muscleblind, a gene required for photoreceptor differentiation in Drosophila, encodes novel nuclear Cys$_3$His-type zinc-finger-containing proteins

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SUMMARY

We have isolated the embryonic lethal gene muscleblind (mbl) as a suppressor of the sev-svp2 eye phenotype. Analysis of clones mutant for mbl during eye development shows that it is autonomously required for photoreceptor differentiation. Mutant cells are recruited into developing ommatidia and initiate neural differentiation, but they fail to properly differentiate as photoreceptors. Molecular analysis reveals that the mbl locus is large and complex, giving rise to multiple different proteins with common S' sequences but different carboxy termini. Mbl proteins are nuclear and share a Cys$_3$His zinc-finger motif which is also found in the TIS11/NUP475/TTP family of proteins and is highly conserved in vertebrates and invertebrates. Functional analysis of mbl, the observation that it also dominantly suppresses the sE-JunAasp gain-of-function phenotype and the phenotypic similarity to mutants in the photoreceptor-specific glass gene suggest that mbl is a general factor required for photoreceptor differentiation.

Key words: Drosophila, photoreceptor differentiation, nuclear protein, zinc-finger domain

INTRODUCTION

Inductive signalling plays a crucial role in the patterning of many tissues and organs in vertebrates and invertebrates. The assembly and patterning of the Drosophila eye has served as a good model system to study such inductions and signalling pathways (for reviews see Wolff and Ready, 1993; Dickson and Hafen, 1993). Ommatidial assembly follows an invariant sequence of cell fate inductions. The photoreceptor R8 cell is the first to differentiate, followed by the sequential and pairwise addition of photoreceptors R2/5 and R3/4. Following a new round of mitosis, the remaining cells are being recruited to the growing ommatidial cluster: photoreceptors R1/6, R7, the non-neuronal cone cells and the other accessory cells (reviewed in Tomlinson, 1988; Ready, 1989). Over the past few years, a wealth of information has been accumulated leading to a fairly good understanding of the signalling pathways during ommatidial assembly and photoreceptor development. Activation of the Ras/EGF-receptor pathway causes induction of precursor cells as photoreceptor neurons (reviewed by e.g. Zipursky and Rubin, 1994; Freeman, 1997). This is followed by the establishment of distinct photoreceptor subtypes, probably achieved by the expression of different specific factors (e.g. Chang et al., 1995; Dickson et al., 1995). Much less is known about the molecular basis of subsequent differentiation that leads to the establishment of functional photoreceptor neurons.

The differentiation of each photoreceptor subtype is accomplished by a series of intercellular signals between differentiating cells and uncommitted precursor cells. Pivotal to this process is the activation of the Ras-mediated signalling pathway via the activation of at least two distinct receptor tyrosine kinases, the Drosophila EGF-receptor (DER; Xu and Rubin, 1993; Freeman, 1996), and Sevenless (Sev; Hafen et al., 1987; Simon et al., 1991; Fortini et al., 1992). DER-mediated activation of the Ras pathway is required for the initial determination of all cells in the ommatidium, including cone and pigment cells (Freeman, 1996). This is reflected in the transient activation or inactivation of the nuclear proteins Pointed (Pnt) and Jun (Brunner et al., 1994; O’Neill et al., 1994; Bohmann et al., 1994; Treier et al., 1995), and Yan (Rebay and Rubin, 1995), respectively (reviewed in Dickson, 1995). The Sevenless kinase is activated specifically in the R7 cell by its ligand Boss (reviewed in Zipursky and Rubin, 1994). It appears that Sevenless enables the R7 precursor to continue differentiation by a second burst of Ras signalling, since a number of genes marking an earlier differentiation are already expressed in this cell prior to Sevenless activation (e.g. klingon (H214) and prospero; Mlodzik et al., 1992; Kauffmann et al., 1996; Butler et al., 1997).
Precursor cells of the outer photoreceptors, R1-6, express specific transcription factors that determine their developmental fate. One of these is Seven-up (svp), a member of the nuclear hormone receptor superfamily, which is required for R3/4 and R1/6 subtype identity (Mlodzik et al., 1990). Svp acts cell autonomously and, in the absence of its function, these precursors do not realise their developmental program, but develop as R7 cells instead. Misexpression experiments have shown that Svp is able to confer neuronal development upon the non-neuronal cone cell precursors, acting as a positive regulator of neuronal fate rather than inhibiting R7 development (Begemann et al., 1995; Kramer et al., 1995). Expression of svp is negatively regulated by the AML/runt family transcription factor Lozenge (lz) (Daga et al., 1996). Lz restricts svp expression to the four outer photoreceptors, as eye disc mutants for a null allele of lz ectopically express svp in precursors of R7 and the cone cells. Lz and svp are also required for the R1/R6-specific expression of Bar/H1/H2, and svp controls the R3/R4 expression of pipsqueak (Higashijima et al., 1992; Weber et al., 1995; Daga et al., 1996).

