Expression of *Drosophila glass* protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein

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**SUMMARY**

The *glass* gene encodes a DNA-binding zinc-finger protein required for the development of *Drosophila* photoreceptor cells and which appears to regulate a number of genes specifically expressed in photoreceptors. We have generated monoclonal antibodies to Glass and used them to examine Glass distribution during development. Glass is expressed in all cell types of the developing eye and in all other organs that contain photoreceptor cells in *Drosophila*, including a small number of cells in the brain. We altered the normal pattern of *glass* expression by placing the gene under the control of the hsp70 promoter. Our results suggest that nonphotoreceptor cells are restricted in their response to Glass expression. In an effort to discover the mechanism of this restriction, we examined the expression of a number of reporter gene constructs. Our results suggest that nonsensory cells are unable to express certain reporter constructs in response to Glass expression because another DNA-binding factor represses Glass activity in nonsensory cells.

Key words: transcription, eye development, neural development, *Drosophila*, photoreceptor, *glass*

**INTRODUCTION**

Development of the *Drosophila* eye depends on inductive interactions between cells to provide the positional information that instructs cells towards their proper differentiated state (Lawrence and Green, 1979; Ready et al., 1976). The terminal events associated with differentiation must result from cells integrating such signals and subsequently executing the appropriate pattern of gene expression to manifest distinct functions and characteristics. The mature *Drosophila* eye consists of a small number of well-defined cell types arranged in a repeating array of subunits or ommatidia. Each ommatidium contains eight photoreceptor cells surrounded by lens-secreting cone cells and optically insulating pigment cells (Ready et al., 1976). Many genes that are involved in the specialized functions of the photoreceptor cells and which are expressed in all or subsets of the photoreceptor cell population have been identified (for review see Smith et al., 1991). Extensive analysis of the *cis* elements that regulate expression of the rhodopsin genes suggests that the combined actions of multiple transcription factors gives rise to the specific patterns of rhodopsin gene expression (Mismer and Rubin, 1989; Fortini and Rubin, 1990).

Several nuclear factors that play a critical role in eye development have been identified, including *seven-up* (Mlodzik et al., 1990), *sina* (Carthew and Rubin, 1990) and *rough* (Tomlinson et al., 1988; Kimmel et al., 1990). These genes all appear to be involved in the specification of neuronal identity in different subsets of developing photoreceptor cells and do not appear to play a direct role in their terminal differentiation. In contrast, *glass* encodes a zinc-finger protein required for the development of all photoreceptor cells in *Drosophila*. *glass* function is not necessary for these cells to become neurons, but rather is required for their subsequent differentiation as photoreceptors (Moses et al., 1989). This requirement appears to be at the level of direct transcriptional regulation of photoreceptor-specific genes (Moses and Rubin, 1991).

In the current study, we have examined the expression and regulation of *glass* during eye development. Moses and Rubin (1991) reported that, despite the specific requirement for *glass* in photoreceptor cells, the protein is expressed in all cells of the developing eye. We have generated monoclonal antibodies specific to the *glass* protein (Glass) that have allowed us to examine Glass distribution in detail. We find that Glass is expressed strongly in the morphogenetic furrow during the very earliest stages of ommatidial assembly and is expressed in all cells posterior to the furrow. Glass continues to be expressed in pupal photoreceptor cells as well as in pigment cells. We have also used these antibodies to examine the distribution of Glass in other non-retinal photoreceptor organs, including the larval Bolwig organ, the ocelli and putative photoreceptors in the brain.
Our results are consistent with previous genetic data suggesting that glass plays a role in the development of photoreceptor cells in all organs in which they occur in Drosophila (Moses et al., 1989).

We have examined the temporal and spatial requirements for glass function by inducing ubiquitous Glass expression via the Drosophila hsp70 promoter. Our data suggest that nonphotoreceptor cells are developmentally restricted in their response to Glass. To gain an understanding of the molecular mechanism of this restriction, we have carried out a series of experiments utilizing Glass-driven reporter genes. Moses and Rubin (1991) showed that a multimer of a Glass-binding site from the Drosophila Rh1 promoter directs expression of a lacZ reporter gene predominantly in photoreceptor cells in the eye disc, providing a direct in vivo assay for the molecular differentiation that occurs in these cells. We have extended these studies and our results suggest that the restriction of Glass activity results from the action of another DNA-binding factor that interacts with a site adjacent to the Glass-binding site in the Rh1 promoter and appears to inhibit the activity of Glass in nonsensory cells.

**MATERIALS AND METHODS**

**Antibodies and immunohistochemistry**

A glass-trpE fusion protein (Moses and Rubin, 1991) was used to generate monoclonal antibodies to Glass according to standard methods (see Ellis, 1992 for details). All data presented in this paper were obtained using mAb9B2.1.

