Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 315 (2008) 535–551
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Differential requirements for the Pax6(5a) genes *eyegone* and *twin of eyegone* during eye development in Drosophila

Jih-Guang Yao [a,b,1,2], Bonnie M. Weasner [c,2], Lan-Hsin Wang [a,d], Chuen-Chuen Jang [a], Brandon Weasner [c], Chiou-Yang Tang [a], Claire L. Salzer [c], Chun-Hong Chen [e], Bruce Hay [e], Y. Henry Sun [a,b,d,*], Justin P. Kumar [c,*]

[a] *Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, ROC*
[b] *Institute of Genomic Sciences, National Yang-Ming University, Taipei 112, Taiwan, ROC*
[c] *Department of Biology, Indiana University, Bloomington, IN 47405, USA*
[d] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, ROC*
[e] *Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

Received for publication 29 August 2007; revised 29 November 2007; accepted 11 December 2007
Available online 4 January 2008

Abstract

In eye development the tasks of tissue specification and cell proliferation are regulated, in part, by the Pax6 and Pax6(5a) proteins respectively. In vertebrates, Pax6(5a) is generated as an alternately spliced isoform of Pax6. This stands in contrast to the fruit fly, *Drosophila melanogaster*, which has two Pax6(5a) homologs that are encoded by the *eyegone* and *twin of eyegone* genes. In this report we set out to determine the respective contributions that each gene makes to the development of the fly retina. Here we demonstrate that both *eyg* and *toe* encode transcriptional repressors, are expressed in identical patterns but at significantly different levels. We further show, through a molecular dissection of both proteins, that Eyg makes differential use of several domains when compared to Toe and that the number of repressor domains also differs between the two Pax6(5a) homologs. We predict that these results will have implications for elucidating the functional differences between closely related members of other Pax subclasses.

Keywords: Pax6(5a); Eyegone; Twin of eyegone; Retina; Eye; Drosophila

Introduction

Pax6 genes play an indispensable role in the development of wide range of retinal systems. Mutations within Pax6 orthologs lead to severe retinal abnormalities in humans, mice and flies (Ton et al., 1991; Hill et al., 1991; Quiring et al., 1994). In contrast forced expression of Pax6 is sufficient to rewrite the developmental program of non-retinal tissues thereby producing an ectopically situated eye (Halder et al., 1995a). Furthermore, the universal presence of Pax6 in all seeing animals examined so far has underscored its importance in eye development and sparked a rethinking of the evolutionary origins of the eye (Halder et al., 1995b; Gehring, 2002, 2005). As a consequence Pax6 has turned into one of the best-studied members of the paired box (Pax) family of transcription factors (Gehring, 1996; Gehring and Ikeo, 1999; Pichaud and Desplan, 2002).

Pax6, like all other Pax proteins, contains a PAIRED DNA binding domain (PD) which itself is comprised of two functionally separable helix–turn–helix motifs, the PAI and the RED domains (Noll, 1993; Jun and Desplan, 1996). In addition Pax6 contains a third nucleic acid recognition motif, the homeodomain (HD). The composition and structure of Pax6 provides for considerable flexibility in its interactions with

* Corresponding authors. Y.H. Sun is to be contacted at Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan, ROC.
E-mail addresses: mbyhsun@gate.sinica.edu.tw (Y.H. Sun), jkumar@indiana.edu (J.P. Kumar).
[1] Present address: Howard Hughes Medical Institute and Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.
[2] These authors contributed equally to this work.

0012-1606/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.12.037

nucleic acids thereby allowing for the combinatorial use of three functionally independent DNA recognition domains. While vertebrates have only a single Pax6 gene, the fruit fly, *Drosophila melanogaster*, contains two Pax6 orthologs *eyeless (ey)* and *twin of eyeless (toy)*. Both play central roles in the specification of the retina (Quiring et al., 1994; Halder et al., 1995a,b; Czerny et al., 1999).

Alternate splicing of vertebrate Pax6 leads to the production of a second isoform, Pax6(5a). This isoform (1) lacks a functional PAI domain; (2) binds to DNA through its RED and HD; and (3) has a different PD binding specificity than canonical Pax6 (Walther et al., 1991; Jaworski et al., 1997). In vertebrates Pax6 and Pax6(5a) appear to play different roles in retinal development. Pax6(5a) loss-of-function mutants have different phenotypes than those of Pax6. Likewise, overexpression of Pax6(5a) induces different developmental defects and patterns of gene expression than Pax6 (Duncan et al., 2000a,b; Chauhan et al., 2002a,b,c; Singh et al., 2002; Haubst et al., 2004).

Pax6(5a) is also found in *Drosophila* but, unlike vertebrates, does not result from alternate splicing of Pax6 but rather is encoded by two separate genes, *eyegone (eyg)* and *twin of eyegone (toe)*. These genes arose from a relatively recent duplication event and together with vertebrate Pax6(5a) represent a novel subclass of Pax genes (Jun et al., 1998; Aldaz et al., 2003). Similar to vertebrates, *Drosophila* Pax6 and Pax6(5a) appear to play different roles in eye development. While *ey* and *toy* act primarily in retinal specification, the main function of *eyg* is to promote cell proliferation (Dominguez et al., 2004; Chao et al., 2004). Each isoform exerts its influence on development through different transcriptional mechanisms: Ey acts as an activator while Eyg has the unique property of acting as a dedicated repressor (Punzo et al., 2001, 2004; Yao and Sun, 2005).