Once a precursor cell fate has been established, an as yet poorly characterised set of genes ensures the subsequent cellular morphogenesis of photoreceptors, which concludes with the differentiation of the highly specialised light harvesting structures, the rhabdomeres. A well-studied gene in this context is the zinc-finger-containing transcription factor Glass, which was shown to directly activate transcription of rhodopsin I (Moses and Rubin, 1991) and chaoptin, another late marker of photoreceptor differentiation (Moses et al., 1989). Glass (Gl) is generally required for photoreceptor differentiation. This is exemplified in weak gl alleles, where photoreceptor cells die in mid-pupal stages when they normally would produce the photosensitive organelles (Ready et al., 1976). Mutations in elav (Homky et al., 1985), ultraspireacle (Oro et al., 1992) and orthodenticle (Vandendries et al., 1996) show defects in photoreceptor cell morphology, such as disorganisation or disruption of the rhabdomeres. The role of these genes in eye development remains, however, unclear.

Transgenic Drosophila lines that ectopically express Svp in R7 and cone cell precursors (sev-svp2) have a dosage-sensitive rough eye phenotype due to the transformation of an increasing number of cone cells into photoreceptors (Hiromi et al., 1993; Begemann et al., 1995). To isolate new genes that are required in photoreceptor development or differentiation, we have taken advantage of the dosage sensitivity of the sev-svp2 phenotype and screened for mutations that dominantly modify this phenotype. This approach has previously identified components of the DER/Ras pathway as essential factors for Svp function (Begemann et al., 1995; Kramer et al., 1995), and also new genes required for photoreceptor development, patterning and differentiation (G. B., Caryl A. Mayes and M.M., unpublished).

Here, we report the identification of the embryonic lethal mutation muscleblind (mbl). We show that mbl acts as a dominant suppressor of the sev-svp2 phenotype. Clones mutant for mbl indicate that it is autonomously required for photoreceptor differentiation. Mutant cells are recruited into developing ommatidia and initiate neural development, but they fail to properly differentiate, e.g. to form rhabdomeres. Molecular analysis reveals that the mbl locus is large (>100 kb) and complex, giving rise to different proteins with common S sequences but different lengths and carboxy termini. Mbl proteins are nuclear and share a Cys/His zinc-finger motif, which is also found in the TIS11/NUP475/TTP family of proteins and is highly conserved in vertebrates and invertebrates. Functional analysis of mbl and the observation that it also dominantly suppresses the se-Jun\(^{Apx}\) gain-of-function phenotype suggest that it is a gene required for photoreceptor differentiation in general.

**MATERIALS AND METHODS**

**Fly strains**

The 2×sev-svp2 line used in the screen (Begemann et al., 1995) carries two insertions of the sev-svp2 construct on chromosome III (Hiromi et al., 1993). The se-Jun\(^{Apx}\) line used for the isolation of mbl\(^{E27}\) is as described (Treier et al., 1995). Several P-element lines were used for the characterisation of the mbl locus: PP2 was a gift of A. Martinez-Arias (University of Cambridge, UK), l(2)k05507 was originally isolated in the screen of the P-element collection (Török et al., 1993) and l(2)k2563 was isolated in the se-Jun\(^{Apx}\) screen from a P-element collection covering both the second and third chromosome (Erdelyi et al., 1995). To confirm that the observed suppression and phenotypes were linked to these P-insertions, the l(2)k05507 and l(2)k2563 P-lines were mobilised by providing an external source of transposase (Robertson et al., 1988) and their progeny was screened for flies that had lost the P[white\(^{+}\)] marker. For both P-elements, several excisions resulted in homozygous viable flies that lost their ability to suppress se-vsvp or se-Jun\(^{Apx}\). In addition, several imprecise lethal excisions were recovered in these experiments. Some of these were used in the present study: mbl\(^{E27}\) and mbl\(^{E27}\) are derived from l(2)k05507, and mbl\(^{E27}\) from l(2)k2563.

cDNA isoforms A and B were cloned into the transformation vector pKB267PL (Basler et al., 1991; Zeidler et al., 1996), which carries the eye-disc-specific sev-enhancer expression module driving expression in the precursors for R3/R4, R7 and the cone cells, and weaker in the mistery cells and R1/6 during ommatidial assembly.

