Molecular Analysis of Drosophila *eyes absent* Mutants Reveals Features of the Conserved Eya Domain

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ABSTRACT

The *eyes absent* (*eya*) gene is critical to eye formation in Drosophila; upon loss of *eya* function, eye progenitor cells die by programmed cell death. Moreover, ectopic *eya* expression directs eye formation, and *eya* functionally synergizes in vivo and physically interacts in vitro with two other genes of eye development, *sine oculis* and *dachshund*. The *Eya* protein sequence, while highly conserved to vertebrates, is novel.

To define amino acids critical to the function of the *Eya* protein, we have sequenced *eya* alleles. These mutations have revealed that loss of the entire *Eya* Domain is null for *eya* activity, but that alleles with truncations within the *Eya* Domain display partial function. We then extended the molecular genetic analysis to interactions within the *Eya* Domain. This analysis has revealed regions of special importance to interaction with *Sine Oculis* or *Dachshund*. Select *eya* missense mutations within the *Eya* Domain diminished the interactions with *Sine Oculis* or *Dachshund*. Taken together, these data suggest that the conserved *Eya* Domain is critical for *eya* activity and may have functional subregions within it.

STUDY of the Drosophila eye provides a system in which to approach, at a molecular genetic level, events involved in patterning, cell fate, and specification of a neural structure (reviewed in Treisman and Heberlein 1998; Bonini and Fortini 1999; Thomas and Wassarman 1999). Whereas much attention has focused on later events of patterning and cell fate determination, more recent study has focused on early genes with roles in eye formation. These genes appear to be highly conserved in vertebrates and include the Pax-6 homologues *eyeless* (*eya*) and twin of eyeless (*toy*) (Qui r i n g et al. 1994; Cze r n y et al. 1999), the homeodomain protein *sine oculis* (*so*) (Che y e t t e et al. 1994; Serikaku and O’Tousa 1994), and novel proteins *dachshund* (*dac*) (M ar d on et al. 1994) and *eyes absent* (*eya*) (Bonini et al. 1993). Whereas loss-of-function of these gene activities in the eye typically leads to loss of the entire eye, directed expression studies have revealed roles in eye specification: the genes, or combinations thereof, can direct the formation of ectopic eyes (H a l d e r et al. 1995; Bonini et al. 1997; Chen et al. 1997; Pignoni et al. 1997; Shen and Mardon 1997).

The genes thought to function earliest in the pathway of eye specification are *toy* and *eya*. *Toy* appears to be a direct activator of *ey* (Cze r n y et al. 1999), which by expression studies appears to function prior to *eya* and so and is critical for their normal expression patterns (Bonini et al. 1997; Shen and Mardon 1997; Halder et al. 1998). Whereas both *dac* and *eya* can direct ectopic eye formation, *eya* functionally synergizes with *dac* and so in directing eye formation. Moreover, the *Eya* protein binds to the *Dac* and *So* proteins, suggesting a possible protein complex involved in specifying target genes for eye formation (Ch en et al. 1997; Despl an 1997; Pignoni et al. 1997). *Eya* is novel but highly conserved in vertebrates (Dunc an et al. 1997; Xu et al. 1997a,b; Zimmerman et al. 1997; Bor sani et al. 1999). Comparison with vertebrate sequences reveals a highly conserved C-terminal region of ~270 amino acids, called the *Eya* Domain. This domain interacts with a conserved domain of *So*, called the Six Domain, which is shared by the *So* class of homeodomain proteins (Oliver et al. 1995; Pignoni et al. 1997). *Eya* has also been shown to interact with *Dac* within a large region of the protein that includes the *Eya* Domain (Ch en et al. 1997).

Most studies have focused on the role of *eya* in eye formation, although *eya*, like most other genes, has functions elsewhere in the animal. *Eya* null mutations are homozygous lethal with defects in head formation, and *eya* has a role in germ cell migration (Bonini et al. 1993, 1998; Boyle et al. 1997). Human and mouse mutants in the *EYA1* homologue display normal eye development, but abnormal development of other organs (Abdelhak et al. 1997b; Xu et al. 1999). In humans, mutations are known throughout the protein, including within conserved *Eya* Domain (Abdelhak et al. 1997a,b; Kumar et al. 1998). To provide greater information on the potential molecular domains of *Eya* critical for its roles in both eye formation and animal development, we...
have sequenced the mutations generated in Drosophila. These studies have revealed that within the highly conserved Eya Domain there are critical residues that may define subdomains of special functional importance.

**MATERIALS AND METHODS**

**Drosophila stocks and transgenic lines:** Flies were grown on standard cornmeal, molasses media, supplemented with dry yeast. All crosses were performed at 25°C unless otherwise noted. 

