Lozenge is expressed in pluripotent precursor cells and patterns multiple cell types in the *Drosophila* eye through the control of cell-specific transcription factors

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Accepted 26 June; published on WWW 25 August 1998

SUMMARY

In the developing *Drosophila* eye, individual cell fates are specified when general signaling mechanisms are interpreted in the context of cell-specific transcription factors. Lozenge, a Runt/AML1/CBFA1-like transcription factor, determines the fates of a number of neuronal and non-neuronal cells by regulating the expression of multiple fate-determining transcription factors. The Lozenge protein is expressed in the nuclei of the cells that it patterns and also in their undifferentiated precursors. An enhancer element located within the second intron of the *lozenge* gene is responsible for its eye-specific expression. Lozenge is not itself a cell-specific transcription factor, rather it prepatterns the eye disc by positioning cell-specific factors in their appropriate locations.

Key words: *lozenge*, AML1, Pattern formation, *Drosophila*, Eye development, Runt, Signal transduction

INTRODUCTION

During animal development, cells acquire specific fates via extracellular cues transduced through signaling pathways. A single class of cell surface receptors can often elicit different cellular responses ranging from cell division and differentiation to migration and programmed cell death. These differing outcomes frequently depend on the developmental context of the cell receiving the signal. In general, a specific cell that expresses a set of transcription factors or cytoplasmic determinants will respond uniquely to a given signal. For example, the signal transduced through the *Drosophila* Epidermal Growth Factor Receptor (EGFR) is reiteratively used for the differentiation of each of the eight neuronal and five non-neuronal cell types in the eye disc (Freeman, 1996; Tio and Moses, 1997). The signal itself relays no fate-determining information but rather acts as a trigger that allows a cell to follow a predisposed differentiation program. Genetic analysis in this system affords a unique opportunity to study the origins of such cell-specific responses and the basis for the predisposition of the different cell types.

Unpatterned cells in the larval eye imaginal disc of *Drosophila* undergo spatially and temporally restricted cell-cell interactions that ultimately specify the fates of the different cell types constituting the approximately 800 ommatidia of the adult compound eye (Tomlinson and Ready, 1987). Each ommatidium contains eight photoreceptor neurons (R1-R8), four lens-secreting cone cells, and a number of pigment and bristle cells. In the third instar of larval development, differentiation begins at a morphogenetic furrow that sweeps across the eye disc from posterior to anterior (Ready et al., 1976). Coincident with the furrow, cells arrest in the G1 phase of the cell cycle (Wolff and Ready, 1991). Immediately posterior to the furrow, the cells that are destined to become photoreceptors R8, R2/R5 and R3/R4 exit the cell cycle and emerge as 5-cell preclusters, while all cells that do not become part of the precluster undergo a synchronized round of mitosis and eventually differentiate into R1/R6, R7, cone, pigment and bristle cells (Ready et al., 1976; Wolff and Ready, 1991). How the equipotent cells within this undifferentiated pool achieve their distinct fates has not yet been fully elucidated and is a central focus of this study.

A number of transcription factors are expressed in cell-specific patterns and function exclusively in determining the fates of the cells that arise from the synchronized round of mitosis at the furrow (reviewed in Kumar and Moses, 1997). These include Seven-up (Svp), a steroid hormone receptor expressed in R1/R6 (as well as in the earlier differentiating R3/R4; Mlodzik et al., 1990), the homeodomain protein Bar, expressed in R1/R6 and primary pigment cells (Higashijima et al., 1992), Prospero (Pros), a homeodomain protein found in R7 and cone cells (Kauffman et al., 1996), and the Pax-2 homolog Sparkling (Spa), which is expressed in cone, primary pigment...
and bristle cells (Fu and Noll, 1997). The developing Drosophila eye disc has thus been described as a mosaic of transcription factors expressed in different yet sometimes overlapping subsets of cells (Kumar and Moses, 1997). To understand the source of specificity of pattern formation, it is important to determine how these cell-specific factors achieve their appropriate expression patterns. Genetic analysis has shown that the transcription factor Lozenge (Lz; Green and Green, 1949) plays a pivotal role in this process (Daga et al., 1996). Lz encodes a transcription factor that negatively regulates 

\[ svp \] in R7 and cone cells and positively regulates \( \text{Bar} \) in R1/R6 in the developing eye disc (Daga et al., 1996; Crew et al., 1997). Recent studies suggest that Lz also positively regulates \( \text{pros} \) in R7 and cone cells (R. Carthew, personal communication) and \( \text{spa} \) in cone and primary pigment cells (G. V. F., H. Duan, M. Noll and U. B., unpublished results).