Glass-driven overexpression was achieved by cloning the cDNA of isoform mblA into the pGMR vector (Hay et al., 1994). Germline transformation was performed by standard procedures (Spradling and Rubin, 1982).

**Somatic recombination and clonal analysis**

Somatic clones were induced by X-ray with 1000 rads in 1st instar larvae, using either a P[white\(^{+}\)] element at 49D as autonomous clonal marker for excision alleles or a w\(^{-}\) background for the P insertion lines. Homozygous clones of mbl\(^{E27}\) and mbl\(^{E27}\) mutants were generated in imaginal discs with the FLP/FRT system (Xu and Rubin, 1993). Larvae of the genotype w;hsFLP1; P[ry\(^{+}\); hs-neo; FRT] 43D, mbl\(^{E27}\) or mbl\(^{E27}\); P[ry\(^{+}\); hs-neo; FRT] 43D, P[mini-w\(^{+}\]; arm-lacZ] 51A were heat shocked at 38°C for 120 minutes at 24–48 and 48–72 hours after egg laying. Clones were marked by loss of arm-lacZ staining (Vincent et al., 1994).

**Histology and immunohistochemistry**

β-galactosidase was detected by either activity or antibody staining as previously described (Tomlinson and Ready, 1987). Anti-Elav and anti-β-Gal double stainings were done in 0.1 M phosphate buffer, 0.2% saponin, 0.3% deoxycholate, 0.3% Triton X-100 and 10% normal donkey serum. The same conditions were used in anti-β-Gal double stainings with anti-Chp and anti-Bar. Elav was detected with a rat monoclonal antibody (gift of G. Rubin), Chp with the mouse monoclonal antibody mAb24B10 (Van Vactor et al., 1988), Bar with a rabbit polyclonal serum (Higashijima et al., 1992), and β-galactosi-
dase with a mouse monoclonal antibody (Promega). Secondary antibodies were purchased from the Jackson Labs. Standard histological methods were used for sections of adult eyes (Tomlinson and Ready, 1987). The enhancer trap line H214 (Mlodzik et al., 1992) was used to detect R7 cells in 3rd instar eye discs. To monitor Rhodopsin expression, we used the Rh3-lacZ and Rh4-lacZ strains (Fortini and Rubin, 1990); detection in whole-mount retinas was performed as described earlier (Weber et al., 1995).

**Molecular analysis**

DNA sequences flanking the P-element insertion points were recovered using the plasmid rescue method (Wilson et al., 1989). All rescued plasmids map to polytene bands 54A1-3.

Lambda and cosmid clones from the 54A1-3 genomic region were isolated from an Oregon R DNA library in λEMBL3 (from J. Tamkun) and from a Canton S DNA library constructed in cosmids (Hoheisel et al., 1991). mbl cDNAs were isolated from several libraries by using different genomic fragments as probes. This led to the identification of four cDNA isoforms: A and B derived from the disc-specific library, and C and D from the embryonic libraries. Genomic and cDNA fragments subcloned in pBluescript or pUC were sequenced by standard procedures. DNA and protein sequences were analysed using the GCG software package (Wisconsin Genetics Computer Group). Similarity searches were carried out using the BLAST network service located at http://www.ncbi.nlm.nih.gov/BLAST/.

**Generation of anti-Mbl sera**

Mbl ORF A was PCR-amplified and cloned in the expression vector pFP98 as an Ndel-EcoRI fragment (gift of F. Peverali). This vector is derived from the pGEX-2T bacterial expression vector and contains a histidine tag. A fusion protein GST-MblA-His of 48 kDa was obtained and injected into rabbits using standard procedures. The serum was used at a 1:100 dilution in 0.1 M phosphate buffer, 0.2% saponin, 0.3% deoxycholate, 0.3% Triton X-100 and 10% normal donkey serum. No staining was detected with the preimmune serum.

**RESULTS**

**Isolation of a dominant suppressor of the sev-svp2 phenotype**

A collection of lethal P-element insertions on the second chromosome (Török et al., 1993) was screened for dominant modifiers of the sev-svp2 phenotype. One of the lines identified as a suppressor carried two P-element insertions at polytene chromosome bands 42D4-5 and 54A1-3. After their separation by meiotic recombination, the insertion at 54A1-3 (subsequently referred to as l(2)k05507) was found to interact with sev-svp2 (Fig. 1). The chromosome carrying this insertion was further associated with embryonic lethality and defects in homozygous mutant eye clones (see below). The second insertion from the original line did not interact with sev-svp2. To confirm that the observed suppression and clonal phenotypes (see below) were linked to the insertion, we remobilised the P-element l(2)k05507 (Robertson et al., 1988) and screened the progeny for flies that had lost the P[white+] marker. Several excisions of the P-element resulted in homozygous viable flies that lost their ability to suppress sev-svp2. Adult eyes of eight such independent lines were sectioned and did not show any aberrant ommatidia, indicating that no second site mutation was responsible for the lethality or the eye phenotype (see below).