For protein immunohistochemistry, eye discs (20 pairs/lane) were dissected and placed directly in SDS sample buffer (Harlow and Lane 1988). Other samples were prepared by standard techniques. Proteins were detected using Enhanced Chemiluminescence (ECL, Western Blotting Kit- Amersham).

Imaginal discs were stained with Glass antibodies using the protocol of Tomlinson and Ready (1987) except that Triton X-100 (0.1%) was used instead of saponin. Signals were detected with diaminobenzidine (DAB) and hydrogen peroxide as described, except that 2.5 mM NiCl₂ was added for intensification. Pupal eyes were dissected at approximately 60 hours postpuparium formation (staging as in Cagan and Ready, 1989), then fixed and stained in the same manner except that they were mounted directly in 80% glycerol/0.1 M sodium phosphate after the DAB reaction. Third instar larval brains were prepared according to Selleck and Steller (1991). Embryos were fixed for 30 minutes in a 1:1 mixture of PLP and heptane, and the vitelline membranes were removed by hand. Subsequent steps were as described by Patel et al. (1989).

** gastrin protein expression was detected using an ascites fluid of the monoclonal antibody 24B10 (Zipursky et al., 1984; gift of L. Zipursky). elav protein was detected using an affinity purified rat polyclonal serum (gift of K. White). A combination of 0.3% Triton and 0.3% deoxycholate was used as detergent. Samples were then incubated with Texas Red-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch), washed and mounted in glycerol. Signals were detected using confocal microscopy.

β-galactosidase was detected essentially as described by Moses and Rubin (1991), except that an FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) was used for double labeling experiments.

Fixation and sectioning of adult Drosophila retinas were done as described (Tomlinson and Ready, 1987). Pharate adults were hand dissected from the pupal case, and their retinas examined using essentially the same protocol.

**Molecular biology**

All DNA manipulations, including restriction digestions, ligations, bacterial transformations, labeling, blotting and DNA purifications were done according to standard techniques (Maniatis et al., 1982; Sambrook et al., 1989).

DNase I protection assays were carried out essentially as described (Heberlein et al., 1989). The DNA probes used for the Rh1 proximal enhancer (Misser and Rubin, 1987) contain sequences from -252 to +67 of the Rh1 gene, end-labeled at nucleotide -252. Probes were incubated in the presence of dIdC competitor with various amounts of a 0-12 hour embryo nuclear extract (0.18-0.4 M KCl Heparin fraction). Embryo nuclear extracts were prepared as described (Dynlacht et al., 1991).

**DNA constructs**

A hsp70-glass fusion gene was constructed as follows: A 2.2 kb Brs7 fragment from glass cDNA 3-2, which extends from position 4320 to 8335 in the genomic sequence and contains the entire open reading frame, was fused to genomic DNA extending from the BrsII site at 8335 to the SalI site at position 9950. hsp70 promoter sequences from the XbaI to the XmnI site of Ingolia et al. (1980) was cloned upstream of the glass minigene in pBlueScript. The resulting plasmid contains the hsp70 promoter fused to the 5′ untranslated leader of a glass minigene which lacks all introns, but includes 3′ untranslated genomic sequences necessary for polyadenylation. The hsgl fusion gene was cloned into pW8 (Klemenz et al., 1987) and transformant lines generated. Heat shocks were performed by placing standard food vials in a 37°C water bath for 30 minutes, then allowing the larvae to recover for 1.5-2 hours at 18°C-25°C before fixation. Multiple heat shocks were done for 10 or 30 minutes every 6-8 hours. Transgenes were introduced into a g690 genetic background by standard crosses.

All reporter genes were made using synthetic oligonucleotides to produce double stranded fragments that were thenimerized and inserted into appropriate lacZ reporter transformation vectors. The orientations of the oligonucleotides in the final constructs were confirmed by DNA sequencing. The sequences of the oligonucleotides used are as follows:

**KMI:** 5′-TCGAGACACGGTGAACCGGTTTGTTGCTACTTATG-3′

**KHB:** 5′-TCGACAAATGGAAGACATGGTTTGCTACTTATG-3′

**BO28:** 5′-GATTCTAACGAGGAAGATGGTTTGCTACTTATG-3′

**BO29:** 5′-GATTTAGGACGAGGAAGATGGTTTGCTACTTATG-3′

**BO40:** 5′-TCGACCCCCGGGTTTGCTACTTATG-3′

**BO41:** 5′-TCGACTTAAAAACGATTTGCTACTTATG-3′

**MEA:** 5′-TCGAGACACGGTGAACCGGTTTGCTACTTATG-3′

**MEB:** 5′-TCGACATTACGAGGAAGATGGTTTGCTACTTATG-3′

**MEC:** 5′-TCGAGACACGGTGAACCGGTTTGCTACTTATG-3′

**MED:** 5′-TCGACATTACGAGGAAGATGGTTTGCTACTTATG-3′

29-1: The oligonucleotides BO40 and BO41 were ligated in the presence of SalI and XhoI, and pentamers were gel-purified and inserted into the Xhol and SalI sites of the kanamycin resistant shuttle vector pHSX (gift of K. Jones), removed as a NotI fragment and inserted into the NotI site of HZ50PL (Hiromi and Gehring, 1987). Two independent transformant lines were generated which showed identical patterns of lacZ expression.

29-2: The oligonucleotides BO28 and BO29 were ligated in the presence of BamHI and BglII, and trimers were gel-purified into the BamHI site of pww9E, a lacZ reporter vector that includes a minimal hsp27 promoter fused to a lacZ gene with a nuclear localization signal in pW8 (pww9E a gift of M. Mlodzik). The trimer was inserted directly upstream of the hsp27 promoter such that the glass sites are in the opposite orientation as they are found in the natural Rh1 promoter. Three independent transformant lines were obtained that showed identical glass-dependent lacZ expression patterns. One line showed weak non-glass dependent lacZ expression most likely due to a position effect.
pentamer of KM1/KM2 cloned into the Not site of HZ50PL via the shuttle vector pHSX.

38-2: The pentamer of KM1/2 as previously constructed (Moses and Rubin, 1991) was removed from pHSX as a SalI/Xhol fragment and inserted into the Xhol site upstream of hsp27 in pwnBE. Ten independent transformant lines were tested and found to have identical lacZ expression patterns.

38-1M1; 38-2M2: The oligonucleotides MEA, MEB, MEC and MED were ligated in the presence of Sall and Xhol. Pentamers of each pairwise combination (A/B and C/D) were inserted into the Xhol site of pwnBE. Two independent transformant lines of 38-1M1 and five of 38-2M2 were tested and found to have identical expression patterns.

RESULTS

glass expression in the developing eye

Differentiation of the cells that will form the adult Drosophila eye begins in the larval eye imaginal disc, a monolayer epithelium, as an indentation called the morphogenetic furrow sweeps across the disc. Cells posterior to the furrow are sequentially recruited into ommatidial clusters (Tomlinson and Ready, 1987). Glass RNA and protein are strongly expressed in the morphogenetic furrow and in most or all cells posterior to the furrow (Moses and Rubin, 1991). In order to facilitate a more detailed examination of Glass expression on fixed tissue and protein immunoblots, we generated monoclonal antibodies to Glass (see Materials and Methods).

Wild-type third instar eye imaginal discs stained with a glass monoclonal antibody are shown in Fig. 1. Glass is first expressed in cells along the anterior edge of the morphogenetic furrow (Fig. 1G). Expression is seen in all cells posterior to the furrow, including the photoreceptor cells (Fig. 1C-E), cone cells (Fig. 1F) and undifferentiated basal cells (Fig. 1G). Ommatidial assembly is completed by 60 hours after pupation at 20°C (Cagan and Ready, 1989). Glass expression persists through this stage of development (Fig. 1H). At this time, Glass is expressed in all photoreceptor nuclei (Fig 1J), but cannot be detected in cone cells (Fig. 1I). Expression is also observed in primary pigment cells (Fig. 1J, arrow), and the more basally located secondary and tertiary pigment cells (Fig. 1K).

glass expression in nonretinal cells

In addition to the retina, photoreceptor cells are found in three other organs in Drosophila - the larval Bolwig organ, the ocelli and possibly the brain (Dushay et al., 1989). The Bolwig organ and the ocelli have been shown to be affected by the glass mutation (Moses et al., 1989) and a glass-responsive lacZ reporter gene is expressed in all three organs (Moses and Rubin, 1991). The Bolwig organ arises during late embryonic development and, by 16 hours of development, these cells express the photoreceptor-specific chaoptin protein (Steller et al., 1987). Fig. 2A,B shows two late stage embryos stained with the anti-Glass monoclonal antibody. Glass is clearly expressed in two groups of anteriorly localized nuclei that correspond to the developing Bolwig organ. Expression is also seen in groups of nuclei in the presumptive brain and some nuclei along the midline of unknown fate (Fig. 2B).

