

# A transcriptional chain linking eye specification to terminal determination of cone cells in the *Drosophila* eye

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## Abstract

Lozenge (Lz) is a multifunctional transcription factor that is activated in a pool of pluripotent cells at the beginning of a wave of morphogenesis during *Drosophila* eye development. Lozenge belongs to the Runx class of transcription factors that includes the mammalian proteins AML1, Runx 2, and Runx 3. These proteins allow a tissue-specific precursor population of cells to attain multiple terminally differentiated fates. We investigated the transcriptional control of *lz* to determine the mechanism by which precursor populations achieve their identity. We have identified a 251-bp region in the second intron of the *lz* gene that functions as a minimal eye-specific enhancer. We provide evidence that Sine oculis and Glass are the two major activators of Lz expression during eye development. This work establishes a bridge between early eye specification genes and late cell-specific transcription factors required for terminal determination of cone cells in the eye. © 2003 Elsevier Inc. All rights reserved.

**Keywords:** Lozenge; Sine oculis; Glass; Cell fate; Eye development

## Introduction

The generation of cellular diversity in a developing organism is a multi-tiered process that involves many crucial steps where each event is dependent on the success of the preceding step. Ultimately, this process generates cell-specific patterns of gene expression that define unique cell types and functions. Transcriptional regulation is a key component of this process; transcriptional regulators expressed in pluripotent and undifferentiated precursor cells cause cell-specific expression of other transcription factors that uniquely define cell types. For example, the mammalian Runx protein AML1 (Acute Myeloid Leukemia 1) is expressed in a pool of hematopoietic precursor cells and regulates the expression of many other cell-specific factors (reviewed by Cohen, 2001). A salient feature of Runx protein function is that they control both the initial step of commitment to cell fate choice, as well as later developmental events, such as AML1's regulation of T-cell and

B-cell antigen receptors, GM-CSF, and IL-3 (Lutterbach and Hiebert, 2000). In the absence of proper AML1 function, all definitive hematopoiesis is blocked and proper regulation of downstream cell-specific factors is lost (Okuda et al., 1996; Wang et al., 1996). The *Drosophila* Runx protein Lozenge (Lz) is similarly expressed in the developing hematopoietic system (Lebestky et al., 2000) as well as in undifferentiated precursor cells in the developing visual system (Flores et al., 1998). Through both positive and negative transcriptional regulation, Lz controls the expression of several other cell-specific factors that specify various unique cell fates (Daga et al., 1996). There have been many studies elucidating the mechanisms by which widely expressed proteins such as Lz and AML1 control the expression of cell-specific proteins (Siddall et al., 2003; Canon and Banerjee, 2003; Behan et al., 2002; Flores et al., 2000; Lutterbach and Hiebert, 2000). However, less is known about the mechanisms that control the expression of these proteins in the precursor cell population in which they function. Understanding the mechanisms by which these transcriptional regulators are controlled is of clear importance since they occupy some of the highest ranks in the

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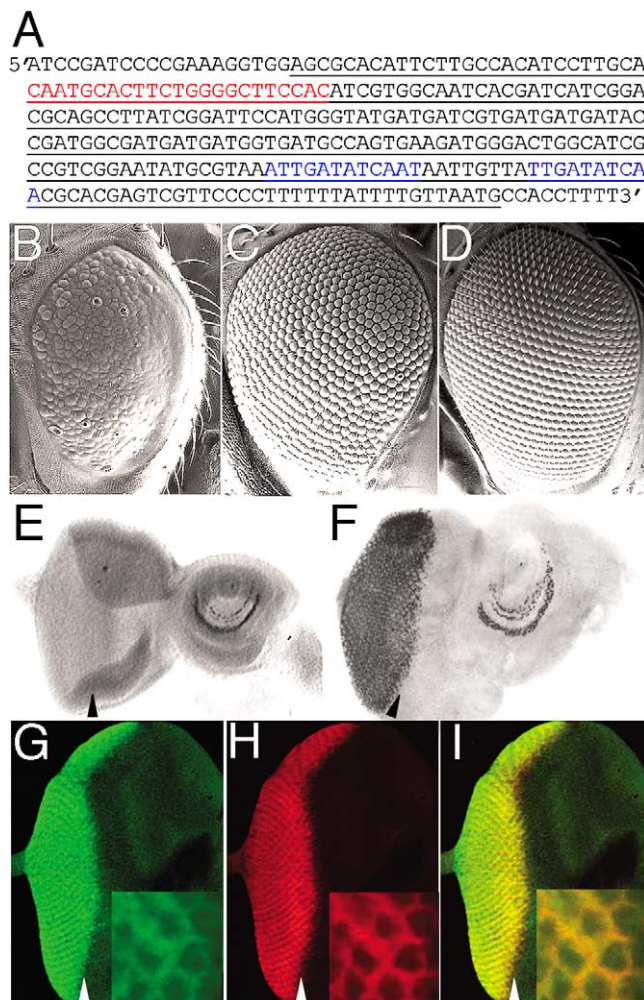