**eya alleles:** are described in Bonini et al. (1993, 1998). Additional alleles were generated by ethyl methanesulfonate (EMS) mutagenesis by Dr. Ilaria Rebay (Department of Biology, M.I.T., Cambridge, MA) and were a gift; these alleles are designated the eya<sup>II</sup> mutants. To determine embryonic lethality, the appropriate eya allele was outcrossed to the Oregon-R wild-type strain and then backcrossed to an eya deficiency (eya<sup>II</sup>) in trans to Oregon-R. Eggs were laid on grape plates, and at least 200 eggs per cross were counted to determine embryonic hatching rate. To determine degree of interallelic complementation, alleles were outcrossed to Oregon-R and then backcrossed to each other, and at least 300 adult flies were counted per cross.

For transgenics, eya cDNAs and mutant forms were subcloned into the pUAST transformation vector (Brand and Perrimon 1993) and transgenic lines were made in the w<sup>1118</sup> fly strain following standard protocols. The transgenic insertions were mapped to a chromosome and then crossed into the appropriate eya mutant backgrounds. Gene activity was directed to the eye using eya-GAL4 promoter lines (Bonini et al. 1997). UAS-dac and UAS-so transgenic lines are as described (Chen et al. 1997; Pignoni et al. 1997). The dpp-GAL4 promoter line directs expression to the eye using the imaginal discs in the pattern of the dpp gene (Stehling-Hampton et al. 1994). Mouse Eya clones used in in vivo rescue experiments were as follows, subcloned into the pUAST transformation vector: Eya1 (GenBank accession no. U61110; Xu et al. 1997); Eya2 (U81603; Zimmer man et al. 1997); and Eya3 (U81604; Zimmer man et al. 1997). Western analysis was performed on adult fly heads of the designated genotypes (see legend to Figure 5) following previously described protocols (Warnick et al. 1999). The antibody used to detect Eya protein was EyaMAb1F7. 

**Sequence analysis of eya mutant alleles:** Genomic DNA was amplified from the eya alleles and sequenced. All polymorphisms were confirmed on independent amplification events. For genomic DNA isolation, 20–100 flies were placed in a 3-ml Dounce homogenizer on ice and 1 ml of homogenization buffer (HB: 10 mM Tris-HCl, pH 8.0, 60 mM NaCl, 10 mM EDTA, 150 μM spermidine, and 0.5% Triton X-100) was added. The homogenate was filtered through a fine mesh screen. After centrifugation at 5000 rpm for 5 min, the supernatant was removed and the pellet resuspended in 1 ml of HB. Centrifugation was repeated and the pellet was resuspended in 450 μl of HB. 10 μl 10 mg/ml proteinase K and 50 μl 20% Sarkosyl were added and the reaction incubated at 50°C overnight. A total of 50 μl of 3 mM sodium acetate, pH 5.5, was added and the reaction was extracted with equal volumes of chloroform/phenol twice, then chloroform once. The DNA was precipitated by addition of 1 ml of 100% ethanol and centrifuged at 1000 rpm for 1 min. The genomic DNA pellet was washed with 70% ethanol and resuspended in 25-50 μl distilled H<sub>2</sub>O. Ten samples were incubated at 60°C for 30 min and then stored at −20°C.

Two procedures were used to amplify the conserved region of the eya alleles. Genomic DNA was isolated from flies that were heterozygous for the lethal allele. To distinguish the normal from the mutant chromosome for sequence analysis, restriction-enzyme polymorphisms were used. A polymorphic Nol site was found on the parental spd<sup>b</sup> chromosome and the EMS alleles derived from that parental chromosome (Bonini et al. 1993). The eya<sup>II</sup> alleles do not contain this polymorphic Nol site, nor does the balancer chromosome CyO. The eya alleles generated on the parental spd<sup>b</sup> chromosome were thus placed in trans to the CyO chromosome and the eya<sup>II</sup> alleles were placed in trans to the spd<sup>b</sup> chromosome. Amplification products derived from genomic DNA of heterozygous stocks were then distinguished by endonuclease digestion using NolI. A number of polymorphisms were found between the parental strains and control strains (Table 1). Most of these were silent base-pair mutations.

Initial amplifications were performed using standard Taq polymerase ( Gibco, Gaithersburg, MD) and primer NB85, which recognizes a sequence within the 3rd exon, and primer NB189, which recognizes a sequence beyond the polyadenylation signals. The amplification protocol was 94°C 5 min; 45 cycles of 94°C 15 sec, 57°C 30 sec, and 72°C 2 min; 72°C 10 min and a 4°C soak. Additional amplifications were performed using a mixture of thermostable Taq and Pwo proofreading Taq (Boehringer Mannheim, Indianapolis). The primers used were NB189 and NB77. NB77 recognizes genomic sequences located within intron 2. The amplification protocol was as follows: 10 cycles of 94°C 2 min; 92°C 10 sec, 57°C 30 sec, and 72°C 8 min 20 sec; 20 cycles under the same conditions except the extension step was increased by 20 sec each cycle; and a 4°C soak. After amplification, products were restriction digested with NotI. The fragments were recovered from a low-melt 1% agarose gel and purified using affinity resin columns (Promega, Madison, WI) according to manufacturer’s instructions. The purified product was washed and concentrated using microconcentrators (Amicon, Beverly, MA).