Thus, Lz controls the expression of all known cell-type specific transcription factors expressed in cells that differentiate after the formation of the 5-cell precluster. Consistent with this role, Lz function has been shown to be required for the proper specification of photoreceptor neurons R1/R6, R7 and for determination of cone and pigment cells (Daga et al., 1996; Batterham et al., 1996). It is not clear from these previous studies, however, whether Lz is a cell-specific transcription factor like the ones that it regulates, or whether it is a ubiquitously expressed transcriptional regulator that responds differentially to incoming signals in different cell precursors. In this paper, we use immunolocalization, promoter analysis and germline-mediated transformation to show that Lz expression is controlled by an intronic enhancer and is exclusively required in the pool of undifferentiated cells posterior to the morphogenetic furrow for the proper specification of all cell types that are derived from this pool. Lz therefore functions as a fundamentally important prepatterning molecule during eye development.

**MATERIALS AND METHODS**

**Scanning electron microscopy**

Adult flies were anesthetized with carbon dioxide and attached to metal mounts as wet, uncoated samples using fingernail polish (Wet ’n’ Wild #401). Images were acquired digitally using a Hitachi S-2460N Scanning Electron Microscope at a ‘high pressure’ setting of 30 Pa using a Robinson detector.

**Immunohistochemistry**

A rabbit polyclonal antibody was raised against a peptide including residues 730-741 in the C-terminal portion of the predicted Lozenge amino acid sequence (Daga et al., 1996). The α-Lz antibody was affinity-purified using the peptide conjugated to a Biorad Affigel 10 column. The purified antibody was preabsorbed against fixed affinity-purified using the peptide conjugated to a Biorad Affigel 10 in Daga et al. (1996). PCR primers 5′-TACAGAAGCGACAACAG-3′ and 5′-GTCGAATTCCGCAGATTG-GTACAGAAGCGACAACAG-3′ were used to amplify intron II from wild-type and mutant genomic fly DNA. All constructs for rescue of the \( \text{ic}^\prime \) phenotype were subcloned into the transformation vector HS-Casper. Construct 1 was generated by fusing an 11 kb EcoRI-SstI genomic DNA fragment to the \( \text{SalI} \) site in position 216 of the cDNA. Construct 2 was made by ligating an EcoRI-NorI genomic fragment containing the endogenous promoter and introns I, II, III and IV into the NorI site in position 1632 of the cDNA. Construct 3 was engineered by cloning the PCR amplified intron II upstream of a 3.8 kb EcoRI-SalI genomic fragment fused to the SalI site in position 216 of the cDNA. Construct 4 was generated by placing the PCR amplified intron II ligated to a genomic fragment beginning at the XmnI site (position -1101 from the gene start) and containing the endogenous promoter upstream of the full-length cDNA.

**RESULTS**

Lz expression pattern

A polyclonal antibody raised against Lz was used to examine its expression pattern in wild-type third larval instar eye-antenna discs. At low magnification, Lz expression is seen to be restricted to cells posterior to the morphogenetic furrow (Fig. 1A). This antibody is specific since no staining is detectable in null alleles of \( \text{lz} \) (Fig. 1B). At higher magnification, Lz expression is visible basally in the nuclei of all undifferentiated cells posterior to the furrow (Fig. 1C). This is in contrast to the undifferentiated cells anterior to the furrow, in which Lz is not expressed (Fig. 1A). Immunolocalization of Lz and X-gal show that Lz and β-galactosidase indeed colocalize in R1/R6 but not in R3/R4 (not shown). Furthermore, Lz is expressed in a cell positioned between R1/R6 (Fig. 1D), which is consistent with its being the R7 precursor (Tomlinson and Ready, 1987). At 20-30 hours after puparium formation (APF), Lz expression is seen in primary pigment cells (Fig. 1E) and, at 30-40 hours APF, Lz is expressed in secondary and tertiary pigment cells (Fig. 1F). At this late stage, Lz ceases to be expressed in the other cells of the eye disc.