The ocelli develop from an anterior-medial localized group of cells in the eye disc (Haynie, 1975). Fig. 1A shows that these cells are Glass positive in a late third instar eye disc. Finally, there is evidence that photoreceptor cells may exist in the Drosophila brain. Hall and coworkers have shown that eyelless and ocelliless flies retain light-sensitive rhythmic behavior (Dushay et al., 1989). Late third instar larval brains stained with the anti-Glass monoclonal antibody show two groups of Glass-positive nuclei in each brain hemisphere (Fig. 2C). The location of these cells does not correspond to the optic lobe precursors, nor do they lie near the position in which the photoreceptor axons innervate the brain (data not shown). Chaoptin expression has been observed in the brain (L. Zipursky, personal communication), although it is unclear whether the same cells express both glass and chaoptin.

Effects of ectopic expression of glass

While glass function is clearly required by Drosophila photoreceptor cells, the protein is expressed in other cell types in the developing eye, where it has no apparent function. To test the temporal and spatial requirements for glass expression, we altered the normal expression of glass by placing the gene under the control of the Drosophila hsp70 promoter. A glass minigene was fused to the hsp70 promoter in the S' untranslated leader (see Materials and Methods) to create the fusion gene hsgl. Two hsgl transformant lines were generated and both showed similar levels of heat-inducible ubiquitous Glass expression. These hsgl transformants produce glass protein of the correct size upon heat induction (37°C for 30 minutes), as demonstrated by protein immunoblotting (Fig. 3A). This protein is of a heterogeneous size, as there are multiple bands seen in both wild-type and the hsgl transgenic flies that are absent in a glass null mutant (Fig. 3A). The multiple forms of Glass expressed in the eye disc have slower mobility on SDS-PAGE than full-length glass protein expressed in bacteria (Fig. 3B). These results suggest that Glass is post-translationally modified; we do not know the molecular nature of these modifications. The spatial distribution of the heat-induced protein was examined by tissue staining with Glass antibodies. An eye disc taken from a hsgl transformant and stained with Glass antibody after heat shock is shown in Fig. 3D. Glass is strongly induced in nuclei of cells ahead of the morphogenetic furrow and in the antennal disc at levels that appear to be similar to cells that express Glass normally. When placed in a glass null genetic background, these transformants are still capable of directing high levels of Glass expression (data not shown), indicating that induction of the endogenous glass gene is not responsible for the observed heat-induced ectopic expression, as has been observed for the Deformed gene (Kuziora and McGinnis, 1988). The hsgl fusion genes induce high levels of Glass expression in all tissues of the third instar larva (see for example, Fig. 3F).

To test the effects of ectopic Glass expression, single 30 minute heat shocks were given to hsgl-carrying third instar larvae, which were then allowed to complete development normally at 18° or 25°C. This single heat shock had little or no effect on viability (data not shown). In a wild-type background, a single burst of ectopic Glass expression has no discernible effect on eye structure. The exterior of the eye
appears normal and, internally, the structure of the ommatidia and the surrounding pigment cell lattice is unperturbed (Fig. 4B). Thus, a single burst of Glass expression in ectopic cells does not perturb their differentiation.

The same tests were performed on hsgl transformant lines in a glass null genetic background. A single heat shock during third instar has no obvious effect on the mutant phenotype (Fig. 4C). If these flies are given multiple short heat shocks (10 minute heat shocks every 6 to 8 hours) beginning in third instar, most of the flies die as pupae. Flies that lack the hsgl transgene and receive the same treatment show no decrease in viability (data not shown). A small number of hsgl flies (less than 10%) develop to the pharate adult stage. Sections of the eyes from these animals (Fig. 4D) show some rescue of the glass phenotype. In particular, photoreceptor cells with rhabdomeres are present and are arranged within a pigment cell lattice. These results indicate that periodic pulses of widespread Glass expression during

Fig. 1. glass protein expression in the developing eye. The third instar eye imaginal discs in A-G and the 60 hour pupal eye in H-K are shown stained with an anti-Glass monoclonal antibody. Anterior is to the left. (A) In the third instar disc, glass is expressed strongly in the morphogenetic furrow (arrow) and in all cells that arise from the furrow. Several Glass-positive nuclei are found in the anterior-medial margin of the disc that gives rise to the ocelli (arrowheads). (B) The antibody is specific to Glass, as it has no reactivity on a disc taken from a g650 null allele. In a wild-type disc, Glass is expressed at high levels in the developing photoreceptor cells in a sequence that reflects their acquisition of neuronal identity. (C) The first photoreceptors to express Glass are 8, 2 and 5. (D) Shortly thereafter the more apically located cells 1, 6, 3 and 4 are seen to express Glass. (E) Finally, the R7 photoreceptor cell expresses Glass near the back of the disc. (F) The four cone cells express low levels of Glass (arrows), beginning 10–12 rows behind the furrow. (G) The anterior edge of expression is characterized by regularly spaced groups of Glass-positive nuclei separated by nonexpressing cells. Behind the furrow, all basal nuclei express somewhat lower levels of Glass. (H) Glass expression persists during pupal eye development. (I-K) Detailed views in three focal planes of the disc in H. (I) No Glass expression is seen in the apical cone cells at this stage. (J) All eight photoreceptor cells continue to express Glass, and the primary pigment cells express low levels of the protein (arrow). (K) In addition, the basal secondary and tertiary pigment cells strongly express Glass.
Regulation of Glass activity

eye development is sufficient to induce differentiation of some photoreceptor cells, although the rescue is not complete.

