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The Basic Helix-Loop-Helix Leucine Zipper Transcription Factor *Mitf* Is Conserved in Drosophila and Functions in Eye Development

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ABSTRACT

The MITF protein is a member of the MYC family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors and is most closely related to the TFE3, TFEC, and TFEB proteins. In the mouse, MITF is required for the development of several different cell types, including the retinal pigment epithelial (RPE) cells of the eye. In *Mitf* mutant mice, the presumptive RPE cells hyperproliferate, abnormally express the retinal transcriptional regulator *Pax6*, and form an ectopic neural retina. Here we report the structure of the *Mitf* gene in Drosophila and demonstrate expression during embryonic development and in the eye-antennal imaginal disc. *In vitro*, transcriptional regulation by Drosophila Mitf, like its mouse counterpart, is modified by the Eyeless (Drosophila Pax6) transcription factor. *In vivo*, targeted expression of wild-type or dominant-negative Drosophila Mitf results in developmental abnormalities reminiscent of *Mitf* function in mouse eye development. Our results suggest that the *Mitf* gene is the original member of the *Mitf-Tfe* subfamily of bHLH-Zip proteins and that its developmental function is at least partially conserved between vertebrates and invertebrates. These findings further support the common origin of the vertebrate and invertebrate eyes.

EYE development in both vertebrates and invertebrates involves precise patterning and cell fate decisions that require the activities of several evolutionarily conserved transcription factors. Among these, the *Pax6* gene provides a striking example (Quiring *et al.* 1994; Halder *et al.* 1995). The DNA-binding domains of *Pax6* (paired box and homeodomain) are highly conserved through evolution and mutations in the gene affect eye development in species as diverse as humans, mice, and Drosophila (reviewed in Gehring and Ikeo 1999). In addition to Pax6, other genes expressed and/or required during early eye development are conserved between flies and vertebrates, including the transcription factors *sine oculis*, *optix*, *eyes absent*, and *dachshund* (reviewed in Wawersik and Maas 2000). Genetic, molecular, and biochemical investigations suggest that some of these nuclear factors assemble into transcriptional complexes and form a specific hierarchy involved in the establishment of a "retinal" fate. Studies performed in mammalian systems suggest that some of these regulatory relationships are conserved (reviewed in Wawersik and Maas 2000). Thus, early aspects of retinal development in both vertebrates and invertebrates may be regulated by a conserved set of transcription factors.

An important regulator of early eye development in the mouse is the microphthalmia-associated transcription factor (*Mitf*). The *Mitf* gene is a member of the MYC supergene family of basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors (Hodgkinson *et al.* 1993; Hughes *et al.* 1993) and can regulate target gene transcription through the canonical CANNTG E-box sequence (Hemesath *et al.* 1994). *Mitf* function is required not only during eye development but also in different cell types, including melanocytes, osteoclasts, and mast cells (reviewed in Moore 1995). *Mitf* has been shown to regulate genes controlling pigment synthesis, such as *tyrosinase*, *tyrosinase-related protein-1* and *-2* (reviewed by Goding 2000), osteoclast-specific genes, such as *TRAP* (Luchin *et al.* 2000) and *cathepsin-K* (Motyckova *et al.* 2001), and mouse mast cell proteases (Kitamura *et al.* 2000). Although the role of this gene during eye development has been characterized, no tissue-specific targets of *Mitf* have been identified.

The lack of *Mitf* function during mouse eye development results in reduced eye size or microphthalmia in the adult animal. In mouse embryos homozygous for loss-of-function mutations at the locus (such as $Mitf^{mi\text{-}vga9}$ and

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Mitf$^{mi\text{-}eyless\text{-}white}$), formation of the optic vesicle and cup occurs at the expected stage. However, the partitioning of the eye tissue into neural retina and retinal pigment epithelium (RPE) is disturbed, leading to the transformation of regions of the RPE into stratified neural retina; mutant RPE cells hyperproliferate and acquire columnar shapes as compared with the cuboidal shape observed in wild-type and form cells, which express neuroretinal marker genes (Nguyen and Arnheiter 2000). Thus, *Mitf* appears to play a role in the establishment of the RPE within the presumptive eye region.

Recently, it was shown that Mitf interacts with Pax6 *in vitro*, resulting in transcriptional inhibition (Planque *et al.* 2001). The significance of these effects to *in vivo* gene function is unknown. During eye development the *Mitf* and *Pax6* genes are initially expressed across the entire optic vesicle (including both presumptive retina and RPE regions) and are then restricted in expression such that *Pax6* is expressed in neuroretinal cells while *Mitf* is restricted to RPE cells (Bora *et al.* 1998; Nakayama *et al.* 1998; Nguyen and Arnheiter 2000). These expression patterns suggest that the transcriptional inhibition observed *in vitro* may be involved in determining neural *vs.* nonneural fate.

Here we report the identification of the Drosophila *Mitf* (*Dmel/Mitf*) gene and show that transcriptional activation by Drosophila *Mitf* is inhibited by Pax6 *in vitro*, similarly to the vertebrate proteins. Expression of *Dmel/Mitf* in the developing fly eye raises the possibility that it is yet another conserved component in the genetic control of early eye development in fly and vertebrates. Moreover, its expression in the peripodial membrane, a tissue that is continuous with and overlies the presumtive retinal epithelium, suggests that peripodial and RPE tissue may be evolutionarily related. Targeted expression of wild-type and dominant-negative *Mitf* confirms a likely role for the gene in the development of eye-antennal disc derivatives. This evidence lends further support to the proposal that vertebrate and invertebrate eyes share a common origin.