The above data show that all cells that express Lz share the property that they arise from the synchronous round of cell division at the furrow. However, when this mitosis is prevented by ectopic expression of the human cell-cycle inhibitor \( p21^{\text{Cip1/Waf1}} \) under the control of the pGMR promoter (de Nooij and Hariharan, 1995), we observed that Lz is still expressed in R1/R6 but not in R3/R4 (not shown). Furthermore, Lz is expressed in a cell positioned between R1/R6 (Fig. 1D), which is consistent with its being the R7 precursor (Tomlinson and Ready, 1987). At 20-30 hours after puparium formation (APF), Lz expression is seen in primary pigment cells (Fig. 1E) and, at 30-40 hours APF, Lz is expressed in secondary and tertiary pigment cells (Fig. 1F). At this late stage, Lz ceases to be expressed in the other cells of the eye disc.

Lz is expressed in all cells posterior to the morphogenetic furrow except for those belonging to the initial 5-cell precluster. The exclusion of Lz from photoreceptor cells R8, R2/R5 and R3/R4 is functionally important for proper cell fate specification. Indeed, when Lz is misexpressed in R3/R4 in the \( \text{ic}^\prime \) allele (Fig. 1H), the presumptive R3/R4 cells differentiate as R7s (Fig. 1I). This phenotype results from the repression of \( \text{svp} \) transcription caused by the misexpression of Lz in R3/R4 (Daga et al., 1996). It is
interesting to note that, in wild-type flies, Lz is expressed in R1/R6 yet it does not repress transcription of svp in these cells, suggesting that Lz controls its target genes in a context-dependent manner.

**Eye-specific enhancer of lozenge**

A group of lz alleles including lz\(^{50e}\), lz\(^{6b}\) and lz\(^{77a7}\) exhibit eye phenotypes that are similar to that seen in lz null alleles (Fig. 2A,B) and fail to complement deletions of the lz locus as would normally be expected of all lz alleles. However, these alleles belong to a unique class in that they can fully complement the eye phenotype of many other lz alleles (Batterham et al., 1996). When stained with the Lz antibody, lz\(^{77a7}\) shows no expression in the eye disc, yet expression in the antenna portion remains wild type (Fig. 2C,D). This suggests that lz\(^{77a7}\) is a mutation in an eye-specific enhancer of the lz gene. We used a genetic approach that enabled us to map this enhancer. lz\(^{77a7}\) was crossed to a series of nested lz deletions in which varying extents of the gene are missing. In lz\(^{-1}\), the entire coding region of the gene is deleted while, in lz\(^{77}\) and lz\(^{68}\), the first two introns are intact while more 3' sequences are deleted (Fig. 3A). lz\(^{77a7}\) can completely rescue the mutant phenotype of lz\(^{77}\) and lz\(^{68}\), as lz\(^{77a7}\)/lz\(^{77}\) and lz\(^{77a7}\)/lz\(^{68}\) transheterozygotes have wild-type eyes (Fig. 3C,D,F,G). In contrast, lz\(^{77a7}\)/lz\(^{-1}\) flies have eyes resembling those of lz null alleles (Fig. 3B,E). This led us to infer that lz\(^{77}\) and lz\(^{68}\) retain an eye-specific enhancer element that can stimulate transcription from the lz\(^{77a7}\) promoter in trans. Such somatic pairing-induced transactivation events in *Drosophila* have been elegantly described for numerous loci including yellow (Geyer et al., 1990). *Ultrabithorax* (Martínez-Laborda et al., 1992), *eyes absent* (Leiserson et al., 1994) and *vestigial* (Williams et al., 1994), and are likely to form the basis for the transvection phenomenon (reviewed in Müller & Schaffner, 1990). These results enabled us to map the putative eye-specific enhancer mutated in lz\(^{77a7}\) to the region deleted in lz\(^{-1}\) yet present within lz\(^{77}\) and lz\(^{68}\), i.e. within the first 5 kb of the gene. Since the lz\(^{77}\) breakpoint does not remove sequences upstream of the start site, we reasoned that the

![Fig. 1. Lz expression pattern and phenotypic consequences of misexpression.](image)
enhancer element will reside in one of the first three introns of the gene.