To examine more closely the effects of ubiquitous Glass expression on eye development, we used expression of the photoreceptor-specific chaoptin protein (Chaoptin) as a marker for photoreceptor differentiation. In wild-type, Chaoptin is first expressed in the photoreceptor cells about 10 columns posterior to the morphogenetic furrow (Fig. 4E; Zipursky et al., 1984) and the membrane-localized protein was also observed on the axons of the photoreceptors as they travel down the optic stalk (data not shown). Fig. 4F,G show discs taken from a hsgl line in a glass mutant background and stained with anti-Chaoptin antibody. In the absence of hsgl transgene induction, no Chaoptin expression is observed (Fig. 4F). However, if the same line is heat shocked at 37°C for 30 minutes and allowed to recover for 1-2 hours, Chaoptin expression is seen in cells behind the furrow (Fig. 4G). This Chaoptin expression is limited to photoreceptor cells, as all of the Chaoptin-positive cells extend axons down the optic stalk. No ectopic Chaoptin expression is observed in the eye disc or in other tissues upon hsgl expression in either a wild-type or glass mutant background (data not shown). Thus, glass expression alone is not sufficient to induce photoreceptor-specific gene expression. Nonphotoreceptor cells are somehow developmentally restricted in their response to glass expression. This suggests that Glass function is modulated post-translationally such that Glass is either activated in photoreceptor cells or prevented from acting in nonphotoreceptor cells.

Glass-responsive reporter genes define an adjacent cis-acting element that regulates Glass activity

We used Glass-driven lacZ reporter gene constructs to address how the ability of Glass to promote transcription is regulated during eye development. Bacterially expressed glass protein specifically binds to enhancer sequences of the Drosophila Rh1 promoter (Moses and Rubin, 1991). When fused to a Drosophila hsp70/lacZ reporter gene, a pentamer of a 38 bp fragment from the Rh1 proximal enhancer containing a Glass-binding site directs high level lacZ expression predominantly in photoreceptor cells in the eye disc (construct 38-1, which corresponds to construct C of Moses and Rubin, 1991), providing an in vivo assay for Glass activity. We analyzed a series of additional glass-dependent reporter genes in an effort to define more accurately the requirements for cell-specific activation.

The 38 bp fragment from Rh1 used to construct 38-1 includes some flanking sequences that are not protected by recombinant Glass in a DNase I protection assay (Moses and Rubin, 1991; Fig. 5). 16 nucleotides that lie 3′ of this Glass-binding site directs high level lacZ expression predominantly in photoreceptor cells in the eye disc (construct 38-1, which corresponds to construct C of Moses and Rubin, 1991), providing an in vivo assay for Glass activity. We analyzed a series of additional glass-dependent reporter genes in an effort to define more accurately the requirements for cell-specific activation.

The 38 bp fragment from Rh1 used to construct 38-1 includes some flanking sequences that are not protected by recombinant Glass in a DNase I protection assay (Moses and Rubin, 1991; Fig. 5). 16 nucleotides that lie 3′ of this Glass-binding site are absolutely conserved in the Drosophila virilis Rh1 promoter, which suggests functional importance in the regulation of Rh1 expression. The 38 bp Glass-binding site used in 38-1 includes ten of these nucleotides (Fig. 5). To investigate whether these sequences are critical for photoreceptor-specific expression of the reporter gene, we made two additional reporter constructs, 29-1 and 29-2, using multimers of 29 bp that include only the sequences protected by Glass in DNaseI footprinting.

**Fig. 2.** Glass expression in nonretinal photoreceptor cells. All tissues were stained with mAb9B2.1. Embryos are oriented with anterior to the right. Glass is expressed in the embryo in three groups of nuclei. (A) The cells of the presumptive Bolwig organ express Glass in a rosette-like pattern in a stage 14 embryo (arrows). (B) These cells are found more anteriorly in a later embryo (arrows). Glass expression is also observed in nuclei of the developing brain (large arrowheads) and two groups of centrally located nuclei (small arrowheads). (C) In the third instar brain, Glass is expressed in two groups of cells in both brain hemispheres (arrows), which may correspond to photosensitive organ in this tissue.
Surprisingly, constructs based on this 29 bp site behave very differently from those based on the 38 bp site in that they direct \emph{lacZ} expression to all cells of the eye disc in which Glass is expressed. We tested both of these binding sites in different promoter contexts in order to rule out trivial explanations for the observed differences in expression pattern, such as orientation of the binding site relative to the gene, the sequences of the linkers, or the sequence of the basal promoter (see Materials and Methods and Fig. 5). The data summarized in Fig. 5 show that the difference in behavior of the constructs appears to depend solely on the presence or absence of the sequences flanking the Glass-binding site.

