Gene Expression Patterns 20 (2016) 120–129

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: http://www.elsevier.com/locate/gep

Regulation of *twin of eyeless* during *Drosophila* development

John Skottheim Honn, Linn Johansson, Åsa Rasmuson Lestander*

Department of Molecular Biology, Umeå University, S-90187 Umeå, Sweden

ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form
7 March 2016
Accepted 7 March 2016
Available online 11 March 2016

Keywords:
Drosophila melanogaster
Eye development
Pax6
twin of eyeless

ABSTRACT

The Pax-6 protein is vital for eye development in all seeing animals, from sea urchins to humans. Either of the Pax6 genes in *Drosophila* (*twin of eyeless* and *eyeless*) can induce a gene cascade leading to formation of entire eyes when expressed ectopically. The *twin of eyeless* (*toy*) gene in *Drosophila* is expressed in the anterior region of the early fly embryo. At later stages it is expressed in the brain, ventral nerve cord and (eventually) the visual primordium that gives rise to the eye-antennal imaginal discs of the larvae. These discs subsequently form the major part of the adult head, including compound eyes. We have searched for genes that are required for normal *toy* expression in the early embryo to elucidate initiating events of eye organogenesis. Candidate genes identified by mutation analyses were subjected to further knock-out and miss-expression tests to investigate their interactions with *toy*. Our results indicate that the head-specific gap gene *empty spiracles* can act as a repressor of Toy, while *ocelliless* (*oc*) and *spalt major* (*salm*) appear to act as positive regulators of *toy* gene expression.

1. Introduction

Paired box domain (Pax) transcription factors are highly conserved and involved in a wide range of developmental pathways (Chi and Epstein, 2002). There are nine classes of Pax proteins in mammals, one of which (Pax6) is involved in formation of eyes, brain, spinal cord and pancreas during embryonic development. A Pax6 gene deletion or loss-of-function mutation results in haploinsufficiency, manifested in an eye malformation syndrome called aniridia. Although very rare, children lacking both copies of the human Pax6 gene suffer from severe craniofacial defects, including absence of eyes and orbits together with multiple defects in the central nervous system (Glaser et al., 1992). Similarly, homozygous Pax6 mutants in mice are born without eyes (Hill et al., 1991). In *Drosophila*, Pax6 is known as the "master control gene for eye development" and can, as the name implies, induce the entire network of gene activities that create eyes (Gehring and Ikeo, 1999). Flies that are homozygous mutants for either of the two Pax6 genes lack eyes and, occasionally, the whole head (Kronhamn et al., 2002; Quiring et al., 1994). Findings that Pax6 apparently has the same basic function in such diverse organisms has prompted revisions of our understanding of eyes evolution (Fernald, 2004, 2006; Gehring, 2002; Gehring and Ikeo, 1999).

Although most organisms carry only one *Pax6* gene, holometabolous insects have two, probably due to a duplication event during insect evolution (Czerny et al., 1999). Since this putative event the two copies appear to have evolved independently and acquired separate functions (Jacobsson et al., 2009; Kronhamn et al., 2002; Punzo et al., 2004). In *Drosophila melanogaster*, the two paralogues are known as *eyeless* (*ey*) and *twin of eyeless* (*toy*) (Czerny et al., 1999; Quiring et al., 1994).

The *toy* gene in *Drosophila* is expressed within 4–6 h of egg laying, in the anterior region of the fly embryo. Its expression subsequently extends both ventrally and towards the posterior, resulting in Toy's presence in the brain and ventral nerve cord of the early embryo. Ey protein is only detectable after Toy expression is clearly manifested. At embryonic stage 16 (Czerny et al., 1999), Toy-expressing cells form a V-shaped pattern, in dorsal view, near the brain. This is the visual primordium that will give rise to the eye-antennal imaginal discs in the larva (Czerny et al., 1999). At about the same time, the second *Pax6* gene, *ey*, is actively expressed in the eye primordia. Thus, Toy acts upstream of Ey in eye formation but the two genes can partially substitute for each other (Czerny et al., 1999; Jacobsson et al., 2009; Kronhamn et al., 2002).

Toy is the first eye specification gene identified to date in the regulatory network that governs eye formation in eye imaginal discs (Czerny et al., 1999). Binding sites for Toy protein have been found in the *ey* and *sine oculis* (*so*) genes. Ey protein, in turn, activates expression of further eye-specification genes such as *so*, *eyes absent* (*eya*) and *optix* (Halder et al., 1998; Ostrin et al., 2006). All

* Corresponding author.
E-mail address: asa.rasmuson-lestander@umu.se (Å. Rasmuson Lestander).

http://dx.doi.org/10.1016/j.gep.2016.03.002
1567-133X/

these genes are crucial for proper development of the visual system and can also promote eye formation through a feedback mechanism that activates *ey* gene expression (Czerny et al., 1999).

The regulatory mechanism of *toy* gene expression is still unclear. Toy is not activated by any known feedback system, which sets it apart from the rest of the eye specification network (Czerny et al., 1999). According to a proposed model, *toy* expression may be initiated by the maternally deposited products of *bicoid*, *torso* and *dorsal* genes (Blanco and Gehring, 2008). However, these gene products are present in a large portion of the anterior embryo, thus additional activators and repressors presumably participate in the limitation and modulation of *toy* activity in the embryo. Likely candidates are early head-specific genes expressed in the anterior end of the early embryo at or just before Toy expression starts, and genes associated with eye-related mutant phenotypes. Therefore, we selected nine of these genes — *empty spiracles* (*ems*), *spalt major* (*salm*), *ocelliless* (*oc*), *buttonhead* (*btd*), *tailless* (*tll*), *Distal-less* (*Dll*), *sloppy paired* (*slp*) and *staroid* (*std*) — then analyzed Toy expression in embryos carrying homozygous mutations in them.