The first three intron sequences from wild type and \(lz^{77a7}\) were PCR amplified. While amplification of introns I and III from \(lz^{77a7}\) yielded PCR products of identical size to those amplified from wild type, amplification of intron II from the \(lz^{77a7}\) template generated a band approximately 150 bp in length, compared to an approximately 1550 bp band isolated from wild type. This suggested that a deletion within this intron may be responsible for the \(lz^{77a7}\) phenotype. Sequence analysis of the PCR product confirmed that the \(lz^{77a7}\) mutation is indeed a 1398 bp deletion of the second intron (Fig. 4A). In addition, amplification of intron II from \(lz^{50e}\) and \(lz^{58b}\) also generated PCR products shorter than that from wild type. Sequencing of these PCR products demonstrated that \(lz^{50e}\) is molecularly identical to \(lz^{77a7}\) and that \(lz^{58b}\) contains a smaller deletion of 880 bp within intron II (Fig. 4A). The existence of three independently isolated eye-specific alleles harboring deletions of intron II strongly supports our hypothesis that an eye-specific enhancer lies within this intron.

These results do not rule out the possibility that other regions of the \(lz\) gene may additionally be required for eye-specific expression. To identify all of the regions necessary for proper \(lz\) expression in the eye, a number of \(lz\) minigene constructs were engineered, transformed into flies and assessed for their ability to rescue the \(lz^{r1}\) mutant eye phenotype. Construct 1, which contains 10 kb of upstream genomic sequences, the transcription start site and intron I fused to the cDNA (Fig. 4B), was not able to rescue the \(lz^{r1}\) mutant eye phenotype. Full rescue was obtained with construct 2, which includes introns I-IV, construct 3, which included introns I and II, and most importantly, construct 4, in which intron II alone was fused to the promoter region and the Lz cDNA (Fig. 4B-D). Staining with the \(\alpha Lz\) antibody revealed that Lz is expressed at high levels behind the morphogenetic furrow in \(lz^{r1}\) eye discs carrying the rescuing constructs, and that there is no expression in the antenna discs (Fig. 4E). Interestingly, expression of Lz in these transformants is robust in undifferentiated cells, yet appears to be absent from R1/R6, R7 and cone cells (Fig. 4F). Since these constructs completely rescue the \(lz\) null eye phenotype, we conclude that Lz is only required in undifferentiated cells at the time of cell fate specification. In wild type, Lz expression is maintained in cells whose identities have already been established; however, this later expression does not appear to be required for the proper patterning of the ommatidium. Taken together with the molecular and genetic data, these rescue experiments demonstrate that intron II is necessary and sufficient for Lz function in the eye.
In addition to the eye phenotype, \(lz\) null mutants have antenna and tarsal claw defects (Stocker and Gendre, 1988; Ray and Rodrigues, 1995; Batterham et al., 1996). Consistent with these observations, \(Lz\) was also found to be expressed in very specific patterns in the antenna (B. P. Gupta, B.P., G. V. F., U. B. and V. Rodrigues, unpublished results) and leg discs (not shown). In the eye-specific alleles, which are deleted for most of intron II, \(Lz\) continues to be expressed at wild-type levels in the antenna (Fig. 2D) and leg discs (not shown). In the eye-specific alleles, which are deleted for most of intron II, \(Lz\) continues to be expressed at wild-type levels in the antenna (Fig. 2D) and leg discs (not shown). Furthermore, the antenna and tarsal claw phenotypes are not rescued by any of the transformation constructs mentioned above. Thus, the intron II enhancer is eye specific and is required solely to restrict \(Lz\) expression to the pool of undifferentiated cells posterior to the morphogenetic furrow in the eye disc, thereby allowing \(Lz\) to properly regulate the expression of multiple cell-specific transcription factors in the developing eye.