Concentrated and purified amplification products were sequenced using a dye terminator cycle sequencing kit (Perkin Elmer, Norwalk, CT) and manufacturer’s instructions except all reactions contained DMSO. The program for sequencing was as follows: 95°C 3 min; 25 cycles of 96°C 30 sec, 50°C 1 min, and 60°C 4 min; and a 4°C soak. After amplification, unincorporated nucleotides were removed using Centrisep gel filtration columns (Princeton Separations), and the reaction dried down under vacuum. The sequencing reactions were run on ABI 377 sequencers with Stretch upgrade using BigDye Taq chemistry. Sequence was analyzed using Sequencer v3.0 (Gene Codes Corp., Ann Arbor, MI).

The intron-exon structure of the eya gene has two alternative first exons (exon 1a for the typlp transcript and exon 1b for the typlp transcript) and then exons 2–5 in common. The Eya conserved Domain falls within exons 3–5. Primers used for amplifying the eya exons from genomic DNA were as follows: for exon 1a, NB-80 (5′-GAGATATACATCCATTCAAACCCA-3′) and NB-82 (5′-ATTCTGTGTTGCTCAGTGAAGAAACGCC-3′); for the 5′ side of exons 3–5, two primers were used, NB-85 (5′-CCACGATAGTATGTAGTGGTCCCTTGGC-3′) and NB-189 (5′-ACCCGAGGAGTGGTGCAGAAGA-3′) for the 3′ side of exons 3–5, four different primers were used, NB-67 (5′-CACGAGGCTGCTCATCGGG-3′), NB-77 (5′-CATGTAACACATTAGATGAAAGG-3′), NB-263 (5′-TACTGAGCCAGCTGTACGGCAGCGG-3′), and NB264 (5′-ATTCTGTGGTTGCTACTTTAAACCGGT-3′). Sequencing primers included the primers used for amplification and additional internal primers designed by the MacVector sequence analysis program (Eastman Kodak Co., Rochester, NY).

Some polymorphisms were found between the parental strains of the mutants and the published cDNA sequence of eya. These are presented in Table 1. These alleles had no...
TABLE 1
DNA polymorphisms present in parental strains

<table>
<thead>
<tr>
<th>DNA polymorphism</th>
<th>Protein mutation</th>
<th>Parental strains</th>
</tr>
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<tbody>
<tr>
<td>bp555-583, 3-bp deletion</td>
<td>aa54-62, one less Q in oqa repeat</td>
<td>cli, IR, spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>bp677, C to G</td>
<td>aa94, silent (G)</td>
<td>cli, IR, spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>bp678, A to G</td>
<td>aa95, S to G</td>
<td>cli, IR, spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>bp836, T to C</td>
<td>aa147, silent (S)</td>
<td>cli, IR, spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>bp921, T to A</td>
<td>aa176, S to T</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1130, T to C</td>
<td>aa205, silent (S)</td>
<td>cli (eyaZ283 but not eya2D18), IR</td>
</tr>
<tr>
<td>bp1133, G to A</td>
<td>aa206, silent (S)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1136, A to G</td>
<td>aa207, silent (A)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1340, T to C</td>
<td>aa315, silent (G)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1412, C to T</td>
<td>aa339, silent (G)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1541, G to A</td>
<td>aa382, silent (E)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1574, G to C</td>
<td>aa393, silent (V)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1595, T to G</td>
<td>aa400, silent (P)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1640, C to A</td>
<td>aa415, silent (G)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1663, G to T</td>
<td>aa425, A to S (neutral to polar residue)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp1856, A to G</td>
<td>aa487, silent (E)</td>
<td>cli, IR</td>
</tr>
<tr>
<td>bp2281, T to C</td>
<td>aa629, silent (R)</td>
<td>cli, IR, spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>bp2293, A to G</td>
<td>aa633, silent (L)</td>
<td>IR</td>
</tr>
<tr>
<td>bp2312, G to A</td>
<td>aa640, E to K (acidic to basic)</td>
<td>spd&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Sequence numbers are in reference to published sequence of eya type1 cDNA (GenBank accession no. L08501). spd<sup>a</sup> indicates those alleles generated on a spd<sup>b</sup> genetic background (described in Bonini et al. 1993); cli indicates those alleles generated by Nusslein-Volhard et al. (1984); IR refers to those alleles generated by Dr. Ilaria Rebay.