Empty spiracles (*ems*) is a cephalic gap gene that acts as a homeotic selector. The protein it encodes is first detectable at the early cellular blastoderm stage in a single circumferential stripe at the anterior end of the embryo (Dalton et al., 1989; Walldorf and Gehring, 1992; Hartmann et al., 2000). Mutations of *ems* lead to a gap-like phenotype in the anterior head, which includes deletion of cuticular structures and the loss of several cephalic sensory structures (Cohen and Jürgens, 1990; Younossi-Hartenstein et al., 1997; Hartmann et al., 2000).

Spalt major (*salm*) is a homeotic gene encoding a transcription factor. It is also initially expressed in a single circumferential ring in the anterior embryo. Later another ring of expression appears at the posterior end of the embryo and at the same time a second, partial ring appears at the anterior end (Kühnlein et al., 1994). *Salm* is involved in gut development and wing vein organization (Jürgens, 1988; de Celis et al., 1996). In *salm* mutants, elements of the posterior head and the anterior tail are partially transformed into trunk (Jürgens, 1988).

Ocelliless (*oc*), also called *orthodenticle* (*otd*), is a gap gene encoding a transcription factor that is essential for defining the antennal segment, forming both eye and antenna (Royet and Finkelstein, 1996). Oc is expressed in the anterior region of the embryo and *oc* mutants display head defects (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1990; Finkelstein et al., 1990; Royet and Finkelstein, 1995).

Buttonhead (*btd*) is another gap gene required for correct segmentation of the embryonic head (Wimmer et al., 1993). It is expressed in a region of the anterior embryo encompassing the expression of *ems* and *oc*. In *btd* mutants, the antennal, intercalary and mandibular segments are missing and head involution is incomplete (Cohen and Jürgens, 1990; Wimmer et al., 1993).

Tailless (*tll*) is a terminal gap gene necessary for formation of the optic lobe (Strecker et al., 1988; Younossi-Hartenstein et al., 1997; Rudolph et al., 1997). Initially, *tll* is expressed in caps at both poles of the embryo, but the anterior cap is later restricted, resulting in formation of a dorsal stripe (Pignoni et al., 1990).

We found that several of these genes affect the level and/or distribution of Toy expression in the early embryo. We then analyzed their effects in later stages of development, using ectopic expression studies and somatic knock-out clone techniques.

2. Results

2.1. Toy expression in the eye-antennal disc primordia is affected in ems, salm, oc and btd mutant embryos

As already mentioned, during embryonic stage 16 of *Drosophila* development, Toy is expressed in the brain, ventral nerve cord and primordia of the eye-antennal imaginal discs, which form a V-shaped structure, in dorsal view, near the brain (Fig. 1A and A′). We first examined changes to this pattern in our set of homozygous mutant embryos, after staining with an antibody directed against Toy protein (Jacobsson et al., 2009).

In ems^1 mutant embryos the anterior expression of Toy was broadened laterally and extended towards the midline (Fig. 1B and B′). The number of Toy-expressing cells was roughly doubled, from 20 to 40 cells per primordium, indicating that wild-type Ems represses Toy expression. Furthermore, Toy staining patterns in the brain neuromeres indicated that the eye-antennal primordia in ems^1 mutants were slightly more posterior than in wild-type embryos.

Similarly, in $salm^1$ mutant embryos the primordia had a more posterior location than in wild-type embryos (Fig. 1C). In addition, Toy was expressed in the familiar V-shape, but significantly less intensely relative to levels in the brain (Fig. 1C′).

In accordance with previous reports (Blanco et al., 2010), we detected no Toy expression in the eye-antennal primordia of oc^1 mutants (Fig. 1D and D′). In btd^1 mutant embryos, the primordia formed a curved structure, but still retained a strong Toy signal (Fig. 1E and E′), and the number of Toy-expressing cells was not affected. Toy expression patterns in the eye-antennal primordia were also similar to wild-type patterns in tll^{l49} mutant embryos (Fig. 1F and F′), although the anlagen appeared slightly shorter than in wild-type.

Mutations in *Dll*, *slp* and *std* genes did not have any apparent effects on the Toy expression pattern in the eye-antennal primordia (not shown). Moreover, we detected no significant changes in Toy expression in the ventral nerve cord in any of the tested mutant embryos (not shown).

We concluded that four genes (*ems*, *salm*, *oc* and *btd*) encode proteins that might function as Toy regulators and focused on these four in further analyses.

2.2. Ectopic expression of selected genes in imaginal discs of 3rd instar larvae

In an effort to elucidate relationships between the four identified potential regulatory genes and Toy expression we expressed them ectopically using the imaginal disc driver *decapentaplegic-Gal4* (*dpp-Gal4*). This driver is expressed in portions of the leg and eye-antennal discs (Fig. 2A) and in a stripe through the middle of the wing and haltere discs (Figure S1A). Expression of the *UAS-toy* construct via this driver resulted in a wedge of ectopic Toy in the antennal disc, but this did not affect the adult head structures (Fig. 2B and B′). However, in discs forming the thorax, results were more dramatic and ectopic eyes were formed on all legs and wings (Figure S1B).

When *ems* was ectopically expressed using the *dpp-Gal4* driver we observed no ectopic Toy expression in the antennal disc (Fig. 2C). In the dorsal and ventral margins of the eye disc, progression of the morphogenetic furrow appeared to be blocked, resulting in a reduction of the eye field. No effects on the legs and wings were observed (Figure S1C and C′).

Similarly, ectopic expression of *oc* did not activate ectopic Toy expression in the antennal disc (Fig. 2D), or in either wing or leg discs (Figure S1D). Further, it had no apparent effects on adult head