MATERIALS AND METHODS

***Dmel/Mitf* genomic structure:** The *Drosophila melanogaster* genome sequence (Adams *et al.* 2000) was available in GenBank through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and was searched using tBLASTn (Altschul *et al.* 1990). Using the bHLH domain of the mouse *Mitf* gene, a Drosophila genomic region was identified (within clone AE003163) that showed considerable homology to the mouse *Mitf* sequence. By using the resulting Drosophila genomic region in a further blast search, several cDNA clones were identified. The Berkeley *Drosophila* Genome Project cDNA clones LP03467 and LD45277 showed considerable identity to the genomic sequences and were obtained and sequenced in full. Clone LD45277 turned out to be a full-size clone, which included a 5′ ATG with a continuous open reading frame as well as the poly(A) tail of a mature mRNA. The other cDNA clone (LP03467) was a chimeric clone containing the 5′ part of *Mitf* and a totally unrelated cDNA. By blasting the full-length LD45277 cDNA against the Drosophila genome, the structure of the Drosophila *Mitf* gene was reconstructed; the 5′ exon 1 was discovered on genomic clone AE003846, which encodes the first 153 nucleotides of the cDNA. This exon had been annotated as a possible open reading frame and had received the identification number CG17469. Clone AE003846 had been assigned to the chromosome 4 region 102E-F, while genomic clone AE003163, which contains the rest of the *Dmel/Mitf* coding region, has yet to be assigned a chromosomal location. On the basis of this, we presume that the *Dmel/Mitf* gene is located on chromosome 4.

Cotransfection studies: The full-length Drosophila *Mitf* cDNA as well as the *Mitf*^{EA} mutant version were cloned into the pCDNA3.1 eukaryotic expression vector. In Mitf^{EA}, the negatively charged glutamic acid (E) within the basic region is changed to a neutral alanine (A). The mutation was created using mutagenic primers and a two-step PCR approach (Ho *et al.* 1989). The clone was then ligated into the *Bgl*II-*Xho*I-digested pcDNA3.1 vector. The reporter construct MBpluc contains six tandem M boxes (generously donated by Kenji Kasai); each M box contains an E box, which *Mitf* can bind to and from which it can activate transcription. For performing the assay, 293 cells were grown to 80% confluency (as judged by eye) in six-well plates in Dulbecco's Modified Eagle's Medium (DMEM) with fetal BSA but without antibiotics. Before transfection, the BSA was washed off using DMEM and the cells were then transfected using LipoFectamin-PLUS (Invitrogen, San Diego) according to manufacturer's instructions. Each pool of DNA was transfected in triplicate and the luciferase activity assayed using the Promega dual luciferase kit on a Wallac Victor^2 luminometer (Perkin-Elmer Life Sciences), 12 hr after transfection. For normalizing the assay, a cytomegalovirus-driven Renilla luciferase was used. A total of 800 ng of each DNA was used and, where appropriate, empty pCDNA3.1 vector was used to bring the total amount of DNA to equal levels.

Expression analysis: Embryonic *in situ* hybridization was carried out with two different probes, a full-size probe made from the cDNA clone LD45277 and a shorter probe that lacks an *Mfe*I fragment containing the basic helix-loop-helix leucine zipper domains. The latter probe was used to prevent false signals due to cross-reactions with other mRNAs coding bHLH-Zip proteins. The probe was labeled using the Boehringer Mannheim (Indianapolis) DIG RNA labeling kit according to instructions. Whole-mount *in situ*s on embryos were performed according to Brody *et al.* (2002). Both probes resulted in the same expression pattern. Imaginal disc *in situ* hybridizations were also carried out with both probes, and no difference was seen between the two probes. The sense probe was used as a negative control and did not show any specific staining in either embryos or discs.

***In vivo* expression studies:** A *pUAS-Mitf*^{WT} construct was generated by introducing the *Bgl*II-*Xho*I fragment of the LD45277 cDNA clone into *Bgl*II-*Xho*I-digested pUAST vector DNA (Brand and Perrimon 1993). For *pUAS-Mitf*^{EA}, cloning into pUAST was at the *Not*I-*Xho*I sites and the *Mitf* sequence was mutagenized as described above (see *Cotransfection studies*). Transgenic lines carrying these constructs were generated by *P*-element transformation. Expression was achieved using the Gal4/UAS system (Brand and Perrimon 1993) by crossing Gal4 driver lines to flies carrying *pUAS-Mitf*^{WT} or *pUAS-Mitf*^{EA} transgenes as described in the results. Immunostaining with mouse monoclonal anti-ELAV Ab (Iowa Developmental Hybridoma Bank) was carried out using standard protocols (Wolff 2000). Few adult escapers were also observed in the cross to the *ey-gal4* driver. These flies displayed eye phenotypes consistent with the effects seen in the developing discs, *i.e.*,