Fig. 1. Identification of the *lz* minimal eye-specific enhancer (LMEE). (A) Sequence of a 280-bp region from the *lz* 2nd intron. The smaller 251-bp LMEE is underlined. Potential Glass binding site (red) and the palindromic sites (blue) are indicated. (B–D) Scanning electron micrographs of adult eyes. (B) *lz*<sup>77a7</sup>. (C) *lz*<sup>77a7</sup>; *P*[280-*lz*, *w*<sup>+</sup>]. (D) Wild-type control. The 280-bp fragment is able to rescue the *lz*<sup>77a7</sup> mutant eye to nearly wild type. In the transformant line shown in (C), the rescue is nearly complete. In other lines, the anterior 2/3 of the eye is completely wild type, while the posterior 1/3 remains partially rescued. (E, F) Expression pattern of Lz in third instar larval eye discs, posterior to the morphogenetic furrow, as revealed by immunostaining with an  $\alpha$ -Lz antibody. (E) *lz*<sup>77a7</sup>. In this eye-specific allele, Lz is not expressed in the eye disc. The rings of expression in the antenna remain unchanged. (F) *lz*<sup>77a7</sup>; *P*[280-*lz*, *w*<sup>+</sup>]. The 280-bp fragment is able to rescue Lz expression. Posterior is to the left for eye discs in all figures and an arrowhead (black or white) indicates position of the morphogenetic furrow. (G–I) LMEE-*lacZ* reproduces the expression pattern of endogenous Lz in third instar larval eye discs. *w*; *P*[LMEE-*lacZ*, *w*<sup>+</sup>] eye disc is labeled with  $\alpha$ -Lz (G, green) to identify cells expressing Lz protein and  $\alpha$ - $\beta$ -galactosidase (H, red) to identify cells expressing the transgene under the control of the minimal enhancer. (I) Merge of (G) and (H), shows a complete overlap of the protein and reporter gene expression pattern. Inserts show higher magnification views.

hierarchy of factors affecting major developmental pathways. To better understand the control of such widely expressed regulators involved in cell fate determination, we have investigated the transcriptional control of *lz* in the *Drosophila* eye.

In early *Drosophila* development, a set of interacting master-control genes (reviewed by Pappu and Mardon, 2002) and signaling pathways (Kumar and Moses, 2001) set up the identity of the eye field. During larval stages, the eye primordium continues to grow as an epithelium called the eye imaginal disc. Pattern formation in the eye is initiated when a physical groove called the morphogenetic furrow proceeds across the eye disc, marking the onset of cell differentiation (Ready et al., 1976; Wolff and Ready, 1991). There are two pools of undifferentiated precursor cells in the eye, one that is anterior to the furrow and one that is formed in the wake of the passing furrow. Although these two sets of cells are both undifferentiated precursors, they are no longer equivalent as they express different transcription factors (reviewed by Nagaraj et al., 2002). For example, the eye specification gene *eyeless* is expressed in all precursor cells anterior to the furrow (Halder et al., 1995; Hauck et al., 1999; Quiring et al., 1994). Conversely, Lz is expressed in precursor cells posterior to the furrow and make these cells competent to respond to subsequent developmental signals (Flores et al., 1998). In addition to the expression of Lz, cell fate specification requires the reiterative and combinatorial use of the Notch and EGFR signaling pathways. The resulting cell types can be neuronal (photoreceptors, called R cells) or nonneuronal (cone and pigment cells). Many events downstream of Lz have been previously described, including the direct transcriptional activation of *D-Pax2* during cone cell specification by the combined action of Lz and effectors of the Notch and EGFR pathways (Flores et al., 2000). Moreover, Lz regulates all of the known transcription factors that are required to specify every cell type arising from the pool of precursor cells in which Lz is expressed. In addition to *D-Pax2*, Lz is required for the expression of the homeodomain protein Bar in R1 and R6 neurons (Crew et al., 1997; Daga et al., 1996) and the homeodomain protein Prospero in R7 and the cone cells (Xu et al., 2000). Lz also directly represses the transcription of the neural specific gene *deadpan* in cone cells (Canon and Banerjee, 2003). However, how Lz itself is regulated within the precursor cell population to specify diverse cell fates was unknown. Through genetic and biochemical analyses, we have identified the mechanism by which Lz expression is activated. In elucidating the control of Lz expression, we are also able to make a connection between the earliest events in eye development, the specification of the eye field, to a very late event, the terminal determination of cone cell fate.