<sup>a</sup> Mutation not found in any of the EMS alleles generated on the spd<sup>b</sup> background. The spd<sup>b</sup> chromosome, however, was not isogenized prior to the mutagenesis in which the eya alleles were isolated (Bonini et al. 1993).

dNA polymorphisms in genomic DNA of exons 3-5: eya<sup>E12</sup>, eya<sup>E32</sup>, eya<sup>E35</sup>, eya<sup>E36</sup>, and eya<sup>E37</sup> had polymorphisms in intron 3 (bp34, T to A; bp43, T to C). eya<sup>E22</sup> was not completely sequenced because the entire genomic region could not be amplified; this allele is likely a rearrangement.

Yeast interaction studies: Constructs were made using the Matchmaker LexA two-hybrid system (CLONTECH, Palo Alto, CA) with bait constructs in the pLexA vector and prey constructs in the pB42AD vector, following protocols outlined by the manufacturer. β-Galactosidase filter lift assays and auxothroph dependence were used to score positive interactions. To subclone the region encoding the Eya and Six Domains to rescue eye formation in Drosophila, with all functionals from the opa domain within the protein, we addressed whether the other eya homologues had activity to rescue the eye and how well the homologues functioned relative to the Drosophila eya sequence. To do this, we used the GAL4/UAS system (Brand and Perrimon 1993) to make transgenic flies expressing mouse Eya1, Eya2, and Eya3 as UAS transgenes and expressed them in the eye primordia of the eya<sup>a</sup> mutant with the ey-GAL4 promoter line. We found that all three mouse homologues had the ability to rescue eye formation in Drosophila, with all functioning about equally well (Figure 1). Compared to the fly eya cDNA, the mouse genes rescued roughly half as effectively, comparing the best transgenic insertions of all the different transgenic lines. These data indicate that the mouse sequences are sufficiently conserved with the Drosophila gene to functionally complement in vivo.

Sequencing of the fly eya alleles: We sequenced Drosophila eya alleles to define mutations in conserved regions of the predicted open reading frame (ORF) that would lead to identification of amino acids critical to protein function. One set of eya alleles, generated by treatment with EMS, was generated on a parental chromosome marked with spd<sup>b</sup> (Bonini et al. 1993). The spd<sup>b</sup> stock had six polymorphisms compared to the published eya cDNA sequence, five of which were found in all eya alleles generated on the spd<sup>b</sup> parental chromosome (see Table 1). A 3-bp deletion removes one glutamine residue from the oqa repeat near the N terminus of the
predicted protein. One polymorphism leads to a substitution of K (lysine) for E (glutamic acid) at amino acid (aa) 640 of the large conserved Eya Domain. This substitution occurs remarkably near a region defined as critical by eya alleles (below). Nevertheless, the spd9 chromosome produces flies with normal eyes, and therefore this polymorphism yields a protein with apparently normal function. None of the EMS alleles generated on the spd9 chromosome have this alteration; this mutation type.

Missense mutations within the Eya Domain: Three alleles predict missense mutations within the large Eya Domain in amino acids that are conserved between flies and humans. eyaE7 predicts a change of aa643 from a polar residue (T, threonine) to a nonpolar residue (I, isoleucine). By genetic analysis, the eyaE7 mutation is severe, but not null (Table 2; also Bonini et al. 1998; Leiserson et al. 1998). The second missense mutation occurs in eyaE1 and predicts a change of aa497 from a polar residue (T, threonine) again to a nonpolar residue (M, methionine). This change also occurs in an amino acid conserved in humans. By genetic analysis, the eyaE1 mutation is mild, leading to pupal lethality when homozygous. A third missense mutation occurs in the eyaE3 allele within a highly conserved stretch of the C-terminal

Premature terminations of the Eya protein: Subsequent sequencing of eya alleles revealed six mutations that effect changes within the large conserved domain of the Eya protein, the Eya Domain (Table 2, Figure 2). Three of these (eyaE4, eyaE5, and eyaE11) are nonsense mutations within the same codon, predicting a protein that prematurely truncates at aa646. The eyaE4 and eyaE12 mutations are identical DNA changes, although these two alleles were generated in independent mutageneses and were confirmed in independently maintained copies of the fly lines. Interestingly, the truncated protein predicted by these alleles retains some activity, as these mutants are only partially, not fully, embryonic lethal (Table 2; also Bonini et al. 1998; Leiserson et al. 1998). The eyaE11 allele also predicts a prematurely terminated protein at aa597. This allele deletes a larger region of the Eya Domain and appears lethal in trans to other eya alleles, although it also displays partial embryonic viability (Table 2). Together, these alleles indicate that a partially intact Eya Domain appears to retain some activity, rather than the entire complete Eya Domain being essential for any functionality of the protein.