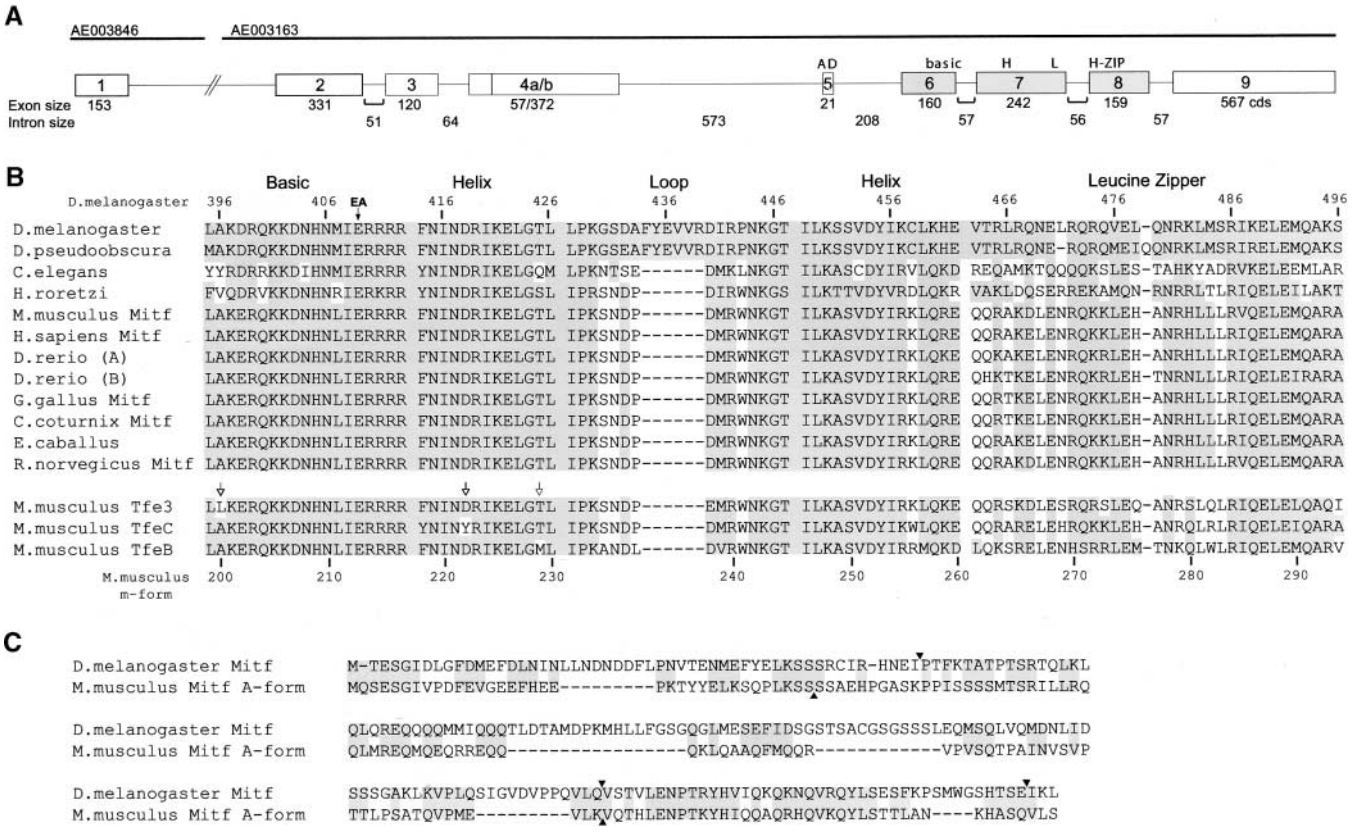

FIGURE 1.—Conservation of the *Mitf* gene in Drosophila. (A) The genomic clones described in the study and the exon-intron structure of the *Dmel/Mitf* gene: exons are shown as boxes. The unsequenced gap region is indicated and the sizes of the different exons and introns are shown. Note that the total size of the *Dmel/Mitf* genomic region is currently not known due to the gap between the two clones. The exon/intron splice sites that are conserved between mouse and Drosophila are indicated by brackets. The exons containing the bHLH-Zip region are shaded. Note that the vertebrate melanocyte-specific alternative first exon (Exon 1M) is not found in the Drosophila gene (see DISCUSSION for comments on this finding). (B) Conservation of the *Dmel/Mitf* amino acid sequence. The bHLH-Zip regions of the Drosophila *Mitf* gene are compared to the *Mitf* gene in most species where the sequence is currently known as well as to the mouse *Tfe3*, *Tfeb*, and *Tfec* genes. Shaded residues indicate amino acids conserved (identical and similar) between Drosophila and the mouse *Mitf*, *Tfe3*, *Tfec*, and *Tfeb* genes. The amino acids are numbered on the basis of the full-length *Dmel/Mitf* protein and on the mouse melanocyte-specific M form (HODGKINSON *et al.* 1993). Arrows above the Tfe sequences indicate differences between the *Mitf* and the *Tfe* genes. The arrow labeled EA indicates the amino acid changed to produce a dominant-negative form of *Dmel/Mitf*. (C) The amino acid sequence of the first three exons of *Dmel/Mitf* compared to the amino terminus of the mouse Mitf A form. This conservation suggests that the A exon is the ancestral 5′ exon. The arrowheads indicate splice junctions.

reduced eyes (*pUAS-Mitf*^{WT}) and split or enlarged eyes (*pUAS-Mitf*^{EA}).

RESULTS

Sequence and genomic structure of the Drosophila *Mitf* gene: The Drosophila *Mitf* gene was identified by comparing the mouse *Mitf* amino acid sequence (bHLH domain) to the *D. melanogaster* genome sequence (ADAMS *et al.* 2000) translated in all six reading frames using tBLASTn (ALTSCHUL *et al.* 1990). The structure of the Drosophila *Mitf* gene is shown in Figure 1A. The gene covers at least 5.5 kb of genomic sequence. However, the total genomic size could not be determined due to a gap in the sequence in the intron between exons 1 and 2 (Figure 1A). At the adjacent ends of the two clones (A0E03846 and AE003164) are repetitive elements, which, when aligned, show an extensive sequence overlap. However, the end sequences of the two clones do not allow perfect alignment and all attempts at closing the gap using PCR have so far failed. The *Dmel/Mitf* gene is composed of nine exons, at least one of which is alternatively spliced (exon 4) as judged from comparing the two cDNA clones LP03467 and LD45277. Alternative splicing is a feature previously demonstrated to be both common and important for the mouse *Mitf* gene (HALLSSON *et al.* 2000) as well as for other members of the mouse *Mitf-Tfe* family (ROMAN *et al.* 1991). The alternatively spliced isoforms (a and b) of *Dmel/Mitf* maintain the same open reading frame and can therefore give rise to protein products that differ only in the first 19 amino acids of exon 4 (Figure 1A). No

functional domains have been identified in this part of the protein and currently it is not clear if these splice forms represent functionally important alternative mRNAs.