## Materials and methods

### DNA constructs for fly germ line transformation

The transformation constructs for *lz* cDNA expression were made as follows: *EcoRI* fragment of *lz* cDNA was first

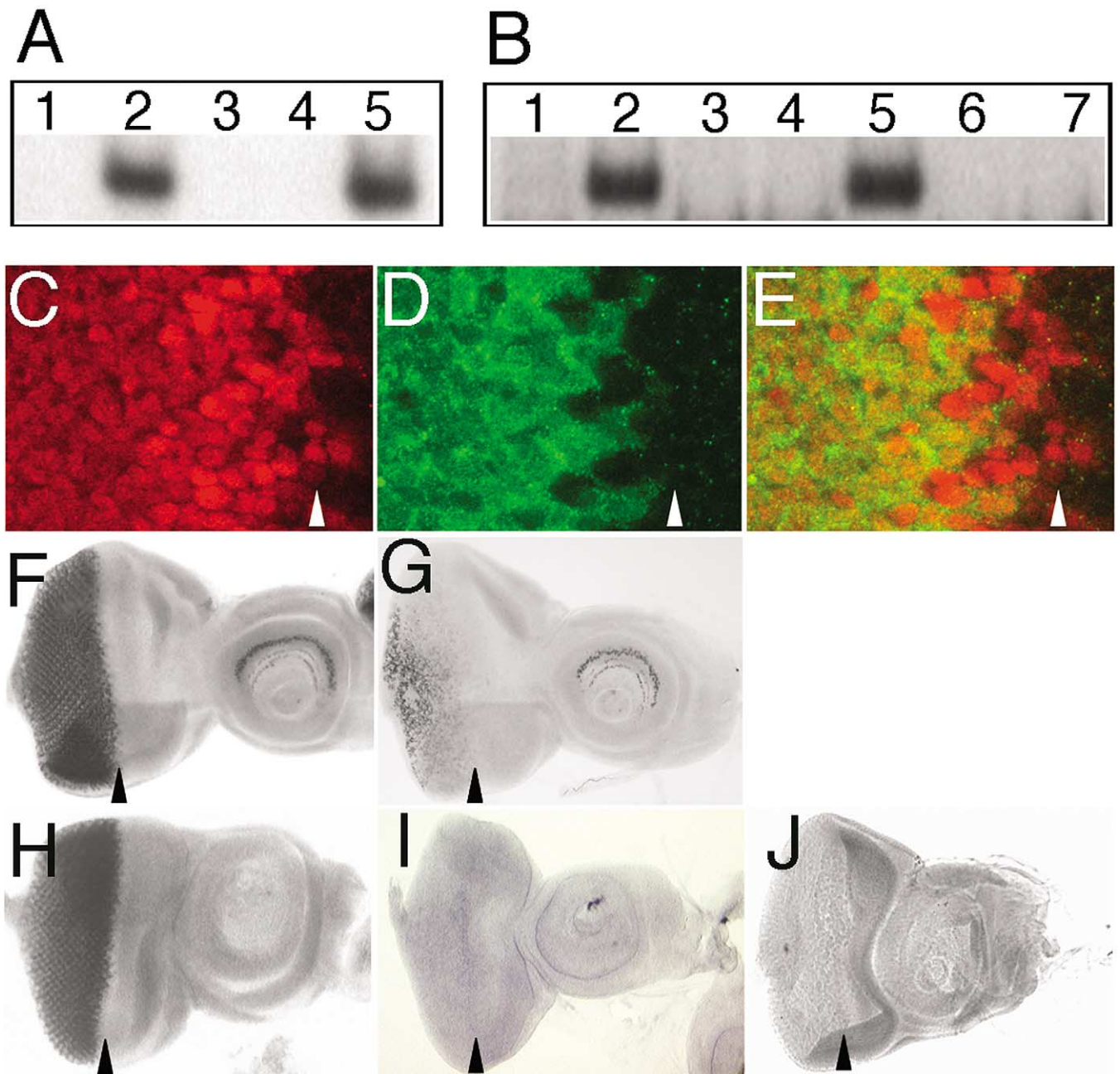


Fig. 2. Glass directly controls *Lz* expression in the eye discs. (A) EMSA analysis. Glass DNA binding domain specifically binds to the putative Glass site oligonucleotide *lzG1* (see Materials and methods). Lane 1, free probe. Lane 2, no competitor, *Gl*/DNA complex shown as a strong gel-shifted band. Lane 3, Cold competitor oligonucleotide *lzG1* competes for binding. Lane 4, *RhlG1*, an oligo corresponding to the Glass binding site in the *Rhl* promoter, competes for binding. Lane 5, cold *mlzG1*, mutated at the putative binding site, does not compete for Glass binding. (B) EMSA analysis. In these experiments, the probe used is either the LMEE (lanes 1–5) or the mutant LMEE mutated for the putative Glass binding site (lanes 6–7). The protein added is the *Gl* DNA binding domain. Lane 1, free probe. Lane 2, no competitor, *Gl*/LMEE complex shown as a strong gel-shifted band. The cold competitor oligos *lzG1* (lane 3) and *RhlG1* (lane 4) efficiently compete for Glass binding, but *mlzG1* (lane 5) fails to do so. Lane 6, free probe. Lane 7, Glass protein added; no competitor added. Mutant LMEE probe fails to form *Gl*/LMEE complex. (C–E) Expression pattern of Glass (C, red), revealed by immunostaining with  $\alpha$ -Glass and  $\alpha$ - $\beta$ -galactosidase (D, green) in LMEE-*lacZ* transgenic eye disc. (E) Merge of (C) and (D). Glass expression (red) is initiated earlier than (anterior to) that of *Lz* (monitored by  $\alpha$ - $\beta$ -galactosidase). Posterior is to the left. (F, G) *Lz* expression pattern in wild-type (F) and *gl<sup>60j</sup>/gl<sup>60j</sup>* (G) eye discs. Expression of *Lz* is grossly reduced in *gl* mutant background. (H–J)  $\beta$ -galactosidase expression pattern in *w*; *P*[LMEE-*lacZ*, *w*<sup>+</sup>] (H), *w*; *P*[LMEE-*lacZ*, *w*<sup>+</sup>]; *gl<sup>60j</sup>/gl<sup>60j</sup>* (I) and *w*; *P*[LMEE<sup>mut</sup>-*lacZ*, *w*<sup>+</sup>] (J) eye discs. LMEE fails to activate reporter expression in *gl* null background (I) or when the *Gl* binding site is mutated in the enhancer (J).

cloned into pCasper-*hs* vector to obtain pCasper-*lz*. Various deletion fragments were generated by PCR with *SacII* and *XbaI* restriction sites added at the 5' and 3' ends, respec-

tively. Construct 280-*lz* was created by cloning the fragment from nucleotide 381 to 660 (the number 1 represents the first nucleotide at the 5' end of *lz* 2<sup>nd</sup> intron, the sequence of

the intron begins with 5'-GTGAGTTGTT-3') as above. All *lacZ* constructs were generated by subcloning appropriate PCR fragments into *PwHZ128* (gift from A. Courey) at *XhoI* and *NotI* sites upstream of *hsp70* promoter and the bacterial *lacZ* gene. Site-directed mutagenesis was performed by overlapping extension PCR strategy. The mutations introduced into the LMEE<sup>mGl</sup> and LMEE<sup>2xmSo</sup> are the same as in oligonucleotide *mlzGl* and *mlzSo*, respectively (see below).