In order to determine whether the different behavior of constructs based on the 29 bp and 38 bp fragments in the developing eye could also be seen in tissues where Glass is not normally expressed, we examined expression of the 38-1 and 29-2 constructs induced by Glass expressed ectopically from the \emph{hsgl} transgene. In a wild-type background, 38-1 is expressed only in the third instar eye disc and a small number of cells in the brain (Fig. 6A and Moses and Rubin, 1991). However, after ubiquitous \emph{glass} expression, \emph{lacZ} is induced in other tissues (Fig. 6C). In particular, \(\beta\)-galactosidase activity is seen in most cells of the larval brain and ventral nerve cord. \emph{lacZ} is also induced in a small number of cells in the leg and wing discs (Fig. 6D-G). No ectopic \emph{lacZ} expression is seen in the eye/antennal disc. Heat shocks of flies carrying 38-1 alone caused no ectopic \emph{lacZ} expression (data not shown). In contrast, 29-2, which directs expression of a \(\beta\)-galactosidase protein that is localized to the nuclei of cells, is more widely induced as a result of ubiquitous Glass expression (compare Fig. 6C,J). Specifically, most cells of the brain, and all cells of the eye/antennal, leg and wing imaginal discs express \emph{lacZ} (Fig. 6J). Thus, in imaginal discs, 29-2 shows unrestricted activation in all cells that express Glass, whereas 38-1 shows a restricted pattern of activation.

In the eye disc, 38-1 is only induced in photoreceptor cells, which are a specialized subset of neural cells. Some of the cells that induce 38-1 in the leg disc in response to ectopically expressed Glass have long axon-like processes and the leg chordotonal organ is \emph{lacZ} positive (Fig. 6D), which suggests that at least some of the cells that fail to repress 38-1 in the leg disc are neural in nature. Fig. 6E,F shows a leg disc from a heat-shocked \emph{hsgl;38-1} larva stained with anti-\(\beta\)-galactosidase and a neuronal-specific antibody to the \emph{elav} protein (Robinow and White, 1990). All \emph{lacZ}-positive cells are closely associated with neuronal cells and are either neurons themselves or are in the correct positions to be accessory or glial cells that will form part of a mature sensory organ (Jan et al., 1985; Tix et al., 1989). This result suggests that the developmental events that occur to permit activation of 38-1 may be restricted to a subset of sensory organ cells.

\begin{itemize}
  \item In wild-type flies, 38-1 is expressed only in the third instar eye disc and a small number of cells in the brain.
  \item After ubiquitous \emph{glass} expression, \emph{lacZ} is induced in other tissues.
  \item \emph{lacZ} is also induced in a small number of cells in the leg and wing discs.
  \item Heat shocks of flies carrying 38-1 alone caused no ectopic \emph{lacZ} expression.
  \item In contrast, 29-2, which directs expression of a \(\beta\)-galactosidase protein, is more widely induced.
  \item In imaginal discs, 29-2 shows unrestricted activation.
  \item In the eye disc, 38-1 is only induced in photoreceptor cells.
  \item In the leg disc, some cells that induce 38-1 are neural.
  \item Heat shocks of flies carrying 38-1 alone caused no ectopic \emph{lacZ} expression.
  \item In contrast, 29-2 shows unrestricted activation.
\end{itemize}
Evidence that a DNA-binding factor interacts with sequences 3′ of the Glass-binding site and represses Glass activity

Our data indicate that a 38 bp fragment from the Rh1 promoter that includes a Glass-binding site is restricted in its ability to activate a heterologous promoter in response to Glass expression, while a 29 bp truncated version of the same binding site directs unrestricted expression of a lacZ reporter in response to Glass. This observation suggests that the sequences adjacent to the Glass-binding site in the 38 bp construct exert a negative effect on the ability of Glass to activate gene expression in nonsensory cells. Such an effect might be mediated by a DNA-binding factor that interacts with these sequences.