**DISCUSSION**

Cells in the *Drosophila* eye disc arise from a common pool of undifferentiated cells that are initially equivalent to one another, yet these cells must express specific transcription factors in order to adopt their unique fates. By the time a cell expresses a specific transcription factor, it is already different from its neighbor and is predisposed to follow a specific developmental fate. It is therefore imperative that this prepattern of differentially expressed transcription factors be itself set up by a protein that is widely expressed. Such a global regulator would not be cell-specific and would control a large number of target genes. \(Lz\) satisfies the above criterion for a global regulator of cell-specific transcription factors within the context of the patterning of all cells that differentiate after the second wave of mitosis. \(Lz\) is expressed not only in the cell types that require it for their proper development but also earlier in the pool of undifferentiated cells that gives rise to these cell types. In fact, our transformant studies indicate that \(Lz\) only needs to be expressed in the undifferentiated cells to carry out its proper function.

\(Lz\) belongs to a growing family of transcription factors whose members contain a DNA-binding Runt domain. The founding member of the family, Runt, is required for *Drosophila* sex determination, segmentation and neurogenesis (Tsai and Gergen, 1994). In the *Drosophila* eye disc, the lack of functional \(Lz\) protein causes an extensive re-programming...
of the fate of a large number of cells. In \(lz\) mutants, \(svp\) is de-repressed (Daga et al., 1996), and the expression of \(Bar\) (Daga et al., 1996), \(pros\) (R. Carthew, personal communication) and \(spa\) (G. V. F., H. Duan, M. Noll and U. B., unpublished) is not initiated, leading to the differentiation of cone cell precursors as neurons and an overall collapse of the ommatidial lattice (Daga et al., 1996). The mammalian proteins that are similar to \(Lz\) include AML1 (acute myeloid leukemia 1) and CBFA1 (core binding factor A1), and, like \(Lz\), are expressed in pluripotent cells in specific tissues and regulate multiple target genes during development. Mutations in these genes lead to severe alterations in the fates of multiple cell types in these tissues (reviewed in Ito, 1997; Rodan and Harada, 1997). For example, AML1 regulates the expression of T cell receptor and cytokines such as GM-CSF and IL3, and represents the most frequent target of translocations that lead to acute myeloid leukemia in humans (Ito, 1997). In mice, a complete knockout of the AML1 homolog PEBP2a results in animals that do not undergo definitive hematopoiesis and lack all cell types that result from this process (Okuda et al., 1996; Wang et al., 1996).

CBFA1 is required for the proper expression of osteoblast-specific genes such as Osteocalcin gene 1 and \(a1(l)\) collagen, and mouse knock-outs lack all bone structures (Rodan and Harada, 1997 and references therein). Thus, \(Lz\), AML1 and CBFA1 share the property that they differentially regulate the expression of several genes within a pool of equipotent cells thereby functioning as prepatterning molecules.

The developmental events in the eye disc can be separated into two stages of patterning. The first occurs within the morphogenetic furrow and leads to the formation of the 5-cell precluster, while the second occurs in the undifferentiated cells posterior to the furrow that gives rise to the remainder of the cells of the mature ommatidium. The first prepatterning event is controlled by transcription factors such as Atonal (Jarman et al., 1995) and Rough (Dokucu et al., 1996). \(Lz\) plays no role in this process as it is not expressed in the 5-cell precluster and \(lz\) mutants show no disruption in the patterning of these cells (Daga et al., 1996). In fact, our results show that misexpression of \(Lz\) at the 5-cell stage leads to a re-programming of cell fates within the precluster. In contrast, proper expression of \(Lz\) is crucial for the second phase of prepatterning that completes the ommatidium by adding the last three photoreceptor cells and the non-neuronal cell types to the precluster. Other transcription factors that play a role in this process include the zinc-finger protein Tramtrack (Ttk) (Xiong and Montell, 1993; Li et al., 1997; Tang et al., 1997) and the Ets domain proteins Yan (Rebay and Rubin, 1995; Rogge et al., 1995) and Pointed (Brunner et al., 1994; O’Neill et al., 1994). The activity of these three proteins is modulated by the EGFR and Sevenless receptor tyrosine kinase (RTK) signaling pathways (Rebay and Rubin, 1995; Brunner et al., 1994; O’Neill et al., 1994; Li et al., 1997; Tang et al., 1997). It seems likely that \(Lz\) may function combinatorially with these transcription factors in order to differentially regulate its target genes in different cells. It is interesting to note that the mammalian homolog of Pointed (Pnt), Ets-1, directly binds to AML1 and, together, they cooperatively activate transcription of the T cell receptor (Wotton et al., 1994; Giese et al., 1995).