#### *Fly stocks and genetics*

The fly strains used include: *Oregon R* (wild-type), *lz*<sup>77a7</sup> (Flores et al., 1998), *gl*<sup>60j</sup> (Bloomington Stocks Center), *hs-flp; FRT42D arm-lacZ* (Pignoni et al., 1997), *FRT42D so*<sup>3</sup>/*CyO* (Pignoni et al., 1997). Clones of homozygous *so*<sup>3</sup> mutant cells were generated by the expression of FLP recombinase under the control of *hsp70* promoter. A 1.5-h heat shock (37°C) was applied in the early 2nd instar to generate small clones in the eye.

#### *Protein preparation and electrophoretic mobility shift assay (EMSA)*

A PCR fragment covering the Glass DNA binding domain (O'Neill et al., 1995) was generated and subcloned in frame into pGEX6p-1 at *BamHI/EcoRI* sites to make construct pGEX6p-Gl for protein expression in *E. coli DHα5*, using primers *gl432Bam* (5'-AATGTAGGATCCAGTGGCGGCGAGATGAAACC-3') and *gl605RI* (5'-AATGTAGAATTCTCATGTGAGCAGGCTGTTGC-3'). The construct for Sine oculis expression (pGEX6p-So) was generated as follows: A PCR fragment of part of *so* cDNA was generated with primers *so-Nde* (5'-CTGGACCATATGTACAGCATCCCGCCAC-3') and *so-Xho* (5'-TTCTGGGCCGAGAAGTGGTG-3'). The PCR fragment digested with *NdeI/XhoI* and an *XhoI/BamHI* fragment from *so* cDNA were subcloned into pGEX6p-Nde at *NdeI* and *BamHI* sites by triple ligation. pGEX6p-Nde was modified from pGEX6p-1 by inserting an *NdeI* site in the multiple cloning sites. Protein expression and purification were performed as described previously (Flores et al., 2000). Procedures for EMSA and competition assays are as described (Flores et al., 2000). The oligonucleotides used as probes and cold competitors include; *lzGl* (with the putative Gl binding site, 5'-TTGCACAATGCACTTCTGGGGCTTCCACATCGT-3'), *mlzGl* (with mutated Gl site, 5'-TTGCACAAATGCTGAGCTACCGGAGTCAATCGT-3', mutated nucleotides are in bold), *RhlGl* (with an established Gl site from the *Rhl* promoter) (Moses and Rubin, 1991), *lzSo* (with the two palindromes, 5'-CGTAAATTGATATCAATAATTGTTATGATATCAACGCAC-3') and *mlzSo* (with mutated So sites, 5'-CGTAAATCCCCCAATAATTGTTATCCCCCAACGCAC-3', mutated nucleotides are in bold). Footprinting assays were performed with the Core Footprinting System following the manufacturer's instructions (Promega). The

probe used is the fragment from nucleotide 301–760 from *lz* 2nd intron.

#### *Immunohistochemistry*

Primary antibodies used include: mouse α-Lz (1:5, anti-lozenge, Developmental Studies Hybridoma Bank, University of Iowa), mouse α-β-galactosidase (1:150, Promega), mouse α-Gl (Ellis et al., 1993), rabbit α-β-galactosidase (1:500). Secondary antibodies: HRP-α-mouse, CY3-α-rabbit, FITC-α-mouse, CY3-α-mouse, and FITC-α-rabbit (1:100, Jackson Labs). Staining of third instar larval eye discs was done as described (Flores et al., 1998).