By aligning the cDNA of Drosophila *Mitf* with mouse *Mitf* and considering the splice junctions, it can be seen that three of eight splice junctions present in the Drosophila cDNA are conserved between the two species. One of these is the splice junction between exons 2 and 3 in Drosophila (corresponding to exons 1B and 2 in the mouse counterpart). The other conserved junctions are between exons 6 and 7 and 7 and 8, which in both organisms contain the highly conserved basic and HLH domains (Figure 1A).

Conservation of the functional domains of the Mitf protein: The *Dmel/Mitf* gene encodes a 729-amino-acid protein, all exons included. This is considerably longer than the 525 amino acids encoded by the A form of the mouse *Mitf* gene, the longest known mouse form. Although overall amino acid homology is only 31%, certain domains of the protein have considerably higher conservation. As expected, the greatest amino acid homology between mouse and Drosophila is found in the bHLH-Zip domain. In both cases the basic domain is contained within exons 6 and 7; of 19 amino acids in this region, 17 are conserved between the two species. The 2 amino acids that differ are conservative substitutions: glutamate (at position 202) in the mouse *vs.* aspartate in Drosophila and leucine (at position 211) in the mouse *vs.* methionine in Drosophila (Figure 1B). The first helix of the HLH domain is completely conserved while in the loop 8 of 14 amino acids are conserved. A striking difference between the two proteins is the length of the loop. The mouse loop is 14 amino acids while in *Dmel/Mitf* it is 20 amino acids long. The second helix contains 16 amino acids and, of those, 11 are conserved between the two species. Interestingly, the leucine zipper of *Dmel/Mitf* consists of only two leucines while the mouse zipper is generally considered to consist of four. A possible explanation for this difference might be that the longer zipper evolved in vertebrates to increase the specificity of protein-protein interactions as more dimerization partners such as the related Tfe proteins became available. Although multiple differences exist between the *Dmel/Mitf* genes in *D. melanogaster* and *D. pseudoobscura* (data not shown), the bHLH-Zip domains are highly similar in the two species (Figure 1B). Comparing Drosophila *Mitf* to known homologs in other vertebrate species leads to the same conclusion as little diversity is found in the bHLH region of the protein. Species as diverse as humans and zebrafish show almost total conservation of the bHLH domains (Figure 1B).

Additional small regions of conservation are present outside the bHLH-Zip region. A block of amino acids at the amino terminus of the *Dmel/Mitf* protein shows considerable homology to the mouse A form, the splice version proposed to be the most ancient of the different *Mitf* transcripts in the mouse, also referred to as the eye-specific form (Amae *et al.* 1998; Figure 1C). Included in this region is a glutamine-rich domain. Although no functional importance has been assigned to this portion of the protein, the conservation of the second splice junction supports the idea that this exon is conserved and may be relevant for normal protein function. A block of conserved amino acids is found in Drosophila exon 3 and shows homology to mouse exon 2A (Figure 1C). Of the first 30 amino acids of this exon, 17 are identical in the two proteins and 7 additional amino acids represent conservative substitutions. No particular function has been assigned to this region of the protein. The *Dmel/Mitf* counterpart to mouse serine 73, an amino acid shown to be phosphorylated by mitogen-activated protein (MAP) kinases in response to extracellular signals, is conserved. Furthermore, serine amino acids corresponding to both Ser298 and Ser409 are conserved even though not much conservation is found surrounding these amino acids (not shown). Close to the C terminus of the protein (25 amino acids from the end of mouse *Mitf* and 50 amino acids from the end of *Dmel/Mitf*) is a stretch of 6 conserved amino acids (DPLLSS). The function of these amino acids has not been determined. Finally, a short stretch of amino acids of *Dmel/Mitf* (DDIFDDIL) is remarkably similar to the DDVIDEII sequence, which in the TFE3 protein has been suggested to play a role in transcriptional activation (Beckmann *et al.* 1990). Although the similarity of these regions suggests functional importance, aligning the two regions creates a big gap in the overall alignment of Drosophila and mouse MITF proteins and thus decreases the overall homology of the alignment. Conservation of a domain may be more important than conservation of its exact location in the protein.

***Dmel/Mitf* functions as a transcriptional activator:** Cotransfection experiments were performed to determine if the Drosophila *Mitf* gene can activate gene expression from promoter elements known to be regulated by the mouse and human *Mitf* genes. The wild-type *Dmel/Mitf* cDNA and a mutant version of *Dmel/Mitf* were cloned into the eukaryotic expression vector pCDNA3.1. The mutant version, $Mitf^{EA}$ (arrow in Figure 1B, labeled EA), changes the most conserved amino acid within the DNA-binding domain. This amino acid is known to be essential for the DNA-binding ability of the related *Tfeb* protein and, since bHLH proteins are known to bind DNA as dimers, this mutation is thought to result in a dominant-negative protein (Fisher *et al.* 1993).