The l(2)k05507 insertion acts also as an enhancer-detector with expression in the eye disc in cells posterior to the mor-
Although the transgenic lines did not show an eye phenotype with one or two copies of the construct on their own, when they were combined with the sev-svp2 tester strain and the mbl 05507 P-element, these constructs reverted the suppression of sev-svp2 normally observed in the presence of mbl 05507 (data not shown). Moreover, when crossed to sev-svp2 in an otherwise wild-type background, the rough eye phenotype of sev-svp2 was dramatically enhanced. While only approximately 15% of ommatidia in a sev-svp2 background have additional outer photoreceptors, many ommatidia in sev-svp2; sE-mblA or sev-svp2; sE-mblB flies contain up to 11 outer photoreceptors (Fig. 1D). This phenotype is reminiscent of 4 copies sev-svp2 or of genotypes, in which sev-svp2 was combined with activated components of the Ras-signalling pathway (Begemann et al., 1995). Taken together with the molecular evidence, we conclude that the isolated gene is disrupted in mbl 05507 and is responsible for the interaction with sev-svp2.

**muscleblind is autonomously required for photoreceptor differentiation**

Homozygous clones of P-element mbl 05507 show phenotypes only in a subset of mutant ommatidia. This relatively variable phenotype and the molecular nature of the allele (insertion in the 5' UTR, Fig. 4) suggested that this original mbl allele was a hypomorphic allele. To isolate additional, possibly stronger, alleles of mbl, we remobilised the P-element and selected for imprecise excisions that affect the respective gene. We obtained several lethal excisions that were allelic to mbl 05507, suppressed sev-svp2 and were mapped by Southern analysis to the genomic map (Fig. 4 and not shown). Excisions E27 and E127 removed (at least partially) exons 1 and 2 or just exon 1, respectively. In particular, excision E27 takes out 2 regions containing the splice-donor site and acceptor sites of exons 1 and 2 and parts of the second exon that encodes the N terminus (Fig. 4). Independently, a new strong allele, mbl2563, was identified in a collection of lethal P-element insertions (Erdelyi et al., 1995). Genetic and molecular analysis of mbl2563 revealed that it was revertible and had inserted in exon 4 within the open reading frame of the gene (Fig. 4). Remobilisation of mbl2563 generated the excision allele mbl E2.

To determine the role of mbl in ommatidial differentiation, we induced mitotic clones (Materials & Methods) of the mblE27, mblE127, mbl2563 and mblE2 alleles (Fig. 3), which were selected because their molecular nature indicated that they
were strong, possibly null alleles. The mutant tissue was analysed both in adult eyes and 3rd instar imaginal discs. Although all these alleles showed stronger phenotypes than the original P-insertion, they did not reveal an external phenotype in adult eyes (Fig. 3D). However, mblE27, mbl2563 and mblE2 revealed an absence of normally differentiated photoreceptors within mutant tissue (Fig. 3B,C). Clones of the mblE127 allele gave similar but weaker phenotypes consistent with its less severe molecular lesion, which does not affect coding sequences (Fig. 4). Mosaic analysis revealed that at the borders of clones all photoreceptors with normal appearance were genotypically wild type (arrowheads in Fig. 3B,C) arguing for a cell-autonomous function of the gene product. Neither pigment cells nor cone cells were affected as judged from the external appearance of the mutant area and their analysis in sections (Fig. 3D and not shown).

To analyse at what stage photoreceptor development was affected in mutant mbl tissue, mosaic 3rd instar larval eye discs for the strong mblE27 allele were stained with different neuronal and photoreceptor-specific markers. The Elav protein, which is expressed in nuclei of all developing neurons, was expressed normally in mutant mbl tissue indicating that mbl+ photoreceptor cells initiate neural development (Fig. 3E). As markers for specific photoreceptor subtypes, we used the enhancer trap line H214, which in wild-type eye discs is expressed in R7, and Bar, which is specific for R1 and R6 photoreceptor cells. mbl mutant cells showed normal expression of both these photoreceptor subtype markers (Fig. 3E and not shown), indicating that the putative transformation of outer photoreceptors to R7 as seen in clones of mbl05507 is due to differentiation defects and not to a mis-specification of photoreceptor precursors. In addition, we analysed the expression of the photoreceptor-specific membrane protein Chaoptin in mbl mutant clones in eye discs. Anti-Chaoptin stainings revealed no significant difference between wild-type and mutant tissue (not shown).