## Results and discussion

#### *Identification of the eye-specific minimal enhancer of lz*

To understand the control of Lz expression, we analyzed the eye-specific enhancer of *lz*, which had been previously determined to lie within the second intron of the *lz* gene (Batterham et al., 1996; Flores et al., 1998). The entire second intron, combined with *lz* upstream promoter sequences and the *lz* cDNA, is sufficient to impart proper Lz expression in the undifferentiated precursors within the eye field and rescue the *lz* mutant eye phenotype (Flores et al., 1998). In order to identify the transcription factors that direct eye-specific expression of *lz*, we undertook a deletion analysis of the second intron to narrow down the minimal enhancer sufficient to support Lz expression in vivo. We identified a 280-bp fragment (Fig. 1A) that can both rescue the *lz*<sup>77a7</sup> mutant eye phenotype (Fig. 1B–D) and restore Lz expression (Fig. 1E and F) in the developing eye disc. Further deletion analysis was carried out in the context of transgenic reporter lines in which enhancer fragments were ligated upstream of a heterologous basal *hsp70* promoter and *lac-Z*. In this way, a slightly smaller fragment (251 bp; underlined in Fig. 1A) was identified that was able to recapitulate the pattern of endogenous Lz expression in the eye (Fig. 1G–I). We refer to this DNA fragment as the *lz* minimal eye enhancer (LMEE). ETS binding sites outside the minimal enhancer identified by Behan et al. (2002) apparently do not contribute to expression of *lozenge*.

#### *Glass directly controls Lz expression*

Within the LMEE is a 23-bp sequence (red in Fig. 1A), which shares high homology (17/23 nucleotides identical) with an established Glass (Gl) binding site within the *Drosophila* rhodopsin *Rhl* promoter (Moses and Rubin, 1991). Gl is a zinc-finger transcription factor that controls the expression of many eye-specific genes (DeMille et al., 1996; Ma et al., 1996; Moses and Rubin, 1991; Treisman and Rubin, 1996). We first tested the ability of Gl protein to bind this sequence in the LMEE through mobility-shift

analysis. G1 protein bound specifically to the putative 23-bp site in the LMEE, as it could be competed away with cold competitor oligonucleotide but not by competitor that was mutated within the 23-bp region (Fig. 2A and B). G1 is expressed in all cells from within the furrow to the posterior of the eye disc (Ellis et al., 1993; Moses and Rubin, 1991), consistent with a possible role in regulating Lz expression. A double staining of LMEE-*lacZ* transgenic eye discs for  $\beta$ -galactosidase and G1 protein showed that G1 expression is initiated prior to Lz expression (Fig. 2C–E). Significantly, endogenous Lz expression is largely eliminated in a *gl* null (*gl<sup>60</sup>*) background (Fig. 2G), and the expression of LMEE-*lacZ* is entirely abolished in this genetic background (Fig. 2I). These in vivo genetic results establish that G1 is a regulator of Lz expression in the eye.

To determine whether the control of Lz by G1 is through direct transcriptional activation of the LMEE, we mutated the G1 binding site in the context of LMEE-*lacZ*, to make LMEE<sup>mG1</sup>-*lacZ* (see Materials and methods). Gel-shift experiments confirmed that G1 could no longer bind the mutated site in LMEE<sup>mG1</sup> (Fig. 2B). When LMEE<sup>mG1</sup>-*lacZ* was transformed into flies, *lacZ* expression was no longer supported in the eye disc (Fig. 2J). Taken together, these in vivo results establish that Glass directly controls Lz expression via the LMEE.

#### *The eye specification protein Sine oculis controls Lz expression through palindromic sequences in the LMEE*

Further analysis of the LMEE revealed a striking feature—two large palindromic repeats (ATTGATATCAAT and TTGATATCAA; blue in Fig. 1A). Interestingly, these two palindromes and the 8 bp between them form a larger nearly perfect palindrome structure (TTGATATCAATA-ATTGTTATTGATATCAA). A previous in vitro study (Hazbun et al., 1997) demonstrated that the eye specification protein Sine Oculis (So) can bind the consensus sequence T/CGATAC, which is similar to the TGATAT core sequence of the LMEE palindromes. In addition, So is expressed earlier than Lz in a pattern consistent with being a potential regulator of Lz (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). Mammalian So-like Six proteins have been shown to bind to a different sequence than that found in the LMEE (Spitz et al., 1998). We therefore first conducted DNase I footprint assays to determine whether recombinant full-length So protein can bind to the LMEE. A 32-nucleotide stretch in the LMEE was protected from DNase I digestion and appeared as a clear footprint (Fig. 3A). The sequence covered by the footprint contains the two palindromic repeats, indicating that these represent So binding sites. A probe containing mutations in the core of the palindromes (GATAT to CCCCC) did not show a DNase I footprint in the presence of So protein (Fig. 3A). Consistent with the footprint analysis, mobility-shift assays also showed that So binds the LMEE palindromes (Fig. 3B). This binding is specific since it is competed away with cold

competitor, but not with mutant competitor (Fig. 3B). Additionally, labeled probe containing mutant palindromes did not cause mobility shift in the presence of So protein (Fig. 3B).