The constructs were cotransfected into 293T human embryonic kidney cells together with a reporter construct containing six tandem M boxes. The M box is an 11-bp sequence that contains an E box; the mouse Mitf protein is known to bind to and activate gene expression from this site. As can be seen from Figure 2A, the wild-type Drosophila *Mitf* construct is able to activate gene expression sevenfold compared to the empty vector,

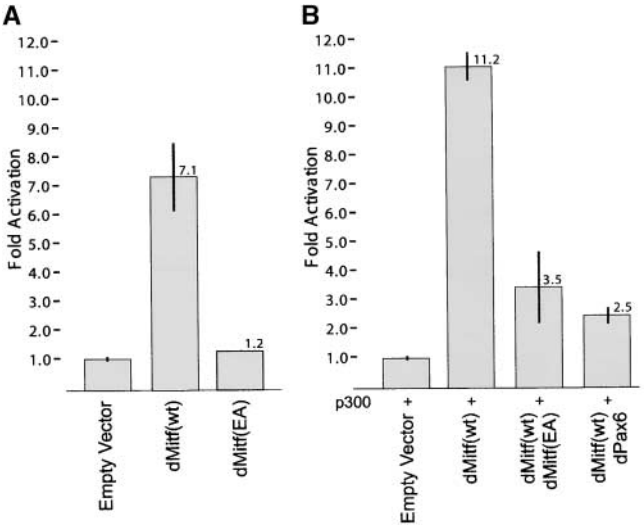

FIGURE 2.—*Dmel/Mitf* activates gene expression. (A) Cotransfection experiments were performed in 293T cells with wild-type and mutant versions of the *Dmel/Mitf* gene and a promoter element containing six M boxes. Each column is based on four individual transformations and two measurements of each sample. Renilla was used as a control for transfection efficiency. The results are shown as fold induction as compared to empty vector. (B) The Eyeless/Pax6 protein interferes with transcription activation by *Dmel/Mitf*. Cotransfection experiments were performed in 293T cells with the wild-type *Dmel/Mitf* and Eyeless/Pax6 genes and a promoter element containing six M boxes.

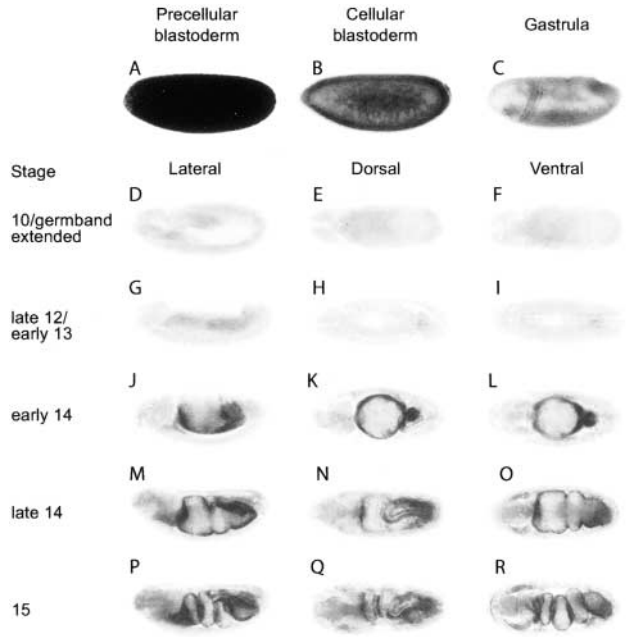

FIGURE 3.—Expression of the Drosophila *Mitf* gene during embryonic development. Whole-mount *in situ* hybridizations: anterior is left in all views and developmental staging is according to HARTENSTEIN and CAMPOS-ORTEGA (1984). (A and B) Maternal *Mitf* transcripts are detected throughout pregastrula Drosophila embryos. (C–F) During gastrulation and through germ-band extension there is a progressive loss of *in situ* hybridization signal in all tissues such that, by late stage 10, little or no *Mitf* transcript is detected. (G–R) Zygotic expression is first detected in cells surrounding the developing midgut and hindgut during late stage 12. By embryonic stage 15, *Mitf* transcripts are observed in most, if not all, midgut and hindgut epithelial cells.

while the mutant version is unable to activate gene expression. Thus, clearly, the Drosophila MITF protein can activate gene expression from this regulatory element, just as the vertebrate factors do. Also, when the $Mitf^{EA}$ mutant version and wild-type *Mitf* constructs are cotransfected with the reporter, less activation is observed (Figure 2B), as would be expected if the EA mutant version acts in a dominant-negative fashion by interfering with DNA binding of the normal protein.

In cotransfection assays, the mouse MITF and PAX6 proteins interact and mutually inhibit transcription activation (PLANQUE *et al.* 2001). To test if this was also true for the Drosophila MITF and PAX6 (Eyeless) proteins, expression constructs containing the two genes were cotransfected into 293T cells together with the 6xM-box reporter construct and p300. As shown in Figure 2B, the presence of the *ey* gene interferes with the transcription activation potential of the *Dmel/Mitf* gene. We conclude that the function of *Dmel/Mitf* as a transcription activator is conserved between vertebrates and invertebrates, as is its potential interaction with Eyeless/Pax6.

***Dmel/Mitf* is expressed in the embryo and in the eye-antennal imaginal disc:** To determine the expression pattern of *Dmel/Mitf* during Drosophila development, *in situ* hybridizations were carried out on embryos and eye-antennal imaginal discs. Two different probes were used, one representing the full-length cDNA and another lacking the bHLH-Zip region. The *in situ*s revealed strong *Dmel/Mitf* expression in the precellular stages of embryonic development (Figure 3), representing maternally deposited *Mitf* message. As cellularization progresses, the amount of *Mitf* message decreases and is considerably reduced by gastrulation. During embryonic stages 9–11 expression is very low or undetectable. Expression then reappears by early stage 12 in the gut and stays high until stage 15, at which time the probe can no longer penetrate the embryo due to cuticular formation. Strong expression is seen in the epithelium of the midgut and hindgut, even though the possibility cannot be excluded that the signal is in the thin layer of visceral mesoderm surrounding the gut epithelium. No signal was detected using the sense control probe.