To further refine the analysis of mbl mutant photoreceptors, we have looked at their axonal projections to the optic lobes and the R7-specific expression of the Rh3 and Rh4...
rhodopsins. Although axonal behavior was largely unaffected with normal projections to the lamina and medulla, the analysis of mutant clones by vertical thin sections revealed that the organization of the neural tissue underlying the retinal clone was affected as compared to the corresponding surrounding wild-type tissue (Fig. 3F;G and not shown). This might suggest that some properties of the photoreceptor axons (e.g. expression of cell adhesion molecules) is not normal in \textit{mbl} mutant cells. The expression of the rhodopsins was analyzed in whole-mount adult retinas, in which \textit{mbl} mutant clones were induced, using the R7-specific Rh3-lacZ and Rh4-lacZ strains (Fortini and Rubin, 1990). The expression of these R7 markers was not affected in adult \textit{mb} \textit{p}^{5507} clones, indicating that no cells other than wild-type R7 have differentiated as R7 cells (not shown). This is in agreement with the analysis of \textit{mbl} mutant clones in eye discs, which has suggested that all photoreceptor cell fate decisions were normal in \textit{mbl} mutant clones (e.g. Fig. 3E).

Taken together, these data suggested that photoreceptor induction, subtype specification and the initial stages of differentiation were not affected in \textit{mbl} mutants, but that some aspects of later differentiation did not proceed normally.

\textbf{\textit{muscleblind} encodes novel nuclear proteins with zinc-finger motives}

Analysis of the different cDNAs with respect to the genomic walk revealed a complex genomic organisation of the \textit{mbl} locus with at least 9 exons spanning over 150 kb of genomic DNA with several large introns (>80 kb). Our data indicate that the \textit{mbl} transcription unit has a single initiation site and that the primary transcript follows a complex splicing pattern giving rise to alternatively spliced mRNAs. All four \textit{mbl} splice forms shared common 5' sequences and the first 2 exons (including the ATG and amino terminal part of the putative peptides) and differ at the 3'-ends giving rise to four open reading frames (ORFs) of different lengths and carboxy termini. The sequences of the cDNAs \textit{mblA}, \textit{mblB} and \textit{mblC} revealed three ORFs of 203, 316 and 243 amino-acids, respectively, sharing the first 179 amino terminal residues (Fig. 5A). The transcript represented by \textit{mblD} also contains an ORF that shares the first 63 amino-acids with the other Mbl isoforms, but due to the use of exon 3, which contains several stop codons, only gives rise to a short peptide of 84 amino-acids (Figs 4, 5A).

\textit{Mbl} isoforms A, B and C are novel proteins that contain two copies of a putative Cys8His zinc-finger structure with a typical spacing of CX7CX6CX3H (Fig. 5A,B). This is similar to the TIS11/NUP475/TTP family of proteins, of which the mouse NUP475 has been shown to bind zinc (DuBois et al., 1990; Lai et al., 1990; Varnum et al., 1991). The short MBID protein contains only one complete finger. Several other proteins contain very similar finger structures and are exemplified by the \textit{Drosophila} proteins Unkempt and Clipper, the zebrafish Clipper homologue No arches and the \textit{C. elegans} protein PIE-1 (Fig. 5B, for references see figure legend). Apart from Clipper, which can act as an endoribonuclease, no specific function has been assigned to proteins containing such finger motives as yet.

To analyse the subcellular localisation of Mbl proteins, antibodies against the complete MblA isoform were raised (Material & Methods). As all Mbl isoforms share the amino termini (see above), this polyclonal antiserum should detect all four protein isoforms. Mbl proteins were specifically detected in nuclei of ommatidial precluster cells and at lower levels in all nuclei of imaginal disc cells (Fig. 5C and not shown). The specificity of the antibody was confirmed in eye imaginal discs of flies expressing the \textit{mblA} cDNA under the control of the