To determine whether the So-binding palindromes are important for Lz activation, we made a *lacZ* reporter construct LMEE<sup>2xmSo</sup>-*lacZ* in which the two palindromes were mutated as above to abolish So binding. In transgenic flies, this mutant construct largely lost the ability to activate reporter expression in the eye disc, showing that the palindromic So binding sites are critical for transcriptional activation of Lz (Fig. 3C, compare with Fig. 2H).

To obtain genetic evidence for control of Lz expression, we made FLP/FRT-mediated clones in the eye mutant for the *so* locus. As in homozygous *so* mutants, large clones of *so* have been shown to block furrow progression (Pignoni et al., 1997), an event which is upstream of Lz activation and would therefore indirectly preclude its expression. We therefore used short heat-shock regimens (see Materials and methods) to induce very small clones that did not block furrow progression. Small clones of *so* posterior to the furrow entirely lacked Lz expression (Fig. 3D–F). Staining with nuclear markers show that the clones contain cells (not shown). These results implicate So and the palindromic repeats in the control of Lz expression.

#### *A transcriptional link from early specification to late terminal determination in the eye*

The identification of G1 and So as regulators of Lz expression in the developing eye provides a link between the earliest event in eye development, the establishment of the eye field, to a very late event, the determination of the terminal cone cell fate (Fig. 4). The eye is derived from a group of about 20 cells set aside during embryonic development. During early larval stages, the cells remain undifferentiated and undergo repeated rounds of divisions to produce virtually all of the cells that will form the adult eye. In later larval stages, a complex of retinal specification genes, including *sine oculis*, function together to initiate cell differentiation (reviewed by Pichaud et al., 2001).

This study shows that Sine oculis is also responsible for the control of a gene that sets up a prepattern for future cell-fate determination beyond the formation of the early five-cell cluster (called precluster) at the furrow. This is achieved by controlling Lz in the precursor population that will give rise to cells R1, R6, R7, cone cells, and pigment cells. Lz prepares a large number of cell specific genes to respond to a combination of signals. In the most extensively studied case, cone cell fate requires, amongst other events, the turning on of a non-neuronal gene *D-pax2* and the repression of the neuron-specific gene *dpn*.

A direct, tripartite input from Lz, Notch and EGFR pathways in the right spatiotemporal event causes D-Pax2 to be expressed in cone cells (Flores et al., 2000). D-Pax2 expression then leads to the activation of Cut, which func-

tions in combination with Lz in the direct repression of Dpn (Canon and Banerjee, 2003).

Interestingly, within the eye-specific promoter of the *gl* gene, two conserved elements containing sequences similar to the TGATAT core in the palindromic sites have been shown to be able to drive reporter expression in a pattern similar to that of endogenous So protein (Liu et al., 1996), suggesting that So may also be involved in Gl expression through these elements. We have confirmed that these *glass*