While it is clear that \(Lz\) regulates the expression of many different transcription factors, it has not yet been determined how the expression of \(Lz\) itself is controlled. We have shown that an enhancer sequence located within the second intron of \(lz\) is necessary and sufficient for proper eye-specific function. The transcomplementation seen at the \(lz\) locus has been previously described as involving two cistrons, in which ‘Cistron A’ alleles (\(lz^{50e}, lz^{hb}\) and \(lz^{77a7}\)) can complement the phenotype of ‘Cistron B’ alleles (\(lz^{27}\) and \(lz^{58b}\) and vice versa (Batterham et al., 1996). Our studies suggest that \(Lz\) is encoded by a single transcriptional unit, with the so-called ‘Cistron A’ alleles representing mutations in regulatory sequences in the eye-specific enhancer located in intron II. ‘Cistron B’ alleles define mutations in the protein-coding region of the gene. Deletions such as \(lz^{1}\) eliminate both the eye-specific enhancer and the coding regions and therefore fail to complement mutations of either class. Further dissection of the eye-specific enhancer element should allow the identification of both the signaling mechanism as well as the specific transcription factors that are responsible for the initiation and maintenance of \(Lz\) expression. We have identified seven consensus AP-1 binding sites within the second intron (Quandt et al., 1995), although the significance of this observation is as yet unclear since Drosophila Jun is either not functional or redundant during eye development (Riesgo-Escovar et al., 1996; Hou et al., 1997; Kockel et al., 1997), and no eye phenotype has yet been reported for Drosophila \(fos\) mutants (Riesgo-Escovar and Hafen, 1997; Zeitlinger et al., 1997).

The determination of a cell with a distinct fate from amongst an initially equipotent group is a fundamental problem in all developing systems. Although such processes have been extensively studied in vertebrate as well as in invertebrate development, the precise mechanisms by which common signaling pathways trigger cells to assume specific and distinct fates has not yet been determined. In the Drosophila eye, RTK signaling pathways have been well described, but the mechanisms by which such signals trigger the differentiation of one cell type rather than another is not well understood. In this study, we have identified \(Lz\) as a transcription factor that is expressed in a population of pluripotent cells and causes the segregation of specific cell types by controlling the expression of different genes in different cells. The mechanism by which \(Lz\) differentially regulates the expression of cell-specific factors is not yet known, but a model in which \(Lz\) and other transcriptional regulators expressed in undifferentiated cells, including Ttk, Pnt and Yan, combine to create unique contexts in which to interpret common signals such as those triggered by the RTK and Notch pathways is appealing. Future studies will begin probing the molecular basis for generating predisposition and patterning in this system. The molecular similarity of \(Lz\) with AML1 and CBFA1, which serve similar functions during mammalian hematopoiesis and bone morphogenesis, furthermore suggests that the pathways elucidated in Drosophila eye development will be generally applicable in understanding mechanisms that underlie patterning of cells in vertebrate systems.

We dedicate this paper to the 40 years of genetic analysis of lozenge by Mel Green and thank him for useful discussions and fly stocks. We are indebted to M. Noll, R. Carthew, J. Pollock and P. Batterham for sharing results prior to publication. We thank I. Harihar, U. Heberlein, G. Rubin and Kathy Matthews and the Bloomington Stock Center for fly stocks, B. Gupta for assistance with pupal disc staining, and R. Flores and members of our laboratory for comments on the manuscript. G. V. F. is supported by a USPHS National Research
Lozenge expression in the *Drosophila* eye