The eyaE alleles were isolated as embryonic lethal mutations with pattern formation defects (Nüsslein-Volhard et al. 1984). Since these alleles are fully embryonic lethal and have a phenotype indistinguishable from deficiencies for the locus, they are predicted to be null for the vital function of the gene. One of these alleles, eyaE201a, leads to a premature stop within Eya Domain 2 at aa335, a weakly conserved region N-terminal to the large conserved Eya Domain (Zimmerman et al. 1997). This mutation predicts a protein missing the entire Eya Domain, indicating that loss of the entire Eya Domain is null for eya function. The eyaE14 allele was also found to be a premature termination mutation with a nonsense mutation within a nonconserved part of the N terminus at aa269. Although this mutation is predicted to be a severe change, leading to loss of most of the protein, the mutant allele displays activity when homozygous and in trans to other eya alleles (Table 2; and Bonini et al. 1998; Leiserson et al. 1998). Downstream of the premature stop is a second methionine that could serve as an initiation methionine; using an antibody raised against a domain just upstream of the Eya Domain (EyaM Ab10H6), we confirmed eya protein expression in whole-mount preparations of this mutant allele (data not shown). This allele therefore appears to generate sufficient protein, presumably from the downstream methionine, to account for the hypomorphic phenotype.
portion of the Eya Domain. This results in a change from nonpolar residue (G, glycine) to a charged residue (E, glutamate). By genetic analysis, this allele appears to be more severe than the other missense mutations, perhaps indicative of a critical residue or generation of an unstable protein.

Select mutations in theeya gene display interallelic complementation of a type called transvection, which defines regulatory regions of the gene critical to expression of the gene (Leiserson et al. 1994, 1998; Zimmerman et al. 2000). However, we noticed that two of the alleles that were missense mutations also showed partial complementation when placed in trans: theeya and eya alleles are lethal, yet sometime yield escapers when in trans to each other (Bonini et al. 1998; Leiserson et al. 1998). We reasoned that this weak complementation may reflect distinct roles ofeya at different developmental times, different functional domains within the protein, or perhaps a combination of both. We pursued the latter possibility by determining whether these mutations, and the subregions of the Eya Domain defined by these mutations, would show differential activity in yeast two-hybrid assays by testing interactions between Eya and two conserved protein partners, Dac and So.

Selective interaction of subregions of the Eya Domain with So and Dac: It has previously been shown that the Eya protein interacts in the two-hybrid system and in vitro with the Six Domain of the So protein and with the Dac protein (Chen et al. 1997; Pignoni et al. 1997). These interactions may be biologically relevant as synergy is observed between Eya and either of the other two proteins in vivo in ectopic eye formation. For interactions with the So protein, the interaction domain of Eya was narrowed to the highly conserved Eya Domain (Pignoni et al. 1997). The Eya Domain is a relatively large domain (274 amino acids) and displays high sequence homology throughout to the vertebrate homologues. Therefore, to define potential subregions within the Eya Domain, we divided the Domain into three parts, EF1 (Eya Fragment 1) defined by theeya mutation, EF2 defined by theeya mutation, and EF3 as the terminal region that shows high conservation with a nematode Eya homologue (Figure 3; see Duncan et al. 1997). We made constructs containing the entire Eya Domain or the three EF subregions in the bait vector of the LexA two-hybrid system (Gyuris et al. 1993). In addition, we used site-directed mutagenesis to make theeya and eya mutations within the conserved Eya Domain to address whether these mutations showed interactions with the Six region of So or the Dac protein. The dac clone used was obtained in a two-hybrid screen for sequences that interact with Eya (Q. Bui and N. Bonini, unpublished results) and turned out to include only amino acid residues 748–826 of the Dac protein. This part of Dac encompasses the DachboxC, a conserved C-terminal domain of the Dac protein (Figure 3; Hammond et al. 1998).

We confirmed that the Eya Domain showed a strong interaction with the Six Domain and showed that the Dac protein interacts with the Eya Domain through the DachboxC in the two-hybrid system (Figure 4). For the Six Domain, a strong interaction with the Eya Domain was also detected with the Eya Domains of a vertebrate homologue (Eya2; MECD) and the Caenorhabditis elegans

### TABLE 2

Mutations ineya alleles

<table>
<thead>
<tr>
<th>Allele</th>
<th>Origin</th>
<th>DNA polymorphism</th>
<th>Protein mutation</th>
<th>Eya Domain</th>
<th>Eye size in trans toeya</th>
<th>Embryonic lethality in trans to Df(2L)eya</th>
</tr>
</thead>
<tbody>
<tr>
<td>eyaE1</td>
<td>EMS G to A, bp2332</td>
<td>W646* ECD (EF2)</td>
<td>○○</td>
<td>○○</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE4</td>
<td>EMS C to T, bp1200</td>
<td>Q269* ECD (EF2)</td>
<td>○○</td>
<td>○○</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE7</td>
<td>EMS C to T, bp2323</td>
<td>T643I ECD (EF1)</td>
<td>●●●●</td>
<td>●●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE8</td>
<td>EMS G to A, bp2333</td>
<td>W646* ECD (EF2)</td>
<td>○○</td>
<td>○○</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE11</td>
<td>EMS A to T, bp1885</td>
<td>T497M ECD (EF2)</td>
<td>●●●●</td>
<td>●●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE12</td>
<td>EMS G to A, bp2323</td>
<td>W646* ECD (EF2)</td>
<td>○○</td>
<td>○○</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eyaE13</td>
<td>EMS C to T, bp1395</td>
<td>Q335* ECD (EF1)</td>
<td>○○</td>
<td>○○</td>
<td></td>
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<tr>
<td>eyaE14</td>
<td>EMS G to A, bp2189</td>
<td>W597* ECD</td>
<td>○●●</td>
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<tr>
<td>eyaIR1</td>
<td>EMS G to A, bp2562</td>
<td>G723E ECD</td>
<td>○●●</td>
<td>○●</td>
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</tr>
<tr>
<td>Df(2L)eya</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>○○</td>
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</tr>
</tbody>
</table>