\begin{figure}
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\includegraphics[width=\textwidth]{fig4}
\caption{Organisation of the \textit{muscleblind} transcription unit. The positions of the enhancer trap P-elements inserted in this region is shown (PP2, P55/7: l(2)k05507, P2563: l(2)2563). The extent of the deleted regions in two lethal mutations (\textit{mb} \textit{E}23 and \textit{mb} \textit{E}127) generated by imprecise excision of the l(2)k05507 insertion are shown as shaded boxes. Only EcoRI sites are shown in the walk. A more detailed restriction map, including \textit{BamHI}, \textit{HindIII} and \textit{SalI} sites, and the relevant cosmid and phage clones are available upon request. The bottom part shows the structure of the isolated \textit{mbl} cDNAs. At least four different \textit{mbl} transcript types with alternative 3' ends have been identified, and are depicted as A, B, C and D. Black boxes indicate the position of the putative translated sequences in each cDNA and white boxes indicate untranslated regions. On top of the map, the coordinates of the genomic walk are indicated in kilobases with the starting point 0 at the insertion site of \textit{mb}^{p5507}.
}
glass response element, which revealed the same nuclear staining in photoreceptor cells, but at higher levels than seen in wild-type eye discs. The nuclear localisation of Mbl proteins is consistent with their molecular nature as members of a zinc-finger family of proteins. Similarly, the analysis of the embryonic expression pattern revealed nuclear localisation in developing muscle cells (not shown). A description of the embryonic expression pattern and phenotype will be presented elsewhere (R. A., unpublished data).

Muscleblind homologues are found in *C. elegans* and vertebrates

Extensive database searches have identified one potential *mbl*-homologue in *C. elegans* and several closely related genes in vertebrates that share the paired zinc fingers and their C-terminal flanking region. Conceptual translation of the *C. elegans* cosmid K02H8 sequence has revealed the existence of a gene with 79% amino acid identity within a region of 89 residues that contains the two zinc-finger domains of *mblC* (Fig. 6). This remarkable sequence conservation, together with the positioning of the finger at the N terminus of the protein, indicates that the K02H8-encoded gene is a true homologue of *mbl*.

At least three different human genes, characterised as expressed sequence tags (ESTs), share regions of extensive homology with *mbl*, all of them encompassing the zinc fingers: four ESTs from human cDNAs (R35479; H69637; H47129; N76537) were found to align and result in a protein of at least 250 amino acids in length and possibly containing two zinc fingers with a spacing of 108 amino acids. Two further human ESTs (Z19309; W16832) share the same overall organisation with two pairs of zinc fingers spaced at an interval of 111 amino acids. A third human gene is characterised by four ESTs (F00133; D31587; D30846; AA186683) and contains one pair of zinc fingers. The highest homology to the human genes is found in a chick EST (Z29350) (Fig. 6) and a mouse-derived EST (AA108023) (data not shown).

**muscleblind** dominantly suppresses the eye phenotype of activated Jun

The sequence of Mbl proteins with the zinc-finger domain and their nuclear localisation (Fig. 5) suggests that Mbl could act as a transcription factor or a co-factor in this context. Since *mbl* suppresses the Svp-induced phenotype, we tested whether Mbl isoforms can directly interact with Svp. The experiments addressing this question, far western (Guichet et al., 1997) and GST-pull down assays (Peverali et al., 1996), however, did not show any molecular interaction (not shown), indicating that Mbl and Svp do not directly interact and that *mbl* might be required in a more general term during photoreceptor differentiation. In support of this notion, an ongoing parallel screen for modifiers of the eye phenotype induced by activated Jun, *sE-Jun*<sup>Asp</sup> (Treier et al., 1995), has identified the *mbl*<sup>2563</sup> allele as a suppressor of this genotype (U. Weber, D. Jackson, D. Bohmann and M.M., unpublished). The *Jun*<sup>Asp</sup> isoform behaves like a constitutively activated Jun protein (Papavassiliou et al., 1995) and, expressed under the control of the *sevenless*-enhancer during eye development, it causes a similar phenotype to *sev-svp2* or activated components of the Ras pathway: transformation of cone cell precursors to photoreceptor neurons (Fig. 7A; Treier et al., 1995). By screening a collection of lethal P-elements (Erdelyi et al., 1995) for modifiers of this phenotype (U. Weber, D. Jackson, D. Bohmann and M.M., unpublished), one of the isolated suppressors was *mbl*<sup>2563</sup>. As in the case of *sev-svp2*, *mbl*<sup>2563</sup> dominantly suppressed the *sE-Jun*<sup>Asp</sup> eye phenotype,
Fig. 6. Sequence alignment of Mbl with related sequences. (A) Mbl homology to vertebrate expressed sequence tags (ESTs). Protein sequence alignment of Mbl with Cys3His fingers and homologous flanking sequences of human and chicken ESTs. The human ESTs 2 and 3 contain parts of two Cys3His finger pairs each (labelled a and b). For the ESTs all finger pairs were aligned individually rather than the complete sequence in order to highlight their conservation to each other and to emphasise the greater homology between the first finger pair in the vertebrate homologues and in Mbl. (B) Protein sequence alignment of MblC and the putative *C. elegans* open reading frame K02H8 (GenBank accession number: U67957). In both panels, large boxes indicate the Cys3His fingers. Identical amino acids are boxed in black, similar ones in grey. The following amino acids were considered similar: A, G, D, E, F, Y and W; I, L, M and V; K, R, N, Q; S, T.

reducing the number of extra photoreceptors (Fig. 7). However, the *mbl* allele specifically and only suppressed the extra photoreceptor phenotype but not the polarity defects associated with *sE-Jun*<sup>Ap</sup> (U. Weber and M. M., unpublished). The original *mbl*<sup>6507</sup> allele and excisions derived thereof also suppressed to a similar extent the *sE-Jun*<sup>Ap</sup> phenotype (not shown). Taken together with the molecular analysis this suggests that *mbl* serves a general function during photoreceptor differentiation.