In the eye-antennal imaginal disc, *Dmel/Mitf* shows a dynamic pattern of expression during the second and third larval stages (L2 and L3). In L2 discs, *Dmel/Mitf* transcripts were detected throughout the eye region (Figure 4A). In L3 discs, *Dmel/Mitf* expression was restricted to two distinct domains. One expression domain lies between the eye and antennal regions (Figure 4B). The second domain (Figure 4C) is located in the region

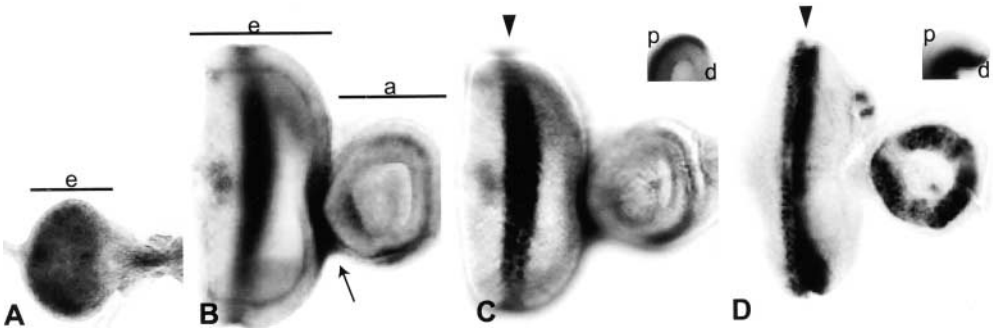

Figure 4.—Expression of *Mitf* in Drosophila eye-antennal discs. Discs are oriented with dorsal up and posterior to the left. *Dmel/Mitf* and *atonal* mRNAs were detected by *in situ* hybridization. (A) L2 disc. (B–D) Late L3 discs. In addition to the domains of expression described below, *Mitf* expression was also detected in the antennal region. However, expression within this region was highly variable from disc to disc, suggesting that expression in this region is dynamic. (A) *Mitf* is expressed broadly across the eye region (bracket). (B) *Mitf* expression is seen in a domain (arrow) lying between the eye (e) and the antennal (a) regions of the disc. (C) A second domain is present in the peripodial cell layer over the morphogenetic furrow (arrowhead). Inset shows a cross-section of the margin of the disc in the area of *Mitf* expression; staining is found in the peripodial cell layer (p) as opposed to the disc proper cell layer (d); compare to inset in D. (D) Staining for *atonal*, a gene expressed within the MF in the disc proper cell layer, is shown for comparison. Inset shows a cross-section of the margin of the disc in the area of *atonal* expression; staining is found in the disc proper cell layer (d) as opposed to the peripodial cell layer (p).

of the morphogenetic furrow (MF), where cells of the disc proper layer stop dividing and a subset commit to a neuronal fate. Posterior to the MF, cells do not divide and neurons differentiate into photoreceptors; anterior to the MF, cells are uncommitted and proliferating. In this second domain, *Dmel/Mitf* is expressed in the peripodial membrane, *i.e.*, the cell layer that overlies the disc proper (inset in Figure 4C).

Expression of dominant-negative *Dmel*/Mitf in the larval disc interferes with eye development: To investigate the potential role of *Dmel/Mitf* in fly eye development, we expressed the wild-type ($Mitf^{WT}$) or dominant-negative ($Mitf^{EA}$) forms in the developing eye-antennal disc. As expression of endogenous *Dmel/Mitf* appears to be restricted to the peripodial layer, we tested two Gal4 drivers to activate expression from UAS transgenes. The *c311* driver (Gibson and Schubiger 2000; data not shown) expresses throughout the peripodial layer of the eye-antennal disc at the L2 stage and is still expressed in the peripodial membrane over the antennal region, but not the eye region, in late L3. The *ey-Gal4* (Hazelet *et al.* 1998; Kenyon *et al.* 2003) driver expresses throughout the disc proper and peripodial cell layers until mid L2. Thereafter, expression is progressively lost from the antennal region and becomes restricted to the peripodial and disc proper layers of the eye region. In L3, *ey-Gal4* expression decreases in the peripodial layer but continues to be robustly expressed in the disc proper. Both drivers are also expressed in other larval tissues. Expression of Drosophila $Mitf^{WT}$ or $Mitf^{EA}$ under the peripodial driver resulted in early larval lethality likely due to expression in tissues other than the imaginal discs. Larvae expressing Drosophila $Mitf^{WT}$ or $Mitf^{EA}$ under ey-Gal4 control most often died later and could be analyzed at the L3 stage. Expression of $Mitf^{WT}$ resulted in eye-antennal discs with smaller eye regions but generally normal or nearly normal antennal regions. Neuronal morphogenesis, as detected by expression of the neural marker ELAV, was always reduced and occasionally absent (compare disc in Figure 5B to wild type in Figure 5A). On the contrary, the eye regions of discs expressing the $Mitf^{EA}$ mutant protein were enlarged as compared to wild type and the developing photoreceptor field appeared correspondingly expanded (compare disc in Figure 5C to wild type in Figure 5A).