DNA base pairs refer to theeya typel cDNA (GenBank accession no. L08501); protein amino acid residue numbers refer to the Eya typel protein. EMS, ethyl methanesulfonate; ECD, Eya Conserved Domain; EF1, Eya Domain; EF2, Eya fragment 1; EF3, Eya fragment 2. EF3, Eya fragment 3. Key for eye phenotype: ○, eyeless; ●, <1/4 eye; ●●, 1/4 to <1/2 eye; ●●●, 1/2 to <3/4 eye; ●●●●, 3/4 to <normal. Key for lethality: ○, >50% lethal; ●●, 50% or less lethal. Lethality includes data from Leiserson et al. (1998), as well as analysis of new alleles from this article.
Figure 2.—Missense and nonsense mutations of the Drosophila Eya protein. (A) Schematic representation of the fly Eya protein, with domains of interest highlighted. The Eya Conserved Domain (Eya Domain or ECD) is the highly conserved domain in the C-terminal portion of the protein. Just N-terminal to the ECD is a cluster of positively charged amino acids that is also found in the vertebrate Eya homologues. Eya Domain 2 is a domain of weak conservation found in the fly Eya protein and M-Eya2 homologue (Zimmerman et al. 1997). The PEST sequence is a domain that, by sequence analysis, may serve in protein turnover, and the opa repeat is a polyglutamine domain (see Bonini et al. 1993). (B) Mutations of the eya alleles that fall within the Eya Domain, highlighted on a sequence alignment of the mouse Eya and fly Eya Domains. Identical amino acids are boxed in black and similarities are shaded. Amino acid similarities were defined using the default symbol comparison table based on the Dayhoff PAM-250 matrix (see materials and methods), with the following amino acids considered similar: F, Y, L, M; I, V, E, D. GenBank accession numbers of the protein sequences are: U61110 (M-Eya1), U81603 (M-Eya2), U81604 (M-Eya3), and L08501 (fly-Eya).
Molecular Analysis of *eya* Mutants

Figure 3.—Domains of the *eya*, *so*, and *dac* proteins used in yeast two-hybrid studies. (A) Domains of *eya*. The top diagram represents entire *eya* sequence and the bottom those constructs made for the yeast system. The EF1 Domain of the *fly* *eya* protein was divided into three subdomains, EF1 (Eya fragment 1), EF2 (Eya fragment 2), and EF3 (Eya fragment 3) on the basis of mutations and conservation with *eya* homologues of other species. Site-directed mutagenesis was used to make the *eya* E11 and *eya* E7 mutations within the ECD. The conserved domains of the mouse *eya*2 homologue (M-Eya2, GenBank accession no. U81603) and the *C. elegans* homologue (W-Eya, GenBank accession no. C38859) were also subcloned into the yeast plexA vectors to test for interactions. The conserved Domain of M-Eya2 shows 66% identity with the *fly* ECD, whereas W-Eya shows 36% identity with the *fly* ECD. (B) Domains of the *so* protein. The So protein has two domains conserved in Six homologues and in vertebrates: the Six domain and the homeodomain. (C) Domains of the *dac* protein. The Dac protein has two domains conserved to vertebrates: an N-terminal domain called Dachbox-N and a C-terminal domain called Dachbox-C. The clone used encompassed the Dachbox-C.

Homologue (W-ECD). For Dac, the interaction was conserved with the vertebrate Eya Domain, but not with the Eya Domain of *C. elegans*. We then tested for interactions with the mutant Eya Domains and with subregions of the Eya Domain. Select interactions with the mutant forms of the Eya Domain or the EF regions were observed, although overall the interactions were considerably weaker than with the wild-type Eya Domain sequence (Figure 4). Therefore, both mutations may disrupt interactions with the Six Domain and the Dachbox-C. However, comparison of the persisting interactions revealed that they could be differentially mapped within the conserved Domain: interaction with the Six Domain was seen with the N-terminal EF1 Domain and not the other EF Domains, whereas a Dac interaction mapped to the EF2 Domain. Moreover, although in yeast interaction with either mutant domain was reduced compared to the wild-type Eya Domain, the Six Domain of So maintained an interaction with the *eya* E11 mutation but not the *eya* E7 mutation—the latter being a mutation within the EF1 Domain, which displayed selective interaction with So. Likewise, the Dac interaction was disrupted by the *eya* E7 mutation, but not the *eya* E11 mutation, with the latter occurring within EF2, which was the subregion of the Eya Domain that displayed a selective Dac interaction.