**DISCUSSION**

We have identified the *muscleblind* (*mbl*) gene as a dominant suppressor of the gain-of-function phenotypes of Svp and Jun. Consistent with these genetic interactions, the subsequent phenotypic analysis revealed an essential and autonomous requirement for *mbl* in photoreceptor differentiation. *Mbl* codes for nuclear proteins that contain Cys3His-zinc-finger domains and are conserved in *C. elegans* and vertebrates.

**muscleblind and eye development**

The phenotypic analysis has revealed that *mbl* is required for the differentiation of all photoreceptors. The initial steps of photoreceptor induction and determination appear normal as judged from the expression of neural- and photoreceptor-specific markers in eye imaginal discs. Photoreceptors mutant for *mbl*, however, fail to differentiate as seen in adult eyes. Nevertheless, even in large clones, the regular ommatidial array is undisturbed and radial sections of *mbl* clones show that (at least partially) photoreceptor cell bodies and their axons are present.

These observations suggest two possible explanations for the *mbl* phenotype. The lack of differentiated photoreceptors in *mbl* mutant tissue could be caused by: (i) failure of the terminal differentiation of the precursor cells or (ii) degeneration of the mutant photoreceptors (in particular their rhabdomeres). The
latter explanation can be largely excluded: degeneration of photoreceptors in known mutants is light induced and slow (reviewed in [Zuker, 1996]) and, in mbl mutant tissue, we see no difference between eyes of flies treated normally (usually aged for a few days before analysis) and very young or old flies that have been raised in the dark (data not shown).

The defects observed in mutant clones of the strong mbl alleles resemble the phenotype of the photoreceptor-specific transcription factor Glass (Moses et al., 1989; Moses and Rubin, 1991). Glass null alleles develop with hardly any eye tissue except for a sea of pigment cells, which is thought to be a secondary defect caused by the lack of a supporting ‘structure’ due to the absence of all the photoreceptors (Moses et al., 1989). Nevertheless, mutant clones of both glass and mbl appear externally normal and affect only photoreceptor cells, but not the cone or pigment cells. In both cases, the initial neural induction of photoreceptors is normal as judged by the extension of axons and the expression of neural markers such as Elav, 22C10 or anti-HRP. A significant difference between glass and mbl is reflected in the expression of the photoreceptor-specific protein Chaoptin (Chp) (Van Vactor et al., 1988): Chp is not detected in glass- eye discs (Moses et al., 1989), but it is expressed in mbl mutant clones. Furthermore, the expression of Rhodopsins appears unaffected in mbl mutant tissue (data not shown). Taken together, this suggests that mbl is required for the terminal differentiation of photoreceptor cells and that it acts at a later stage in this process than glass, affecting a subset of the aspects that are required in this process.

The expression patterns of glass and mbl posterior to the morphogenetic furrow in the eye disc are very similar. Although both genes are exclusively required for photoreceptor development, their nuclear expression is found in all cell types behind the furrow. Moreover, during embryogenesis both genes are expressed in the developing Bolwig organ, the larval visual system (Moses and Rubin, 1991; R. A., unpublished). To test whether mbl expression depends on glass function, we analysed its expression in discs of the glass$^+$ allele. Mbl expression in glass$^-$ eye imaginal discs is indistinguishable from the wild-type expression pattern (not shown), indicating that mbl expression is controlled by a mechanism independent of glass-mediated photoreceptor development.

Although many modifiers of sev-svp2 are part of the Sev/Ras/MAPK pathway or interact with it genetically (Begemann et al., 1995), mbl does not show any genetic interactions with either sev$^{511}$ (Basler et al., 1991) or se-rat$^{519}$ (Dickson et al., 1992), suggesting that mbl is not a component of this signalling pathway. Mutations in mbl suppress the eye phenotypes induced by the nuclear proteins Svp and Jun, which are thought to directly trigger photoreceptor induction and differentiation. Moreover, overexpression of Mbl isoforms from the sev promoter or glass response elements has no effect on eye development itself unless combined with these nuclear proteins (Fig. 1 and not shown). It is also worth noting that mbl suppresses only the ectopic photoreceptor aspect of the Jun$^{ASP}$-mediated eye phenotype and not the polarity and rotation defects (Fig. 7). Taken together with the similarity to the glass phenotype, all the above data suggest that mbl is required downstream of Svp and Jun$^{ASP}$ during the terminal differentiation of photoreceptors.