DISCUSSION

Here we describe the identification and initial characterization of *Dmel/Mitf*, the Drosophila homolog of the vertebrate bHLH-Zip transcription factor gene *Mitf*. Like its vertebrate counterpart, *Dmel/Mitf* can activate a known Mitf reporter *in vitro* and this transcriptional activation is sensitive to regulation by Eyeless/Pax6. Targeted expression of wild-type or dominant-negative forms of *Dmel/Mitf* results in opposite effects on the development of the eye-disc region and suggests that Mitf's role in eye development is at least partially conserved between fly and vertebrates. We discuss below the implications of this conservation at the molecular and developmental levels.

Conservation of Mitf at the DNA and protein levels: Despite extensive genome-wide searches for basic-helix-loop-helix proteins in the Drosophila genome, no *Mitf* or *Mitf*-related genes were found in previous analyses (Moore *et al.* 2000; Peyrefitte *et al.* 2001). This suggests that the *Dmel/Mitf* gene described here is the only family member found in the Drosophila genome. This is in sharp contrast to vertebrate genomes, which, in addition to *Mitf*, contain the three other closely related genes *Tfeb*, *Tfe3*, and *TfeC* (discussed below). Furthermore, the zebrafish genome contains two *Mitf* genes (*nacre/Mitfa* and *Mitfb*; Lister *et al.* 1999, 2001) in addition to a presumed unknown number of *Tfe* genes. Other fish species, including Xiphophorus, *Fugu rubripes*, and *Tetraodon nigroviridis*, also contain two *Mitf* genes in their

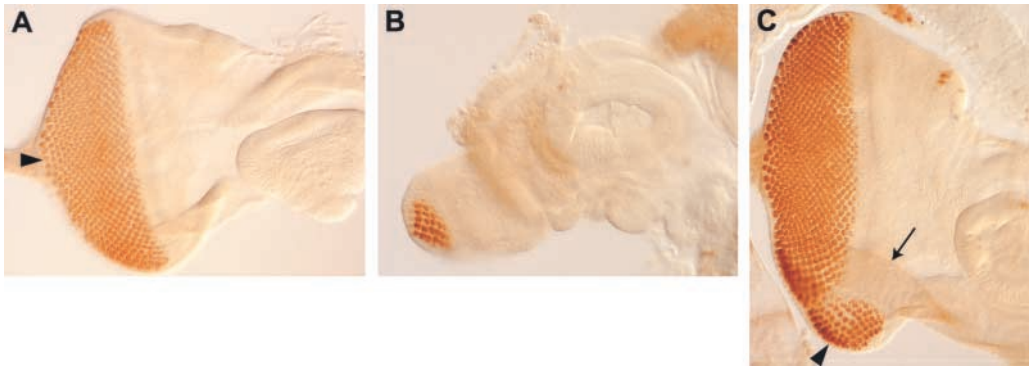

Figure 5.—Targeted expression of wild-type and dominant-negative *Mitf*. Discs are oriented with dorsal up and posterior to the left. Development of the photoreceptor neurons is detected by staining for the pan-neural marker ELAV. (A) L3 wild-type disc. Neuronal development in the eye field initiates at the posterior margin (arrowhead) and progresses anteriorly. (B) L3 *ey-Gal4 UAS-Mitf*^{WT} disc. Disc size is reduced and neuronal field is similarly affected. (C) L3 *ey-Gal4 UAS-Mitf*^{EA} disc. Discs are larger than wild type and often display folds (arrow) within the eye field epithelium. The neuronal field is similarly expanded. Occasionally, neuronal development also initiates from the ventral margin of the disc (arrowhead) as well as from the posterior.

genomes, suggesting a gene duplication event in teleost fish after their separation from the bird/mammalian lineage (Altschmied *et al.* 2002). Studies on *Mitf* function should therefore be greatly simplified in Drosophila as compared to studies in vertebrate species.

Within the MYC supergene family of basic bHLH-Zip transcription factors, the *Mitf* gene is most closely related to the *Tfeb, Tfec*, and *Tfe3* genes. Together these four proteins form the *Mitf-Tfe* subfamily of bHLH-Zip proteins. All four proteins share almost identical basic regions and very similar HLH and Zip regions; the sequence is quite divergent outside these domains. It is likely that the *Mitf* gene is most closely related to the ancestral form of the *Mitf-Tfe* family of proteins since there are more similarities between *Dmel/Mitf* and the mouse *Mitf* genes than between *Dmel/Mitf* and any of the three *Tfe* genes (arrows in Figure 1B). For example, the mouse *Tfec* and *Mitf* genes differ at two positions in the helix 1 domain (YNINY in *Tfec vs.* FNIND in *Mitf*) whereas the mouse and fly *Mitf* genes are identical. Similarly, the mouse *Mitf* and *Tfe3* genes are different in one position in the basic domain (LLKE in *Tfe3 vs.* LAKE in *Mitf*) and the *Mitf* and *Tfeb* genes are different in one position in helix 1 (LGML in *Tfeb vs.* LGTL in *Mitf*). All these residues are conserved in the mouse and fly *Mitf* genes, suggesting that the *Mitf* gene is the common ancestor and that the *Tfe3*, *Tfeb*, and *Tfec* genes arose from the ancestral gene after the separation of the vertebrate and invertebrate lineages.

The Drosophila *Mitf* protein is considerably larger than its vertebrate counterpart. In addition to the highly conserved bHLH-Zip domains, several other conserved regions were identified, suggesting that they represent regions of functional importance. These include a glutamine-rich region at the amino terminus, an amphipathic helical region with a transcription activation function, and a stretch of six amino acids at the carboxy end. In addition, a serine amino acid—which in the mouse MITF protein is phosphorylated by the MAP kinase pathway (Hemesath *et al.* 1998; Wu *et al.* 2000)—is also conserved in *Dmel/Mitf*. Thus, regulation of *Dmel/Mitf* function may involve phosphorylation at this site.