The Pax6 genes in *Drosophila* do not play completely redundant roles in development. There are some differences in the expression patterns of the two genes (Quiring et al., 1994; Czerny et al., 1999; Kammermeier et al., 2001). As a result *ey* and *toy* loss and gain-of-function mutants have some significant phenotypic differences (Kammermeier et al., 2001). Even within the eye specification hierarchy *toy* appears to sit atop *ey* (Czerny et al., 1999; Kronhamn et al., 2002). Interestingly, there are also disparities between the abilities of the two genes to direct eye formation in non-retinal tissues (Halder et al., 1995a; Czerny et al., 1999; C. Salzer and J. Kumar unpublished data). Differences in the activities of individual DNA recognition domains and protein–protein interaction motifs account for these many distinctions (B.M. Weasner and J. Kumar unpublished data).

Since the *Drosophila* genome encodes two Pax6(5a) genes we set out to determine if there are disparities between the roles that *eyg* and *toe* play in eye development. We will show that *eyg* and *toe* are expressed in identical patterns in the eye but *eyg* mRNAs account for the vast majority of Pax6(5a) transcripts. A comparison of the effects that loss of each gene has on eye development demonstrates that *eyg* and *toe* are differentially required in the retina. We have gone on to show that while Toe, like Eyg, is a transcriptional repressor, the number of repressor domains is different. And finally, we demonstrate that each Pax(5a) protein makes use of a unique combination of domains during normal eye development and extra eye field induction. Together, these results suggest that although *eyg* and *toe* arose through a recent duplication event, the two Pax6(5a) proteins likely play non-redundant roles in the eye and exert their influence on retinal development through differential use of combinations of protein domains.

Materials and methods

Fly stocks, reagents and microscopy

The following stocks were used in this study: $eyg^{[1]}$, $eyg^{[22\text{-}2]}$, $eyg^{[M3\text{-}12]}$, $wg^{[W11]}$-GAL4, eyg-$GAL4^{22\text{-}2}$, tub-GAL4, ey-GAL4, GMR-GAL4, dpp-GAL4, CD-Gal4, UAS-ey, UAS-toy, UAS-so, UAS-optix, UAS-eya, UAS-GFP, wg-lacZ and an additional 220 GAL4 lines from the Bloomington *Drosophila* Stock Center (details of these stocks are available upon request). The eyg-$Gal4^{22\text{-}2}$ (also referred to as EM458) carries a P[GawB] insertion 527 bp upstream of the eyg transcript. It is homozygous viable and has no visible phenotype on its own (Jang et al., 2003). CD-Gal4 drives expression mimicking *eyg* expression (LHW and YHS, unpublished results). The following antibodies were used in this study: rat anti-ELAV, mouse anti-Eyg (gift of Ntalia Azpiazu), IgG conjugated Cy3. Adult eyes were prepared for scanning and light microscopy as essentially described in Kumar et al., 1998. Developing imaginal discs, salivary glands and embryos were prepared for light and confocal microscopy as essentially described in Yao and Sun, 2005 and Jang et al., 2003.

Generation of eyg and toe protein variants

Schematic drawings of Eyg deletion, Toe deletion and Eyg/Toe chimeric proteins are diagramed in Fig. 9. An alignment of the Eyg and Toe proteins, along with a demarcation of the individual domains, is provided within the Supplemental Data Section Fig. S1). Our full-length Eyg protein is 525 amino acids in length and represents the shortest functional isoform. Each protein domain was originally defined by Jun and Desplan, 1996. The N-terminal (NT) region consists of residues 1–13, the PD domain consists of residues 14–104, the B region contains residues 105–231, the HD domain contains residues 232–291 and the C-terminal (CT) region contains residues 292–525. The N-terminal deletion (Eyg ΔNT) contains amino acids 14–525, the paired domain deletion construct (Eyg ΔNT+PD) contains amino acids 105–525, the B domain deletion construct (Eyg ΔB) contains amino acids 1–104 fused to residues 232–525, the homeodomain deletion construct (Eyg ΔHD) contains amino acids 1–231 fused to 292–525 and the C-terminal deletion construct (Eyg ΔCT) contains amino acids 1–291. In addition we made two multiple domain deletion constructs. The combined N and C terminal deletion constructs (Eyg ΔNT+CT) contains amino acids 14–291 and the triple N terminal, B domain and C-terminal deletion construct (Eyg ΔNT+B+CT) contains amino acids 14–104 fused to residues 232–291.

Our full-length TOE protein is 640 amino acids in length. The N-terminal region consists of residues 1–142, the PD domain consists of residues 143–233, the B region consists of 234–383, the HD domain consists of residues 384–443 and the C-terminal region consists of residues 444–640. The N-terminal deletion (Toe ΔNT) contains amino acids 143–640, the paired domain deletion construct (Toe ΔNT+PD) contains amino acids 234–640, the B domain deletion construct (Toe ΔB) contains amino acids 1–233 fused to residues 384–640, the homeodomain deletion construct (Toe ΔHD) contains amino acids 1–383 fused to 444–640 and the C-terminal deletion construct (Toe ΔCT) contains amino acids 1–443. In addition we made two multiple domain deletion constructs. The combined N and C terminal deletion constructs (Toe ΔNT+CT) contains amino acids 143–443 and the triple N terminal, B domain and C-terminal deletion construct (Toe ΔNT+B+CT) contains amino acids 143–233 fused to residues 384–443.

We made a series of chimeric proteins in which single or multiple domains of Eyg were replaced with the corresponding domains of Toe. The Eyg/Toe NT chimera contains amino acids 1–142 of TOE fused to residues 14–525 of Eyg,