To test this possibility genetically, we made two versions of the 38 bp oligonucleotide that each contain 4 bp mutations 3′ of the Glass-binding site (Fig. 5F and G). These mutations would be expected to interfere with the protein-DNA interactions of any factor that binds to this site and disrupt its function. These sites were used to make reporter constructs 38-2M1 and 38-2M2 that, except for the mutations, are identical to the nuclear β-galactosidase reporter 38-2. In contrast to the restricted expression pattern observed in 38-2, both 38-2M1 and 38-2M2 direct high levels of lacZ expression to all cells of the eye disc that normally express Glass. Thus, these mutants are insensitive to the repression of Glass activity in nonphotoreceptor cells in the eye disc.

We also carried out DNase I protection assays using nuclear extracts from Drosophila embryos (Fig. 5H) and adult heads (data not shown). Using the Rh1 proximal enhancer as a probe, we observe a protected region corresponding to the 16 bp that lie 3′ of the Glass-binding site. An interesting feature of this 16 bp site is the presence of two repeats of the sequence ATTG, both of which are protected and bounded by hypersensitive sites. This footprint pattern may reflect the presence of two proteins or a dimer of a protein that recognizes the ATTG core element. This sequence is also found near other Glass-binding sites in the Rh1 distal enhancer and in the promoter of the glass gene (data not shown). The 38 bp fragments used in the reporter genes include only ten of these conserved nucleotides (see Fig. 5A), but include a single ATTG repeat which appears to be sufficient for repressive activity.

DISCUSSION

We have shown that Glass is expressed in the developing
Fig. 5. lacZ reporter constructs define a short sequence adjacent to a Glass-binding site that is critical to regulation of Glass activity. (A) The sequence surrounding the Glass-binding site in the Rh1 proximal enhancer is shown. Glass protects a 27 bp site that is highlighted in black in the *D. melanogaster* sequence (Moses and Rubin, 1991). The site coincides with a sequence that is conserved in the *D. virilis* Rh1 promoter at 21 out of 25 positions. Just downstream there is a 16 bp site that is absolutely conserved in *D. virilis*, though the spacing between the sites is different in the two species, with a 4 bp overlap in *D. melanogaster*. The sequence from the *D. virilis* Rh1 promoter is shown below the *D. melanogaster* sequence and the conservations are indicated. A comparison of the 16 bp site to the region 3′ of the Glass-binding site contained in constructs 38-1 and 38-2 is also shown. (B-G) Structural diagrams of glass reporter genes used in this study. In all cases, the sequences from the proximal enhancer that were multimerized and inserted into lacZ reporter vectors are shown in capital letters. The sequences protected by Glass are highlighted. Nucleotides that were added to facilitate cloning are shown in lowercase letters. In the schematic diagrams, each triangle represents a single copy of the sequence shown. Triangles pointing toward the right indicate the sequence is in its natural orientation in the Rh1 promoter, to the left indicates reverse orientation. The type of basal hsp promoter used is indicated. The reporter gene used in the constructs was either the naturally occurring cytoplasmic form of β-galactosidase (lacZ) or a version in which β-galactosidase is fused to a nuclear localization signal (nlacZ). The nucleotides mutated in 38-2M1 and 38-2M2 are shown boxed with the new sequence shown below. (H) Proteins in a *D. melanogaster* embryo nuclear extract specifically protect sequences in the putative repressor-binding site of the Rh1 proximal enhancer from DNase I digestion. Lane 1 (G+A) is purine cleavage markers, and lane 2 (0) is the DNase I digested probe in the absence of added protein. In lanes 3 and 4, 50 and 200 µg of protein respectively were incubated with the probe. A footprint, characterized by areas of protection (open arrows) and two hypersensitive sites (arrows), is observed over nucleotides −196 to −180.