The mutant phenotypes of clones for ultraspiracle (usp) and orthodenticle (otd) share some similarities with those of weak mbl alleles (Oro et al., 1992; Vandendries et al., 1996). Nevertheless, it is unlikely that usp and otd have common functions to or act in concert with mbl during eye development. Although, usp mutant clones show abnormal photoreceptor morphology as analyzed in adult eyes (Oro et al., 1992), it was shown recently that the main role of usp in eye development is during the morphogenetic furrow (MF) movement. Moreover, a pGMR-Usp transgene (expressing Usp in all cells behind the MF) is not capable of rescuing the defects of usp$^-$ clones, suggesting that usp is not required for the differentiation of photoreceptors but rather for early eye patterning (Zelhof et al., 1997). Similarly, in otd mutant ommatidia, rhombomeres also have aberrant morphology (Vandendries et al., 1996) comparable in appearance to those within mbl$^{05507}$ clones. However, other defects observed in otd mutant clones (e.g. misplacement of R7 and R8 photoreceptors and gaps/holes between photoreceptors; Vandendries et al., 1996) indicate that otd is required more generally for eye development and not just for the terminal differentiation of photoreceptor neurons. Our results do not suggest a close link between mbl and otd.

**Molecular function of Muscleblind**

The molecular analysis of the mbl gene has revealed that it encodes for at least four protein isoforms containing two Cys$\times$His-type zinc fingers (DuBois et al., 1990; Lai et al., 1990; Varnum et al., 1991). This motif has been found in many proteins from distantly related animals, although no biochemical function has been assigned to it yet. Some of these proteins have been proposed to be involved in transcriptional regulation, as is the case of the mouse NUP475 protein, which contains two Cys$\times$His regions (DuBois et al., 1990). Its nuclear localisation and the fact that its expression is induced by stimulation with various mitogens and growth factors suggested that it might be a nucleic-acid-binding protein involved in regulating the response to those factors. This is supported by the finding that it binds Zn$^{2+}$ (DuBois et al., 1990). Another protein containing this motif is the C. elegans PIE-1 protein, which is expressed in the totipotent germline blastomere after each division in the early embryo. It has been proposed to act as a general repressor of somatic cell fates (Mello et al., 1996). Other Cys$\times$His finger-containing proteins, such as U2AF35 in mammals (Zhang et al., 1992) and Suppressor of Sable in Drosophila (Voelker et al., 1991), have been implicated in pre-messenger RNA splicing. Nevertheless, the overall structure of these peptides is different from the proteins mentioned above since they contain up to five Cys$\times$His repeats. An endoribonuclease activity has been demonstrated for the Drosophila protein Clipper (Bai and Tolias, 1996). Thus, although it is intriguing to speculate that Mbl might control the transcription of genes required during photoreceptor differentiation, a different role involving binding of RNA or affecting RNA metabolism in this process cannot be excluded. In this context, it is also possible that Mbl acts as a cofactor for several transcription factors required in photoreceptor development.

The mbl locus is very complex, giving rise to several protein isoforms through alternative splicing. Sequence analyses of all these proteins reveal that they share the amino terminus, but contain different carboxy terminal regions. Interestingly, one of the Mbl isoforms (Mbl D) contains only one Cys$\times$His region.
It is thus possible that different isoforms have different properties, specificities or functions as has been proposed in other similar cases. For example, the homeotic gene *Ubx* gives rise to multiple isoforms through alternative splicing and it has been suggested that, depending upon the assay, they can display functional differences (Busturia et al., 1990; Subramaniam et al., 1994). Nevertheless, in the case of *mbl*, the co-expression assay with 2xsev-svp2 and MblA or MblB (Fig. 1) shows indistinguishable properties of these two isoforms.

The embryonic phenotype of *mbl* affects mainly muscle differentiation (R. A., unpublished data) and it is also expressed in the Bolwig organ and in specific cells of the central nervous system. Thus, although *mbl* shares many similarities with *glass* (see above), its function is not solely restricted to the development of the visual system. The existence of distinct Mbl homologues in *C. elegans*, and also in humans and other vertebrates suggests a very general function for this family of proteins. The *mbl* mutants will permit the unravelling of the function of this gene family in a genetically amenable organism.

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