**Interactions in vivo of Eya Domain mutant forms:** The above studies defined two mutations in *eya* that revealed...


**Figure 4.**—Interactions between the Eya Domain and the So and Dac proteins in the yeast two-hybrid system. Picture of yeast cells bearing the So construct (Six domain) or the Dac construct (Dachbox-C domain) and the indicated Eya constructs, grown on a plate containing β-galactosidase. Blue colonies indicate a positive interaction; white colonies indicate no interaction. Eya protein domains are shown in Figure 3. The So Six Domain corresponds to aa98-217 of the fly protein (Pignoni et al. 1997). The Six Domain is distinct from the homeodomain and is conserved between the fly So protein and vertebrate and other Six homologues (Oliver et al. 1995). The Dac Domain is a portion of the Dac protein (amino acid residues 748-826) from a clone isolated in a yeast two-hybrid screen (Q. Bui and N. Bonini, unpublished results). This clone contains a C-terminal part of the Dac protein that includes a domain conserved in vertebrates called the Dachbox-C (aa747-818 of the fly protein; Mardon et al. 1994; Hammond et al. 1998).

partial selective defects in the yeast system in interaction with two genes thought to be critical for eya function: so and dac. To address the degree to which these domains of Eya might define interactions critical to function in vivo, we made UAS transgenic lines expressing the two mutant forms of Eya UAS-eyaE7 and UAS-eyaE11. We then addressed the ability of these mutant forms to function in eye formation, as well as in ectopic eye formation when coexpressed with UAS-so or UAS-dac.

First, we addressed the degree to which these two mutant forms of Eya could function in eye development. To do this, we determined the degree to which they could rescue eye formation of the eya1 mutation. To this end, we coexpressed the wild-type or mutant eya transgene with one copy each of the UAS-eyaE7 and UAS-eyaE11 transgenes. These two transgenes are expressed at similar levels. Lanes 5 and 6 are two different wild-type UAS-eya transgenic lines, lane 5 and 6 being that of Pignoni et al. (1997) and lane 6 that of the present article and Bonini et al. (1997). The wild-type transgenic line used in these studies expresses at a level slightly less than that of the UAS-eyaE7 and UAS-eyaE11 lines.

**Figure 5.**—Activity of the eya1 and eya11 mutations in eye formation. (A) Rescue of eya1 expressing the UAS-eyaE7 transgene. Fly of genotype w; eya1-eyaE7/eya1-eyaE7. (B) Rescue of eya11 with the UAS-eyaE11 transgene. Fly of genotype w; eya11-eyaE7/eya11-eyaE7; UAS-eyaE7/eya2 ey-GAL4; UAS-eyaE11. (C) Rescue of eya11 with one copy each of the UAS-eyaE7 and UAS-eyaE11 transgenes. Fly of genotype w; eya11-eyaE7/eya11-eyaE7; UAS-eyaE7/eya2 ey-GAL4. (D) Immunoblot analysis of Eya protein levels in adult heads, showing that the UAS-eyaE7 and UAS-eyaE11 transgenes express at the same level. Eya was detected with monoclonal antibody EyaMab1F7. Lane 1, Oregon-R; lane 2, deficiency for eya in trans to a normal chromosome, eya11/eya11+. Lanes 3-6 are heads of flies expressing various transgenic insertions driven by the gmr-GAL4 promoter line: lane 3, UAS-eyaE11; lane 4, UAS-eyaE7. These two transgenes are expressed at similar levels. Lanes 5 and 6 are two different wild-type UAS-eya transgenic lines, lane 5 being that of Pignoni et al. (1997) and lane 6 of the present article and Bonini et al. (1997). The wild-type transgenic line used in these studies expresses at a level slightly less than that of the UAS-eyaE7 and UAS-eyaE11 lines.

eye formation comparable to that of the UAS-eyaE11 transgene. The weak interallelic complementation between the two mutants in lethality indicated that we might see an interaction between the two proteins upon directed coexpression. However, we tested but found no special interactions by this assay (Figure 5C). Therefore, coexpression of these two forms of Eya—mutant in different parts of the Eya Domain—did not reveal a synergistic interaction indicative of complementation between the two mutant forms of Eya in this assay for function.

