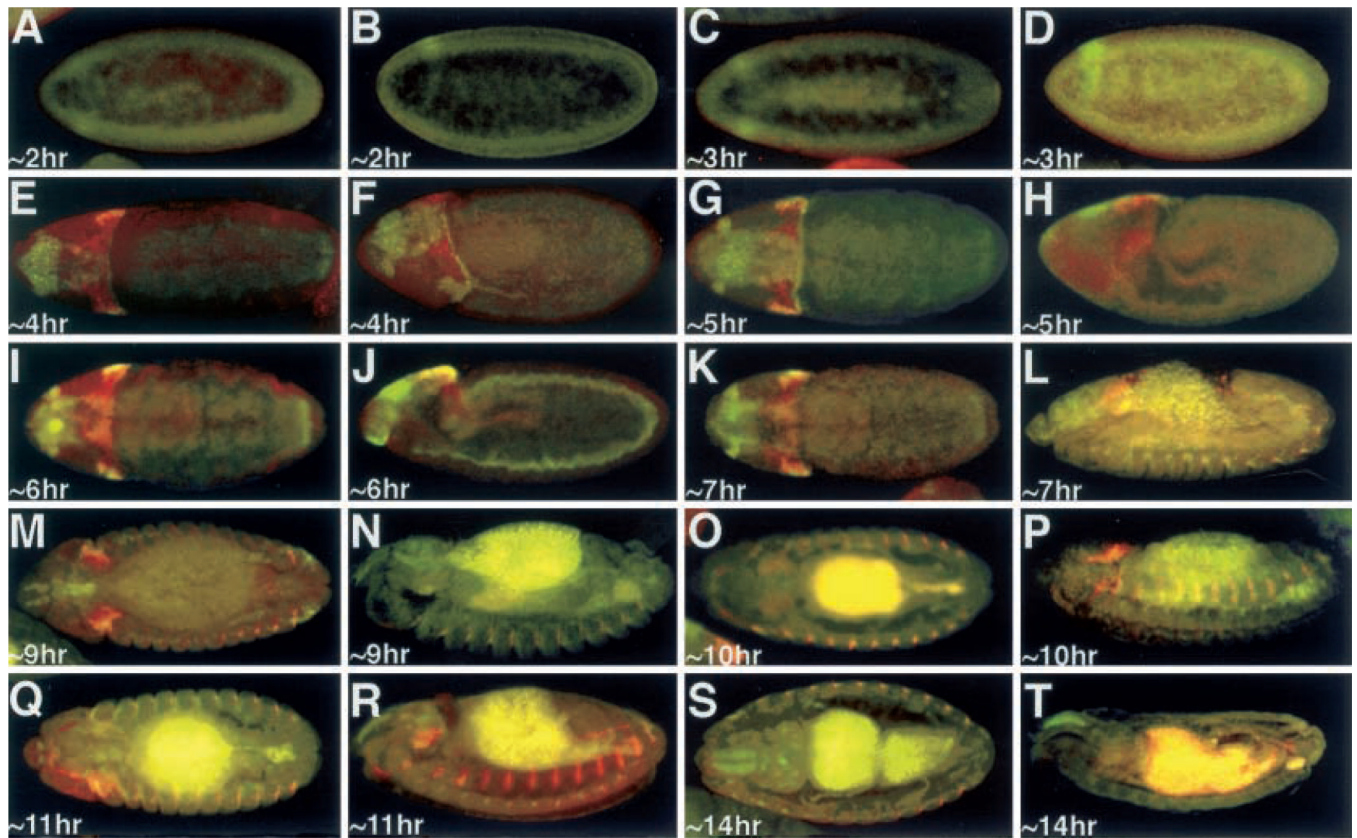
Fig. 5A–T.
Expression of Eya and *so-lacZ*. **A, C, E, G, I, K, M, O, Q, S** Dorsal view. **B, D, F, H, J, L, N, P, R, T** Lateral view. *Arrowheads* denote the eye imaginal discs. Age of embryos listed at *bottom left* of each panel. *Red so-lacZ, green* Eya. Note that Eya and *so-lacZ* is not found within the embryonic eye imaginal disc. Note: each panel has a low level of background autofluoresence, while older embryos have high levels of background staining in the presumptive gut region