Significant differences also exist between vertebrate and fly Mitf. Most notably, in the mouse, an additional first exon (1M) codes for 11 amino acids and is included in a melanocyte-specific form of the *Mitf* mRNA, the M form. We have not been able to find sequences corresponding to exon 1M near the *Dmel/Mitf* gene. If we assume that the order of exons in the gene is conserved between mouse and Drosophila, then exon 1M would be situated between exons 2 and 3. However, the intron between exons 2 and 3 in Drosophila is only 51 nucleotides long and does not include an ATG. This lack of conservation is not unexpected. Vertebrate melanocytes originate from the neural crest, a cell lineage with no counterpart in Drosophila. Although the Drosophila eye does contain pigment cells, these arise from the eye-antennal epithelium and their pigment granules (ommochromes and drosopterins) are chemically distinct from the melanosomes (melanins) of vertebrates. Hence, fly pigment cells are not evolutionarily related to melanocytes. In this respect, exon 1M may reflect an evolutionary modification of the ancestral *Mitf* gene that arose specifically in the vertebrate lineage. Consistent with that, none of the related *Tfe* genes have an M-like exon, suggesting that this exon arose after the *Tfe* genes had arisen from the ancestral *Mitf* gene. Although the recently characterized *Mitf* gene of the ascidian *Halocynthia roretzi* is expressed in pigment lineage blastomeres, it does not appear to contain sequences that resemble exon 1M (Yajima *et al.* 2003). Interestingly, the ascidian *Mitf* gene is expressed maternally, like its Drosophila counterpart.

RPE *vs.* peripodial epithelia—a case of common ancestry? The *Mitf* gene is expressed in the mouse eye during the optic vesicle and optic cup stages of eye development and is required for the normal formation and maintenance of the RPE. The RPE is a single layer of cuboidal cells, which basally displays numerous infoldings while apically abundant microvilli enclose and interdigitate

with rod outer segments (Braekevelt and Hollenberg 1970; Braekevelt 1988, 1990). In *Mitf*$^{mi/mi}$ mutant mice, the RPE apical microvilli are absent and elongated rod outer segments do not develop (Bumsted *et al.* 2001). During development, the retina and RPE cell layers are closely juxtaposed and recent evidence supports an early role for the RPE in morphogenesis of the neural retina. Genetic ablation of the RPE cells early during eye formation prevents lamination of the retina, and later ablation results in loss of laminar organization (Raymond and Jackson 1995). In addition, factors secreted by the RPE have been shown to positively influence the development and maintenance of normal retinal morphology (Gaur *et al.* 1992; Sheedlo *et al.* 1992; Sheedlo and Turner 1998; Jablonski *et al.* 2000). Thus, the RPE is thought to be a source of signaling molecules that lead to proper patterning and maintenance of the vertebrate retina.

The Drosophila eye, albeit structurally very different from the mouse eye, also develops from a bilayered epithelial structure. The progenitor epithelium that gives rise to the adult fly eye and associated head cuticle consists of a flattened sac with a columnar "disc proper" cell layer (from which the retina develops) and a noncolumnar "peripodial" cell layer (from which mostly cuticle, or epidermis, will form). Until recently the peripodial cell layer was not thought to be directly involved in retinal morphogenesis and it does not in fact contribute directly to any part of the adult eye (as mentioned above, these cells give rise to head cuticle). However, two groups (Cho *et al.* 2000; Gibson and Schubiger 2000) have recently shown that peripodial cells are in fact required for proper development of the retina. In addition, Schubiger and colleagues (Gibson and Schubiger 2001; Gibson *et al.* 2002) have shown that cellular projections, named "transluminal" projections, extend from one layer to the other and provide a mechanism for direct interactions between these two layers. Thus, it is now thought that signaling occurs between cells of peripodial and disc proper layers and that these interactions are essential for proper retinal development. The expression of *Dmel/Mitf* in the peripodial cell layer, specifically in the portion of the peripodial membrane that overlooks the site of photoreceptor neuron formation (MF), suggests that Mitf may be involved in this process.

To investigate the potential role of *Dmel/Mitf* in eye development, we expressed the wild type and dominant-negative Mitf^{EA} mutant in the developing eye-antennal disc. Discs expressing wild-type *Dmel/Mitf* were variably reduced in size and neuronal morphogenesis was always reduced and occasionally absent. On the contrary, discs expressing the Mitf^{EA} mutant version were larger than wild-type discs and the developing photoreceptor field appeared correspondingly expanded. The striking effect on disc size likely reflects changes in proliferation, whereas the variation in neuronal field size may be secondary to this or result from effects on primordia formation (cuticle/peripodial *vs.* eye) within the epithelium. As vertebrate *Mitf* has been implicated in proliferation and RPE specification (Nguyen and Arnheiter 2000), our observations strongly suggest significant conservation of *Mitf*'s role in eye development. Further investigation of *Dmel/Mitf* function awaits the generation of *Dmel/Mitf* mutant alleles and better peripodial-specific drivers. Nonetheless, the similarities we have uncovered between the peripodial membrane of the fly eye-antennal disc and the RPE of the vertebrate optic vesicle/cup are very intriguing. The expression of *Mitf* in both epithelia raises the possibility that these tissues are evolutionarily related. In such a scenario, the ancestral tissue from which the eye eventually formed may have already displayed a partition into two fields: a nonneural Mitf^{+}/Pax6^{-} field and a neural Mitf^{-}/Pax6^{+} field. Moreover, development of these two fields may have already involved inductive events between juxtaposed cell layers. Parallel investigations of Mitf function in mouse and fly will provide useful insights in evaluating these hypotheses.

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