Fig. 6. Reporter constructs show different responses to ectopic glass expression. β-galactosidase expression detected by activity staining in A-C and H-J, and by anti-β-galactosidase antibodies in D,E and G. (A) Under wild-type conditions, 38-1 directs lacZ expression to cells in the eye disc and two groups of cells in the brain (see also Moses and Rubin, 1991). (B) No lacZ expression is detected in the leg discs from 38-1 larvae in the absence of heat shock. In the presence of the hsgl transgene following heat shock, 38-1 directs lacZ to: (C) many cells in the brain and ventral nerve cord and (D) small numbers of cells in the leg discs. Some of these cells appear to be neurons, such as the chordotonal organ (large arrow), and cells that appear to have axons (small arrows). (E,F) Confocal images of the distal tip of a leg disc from a hsgl; 38-1 animal following heat shock and stained with rat antibodies to Elav and mouse anti-β-galactosidase (see Materials and methods). (E) 38-1 lacZ expression as a result of ectopic glass expression. Some cells around the distal tip of the disc induce the reporter that are also Elav-positive (small arrows). There are also cells that induce the reporter that do not express elav (large arrow). (F) Elav expression in the leg disc. In this plane of focus, neurons are arranged as a crown around what will become the distal tip of the leg. (G) 38-1 directs β-galactosidase to cells that correspond to sensory organs in the wing disc in response to ubiquitous Glass. (H) Under wild-type conditions, 29-2 directs lacZ expression in the eye disc and to small numbers of cells in the brain. (I) No expression is observed in the 29-2 leg disc. (J) In the presence of the hsgl transgene after heat shock, lacZ from 29-2 is induced in many cells in the brain and in all cells of the eye/antennal disc and leg disc.
retina, ocelli and Bolwig organ, consistent with their requirement for glass gene function and expression of lacZ reporter genes (Moses and Rubin, 1991). To examine the temporal and spatial expression requirements for glass function in the eye, we generated transgenic flies that carry glass under the control of the hsp70 promoter. We have shown that ubiquitous expression of Glass does not affect normal eye development. In a glass mutant background, repeated pulses of ubiquitous Glassexpression rescues some photoreceptor cells without appearing to affect the fate of other cell types in the eye. During development, ubiquitous Glass induces Chaoptin expression only in photoreceptor cells. These data suggest that only developing photoreceptor cells receive the proper inductive cues to be competent to respond to Glass expression.

One possible molecular mechanism for regulating Glass activity is direct covalent modification of the Glass protein itself, which might modulate the activity of Glass as a transcriptional activator (see also Moses and Rubin, 1991). We have presented evidence that Glass is post-translationally modified in vivo. The molecular nature or functional significance of these modifications are currently unknown.

Our experiments using Glass-driven lacZ reporter genes suggest an alternative mechanism in which Glass activity is modulated by another DNA-binding factor(s). A multimerized 38 bp Glass-binding site from the Rh1 proximal enhancer directs lacZ expression in photoreceptor cells in the eye disc (Moses and Rubin, 1991; this report). This 38 bp fragment includes 10 bp of a 16 bp element that lies immediately 3' of the Glass-binding site and is absolutely conserved in Drosophila virilis. Previous studies have shown that most sequences that are highly conserved between Drosophila melanogaster and Drosophila virilis promoter elements have regulatory significance (Fortini and Rubin, 1991; Scholnick et al., 1986). When a multimer of a 29 bp Glass-binding site that does not include this sequence, or versions of the 38 bp site in which this sequence is mutated, are used in reporter gene constructs, lacZ is induced in all Glass-expressing cells in the eye disc. The 38 bp element appears to be similarly regulated in other tissues. In particular, when Glass is ubiquitously expressed, the 38 bp binding site directs lacZ reporter expression to a small subset of cells in the leg disc whereas the 29 bp site induces lacZ expression in all cells of the leg disc. An activity from Drosophila nuclear extracts binds to the 16 bp site in vitro.

Our data suggest a simple model of combinatorial gene control in which the sequences surrounding the Glass-binding site in the Rh1 proximal enhancer define an element that interacts with two DNA-binding factors: constitutively active Glass and an unidentified factor that acts to repress Glass activity in nonphotoreceptor cells. We currently do not have any information as to the molecular nature of this other factor, but it appears to be functional in tissues throughout the fly, implying that it has a widespread role during development. Our analysis of the expression of the 38 bp reporter gene in the leg disc suggests that some sensory organ cells allow transcription of this construct in response to Glass. This result is intriguing in that the cells in the eye disc that activate this reporter gene are subsets of neural cells. It may be that the signals that prevent the action of the putative repressing factor and allow Glass to activate gene expression are acquired by cells that differentiate as particular classes of neural cells.

In the eye disc, Glass is expressed in all differentiating cells, and our data imply that the other factor is expressed in most tissues. We propose that the 38 bp element is capable of integrating the actions of these two broadly expressed factors and refines this information to give a very specific pattern of gene expression. The simplest model consistent with our data is that the repressing activity of the adjacent factor is downregulated in the presumptive photoreceptors. It is also possible that cell-specific modification of Glass may modulate how these proteins interact and thus function in different cells.

The combinatorial action of transcription factors to increase specificity of gene expression has been documented in several developing systems. For example, the products of the broadly expressed Drosophila gap genes regulate the specific stripe expression of the pair rule genes via discrete enhancer elements (Howard and Struhl, 1990; Small et al., 1991). In mammalian systems, the glucocorticoid response element mediates both activation and repressive functions depending on the context of the factors that interact with it (Diamond et al., 1990). Because of the detailed understanding that is emerging of the inductive events that lead to cell fate determination in the Drosophila eye, further study of the action of Glass and its role in photoreceptor gene expression may lead to a greater understanding of how cells integrate the external signals that lead to specific gene expression during development.

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