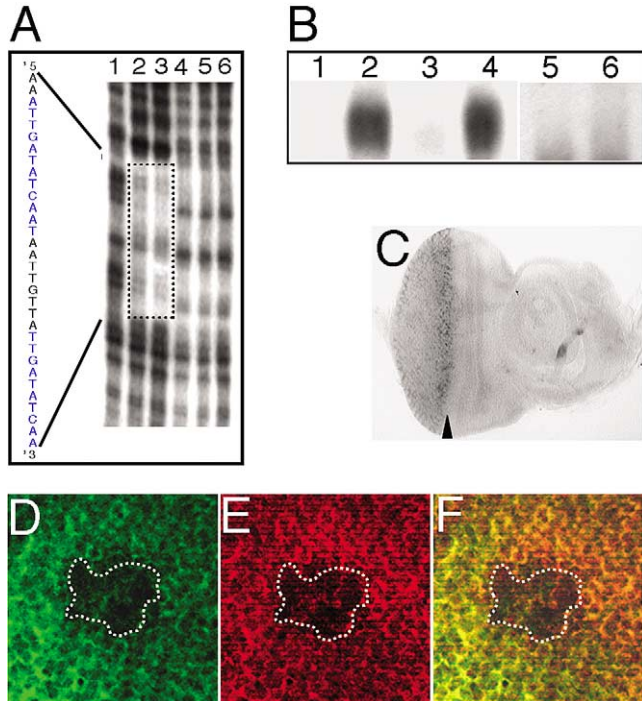


Fig. 3. Sine oculis regulates Lz expression. (A) DNase I footprinting assay. Wild-type (lane 1–3) and palindrome mutant (lane 4–6, see text or Materials and methods for the mutations introduced) probes were incubated with 0 ng (lanes 1 and 4), 100 ng (lanes 2 and 5), and 200 ng (lanes 3 and 6) So protein and then partially digested with DNase I. The sequence in wild-type probe protected by So protein from digestion, not protected in the mutant, is demarcated with dotted lines. The corresponding sequence determined on the same gel is shown in the left. The DNase I banding patterns are different between wild-type and mutant probes in the region that is mutated. The protected region corresponds to the palindromic sequences (blue). (B) EMSA assay using So protein with either a wild-type (lane 1–4) oligonucleotide, *lzSo* (containing the two putative So binding sites, see Materials and methods), or a mutant oligonucleotide, *mlzSo* (lanes 5 and 6). Lane 1, free probe. Lane 2, no competitor. The So/DNA complex shown as a strong gel-shifting band. Cold competitor *lzSo* (lane 3) but not cold *mlzSo* (lane 4) compete for Sine oculis binding. Lane 5, free probe. Lane 6, So protein added to mutant probe, no competitor added. The probe mutated for the palindromic sites does not bind So. (C)  $\beta$ -Galactosidase expression pattern in *w; P[LMEE<sup>2xmSo</sup>-lacZ, w<sup>+</sup>]* eye disc. Compare with Fig. 2H. LMEE fails to efficiently activate reporter expression when the So sites are mutated. (D–F) Lz expression is lost in mutant *so* clones in the eye. (D)  $\beta$ -Galactosidase (green) staining marks wild-type tissue surrounding a *so* clone and the *so* mutant tissue lacks  $\beta$ -galactosidase expression (no green). (E) Lz (red) is not expressed in the small *so* clone in cells that are not green. (F) Merge of  $\beta$ -galactosidase and Lz staining in *so* clone. The dotted line outlines the homozygous *so* mutant clone. All Lz-positive cells are also *so*<sup>+</sup>.

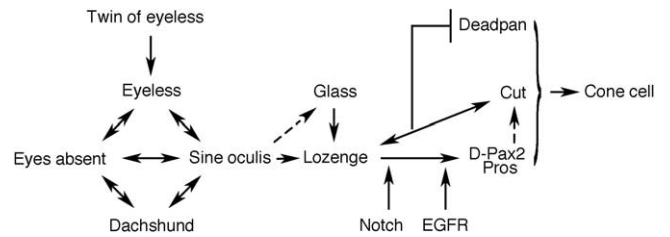


Fig. 4. A genetic circuit linking early specification to the late determination of the nonneuronal cone cell type. Hierarchy of five core members of eye specification factors summarized on the left (reviewed Pappu and Mardon, 2002) prepare the eye primordial field. So and Gl together activate Lz expression. Together with activated EGFR and Notch pathways, Lz plays a key role in the control of cell specific expression of D-Pax2 (Flores et al., 2000) and Prospero (Pros) (Xu et al., 2000). Lz also functions with Cut to repress expression of proneural protein Deadpan in cone cell precursors (Canon and Banerjee, 2003). The presence of D-Pax2 (a “non-neuronal” protein) and absence of Dpn (a “neuronal” protein) are required for the determination of cone cell fate.

upstream elements can bind So protein in gel shift experiments (not shown). This raises the possibility that, in addition to directly controlling Lz, So also controls Gl. Upon Gl expression, So and Gl can together activate Lz expression. This scheme (Fig. 4) further solidifies the concept that a very small number of transcription factors and signaling pathways are used reiteratively and combinatorially to generate a patterned array of various cell types from a population of equivalent pluripotent cells.

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