We then tested for distinctions between the mutant forms and wild-type eya in ectopic eye formation upon coexpression with UAS-so or UAS-dac. For these studies, we coexpressed the wild-type or mutant UAS-eya insertion alone or with UAS-so or UAS-dac by the dpp-GAL4 promoter line. The dpp-GAL4 line directs expression to all of the imaginal discs, including the antennal disc, the leg discs, and the wing discs. Previous studies have shown that UAS-eya on its own can direct ectopic eye development when expressed sufficiently strong, but shows synergy in ectopic eye formation when coex-
We describe molecular analysis of mutations in the Drosophila Eya protein. Coupled with genetic analysis of the alleles, these data reveal critical amino acid residues within the highly conserved Eya Domain that may be relevant to the function of the protein. These results

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ectopic eye development (%)</th>
<th>Abnormal leg development (%)</th>
<th>Reduced-eye phenotype (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAS-eyaE7/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UAS-eyaE11/+</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>100±1</td>
<td>0</td>
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<tr>
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<td>100±1</td>
<td>12</td>
</tr>
<tr>
<td>UAS-eyaE11/UAS-so</td>
<td>51±1</td>
<td>100±1</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Ectopic retinal development ranged from specks of pigment to discernible ommatidia and were seen on the antennae, legs, and wings. In the antennal region, ectopic ommatidia were seen.
* b Ectopic retinal tissue consisted of discernible ommatidia and was limited to the antennal region.
* c Ectopic retinal tissue was limited to specks of pigment on the tips of the antennae.
* d Abnormal leg morphology only; no ectopic retinal structures were seen.

Figure 6.—eyaE7 mutant form of eya synergizes with so. (A) Ectopic eye development upon coexpression of so and eya. Ectopic eyes develop on the antennal region of the head (A, arrows). Fly of genotype w; dpp-GAL4 UAS-eya/UAS-so. (B-D) Ectopic eye development upon coexpression of so and the mutant form of eya, eyaE7. Ectopic eyes develop on the antennal region of the head (B, arrows), although with lower penetrance than upon coexpression of normal eya. In some animals, the eye shows a new phenotype of a reduced eye (C). In the leg, although no ectopic ommatidial development is seen, the leg structure is deformed (D). Fly of genotype w; dpp-GAL4 UAS-eyaE7/UAS-so. (E) Ectopic expression of the UAS-eyaE7 transgene in the absence of coexpressed so has no phenotype in the leg (E) or elsewhere. Fly of genotype w; dpp-GAL4/ UAS-eyaE7.
provide new insight into functional aspects of Eya that are likely to be conserved to the vertebrate counterparts. With respect to known interactions of the protein with So and Dac, our evidence indicates that these proteins may have special interactions with different subregions of the Eya Domain. This would suggest that So and Dac, rather than competing for interaction with Eya, may interact with Eya at once, providing evidence in support of a model whereby the three proteins may indeed form a single complex critical for eye development (but see below).

Conservation of Eya function in Drosophila eye development: We demonstrated that Eya function in eye development is shared between the vertebrate Eya homologues and the fly protein: all three murine Eya genes were able to restore eye development to an eyeless allele of eya (see Figure 1). This demonstrates functional conservation of the pathway of eya in eye development between the vertebrate proteins and the fly protein. Among the different homologues, the only conserved domain is the Eya Domain in the C terminus (Duncan et al. 1997; Xu et al. 1997b; Zimmerman et al. 1997). The N-terminal regions differ significantly in sequence among the homologues and with the fly protein. These N-terminal regions of the mouse proteins have been shown to have transcriptional activation activity (Xu et al. 1997a), however, suggesting a potentially similar function if that part of the protein is critical for Eya activity.

eya mutants reveal critical residues within the conserved Eya Domain: We sequenced a number of eya mutant alleles and found several with nonsense or missense mutations within the Eya Domain. Comparison of the site and type of mutation with the genetic nature of the allele revealed that there may be functional subregions within the Eya Domain. Analysis of nonsense mutations revealed one premature termination prior to the Eya Domain and a second set of mutations within the Eya Domain. The allele that terminates early (eya

Figure 7.—Summary of potential interactions revealed by analysis of Drosophila eya mutations. Schematic of Eya protein structure with potential subdomains of the Eya Domain and possible special interaction regions for the Six domain and Dachbox-C indicated, as suggested by these studies. The first part of the Eya Domain may be more selective for interactions with the Six domain, whereas the second part of the Eya Domain may be more selective for interactions with Dachbox-C. Studies of embryonic activity of the premature termination mutations of eya suggest that mutations leaving the first part of the Eya Domain intact appear to display some embryonic function, whereas loss of the entire Eya Domain is null for embryonic function, as demonstrated by the eya

Eya comprises two domains of high conservation to vertebrates: an N-terminal domain called Dachbox-N and a C-terminal domain called Dachbox-C (Hammond et al. 1998). These domains are predicted to have structural homology with the Ski/Sno family of genes, with the Dachbox-C having a potential role in dimerization. Our analysis revealed that the Dachbox-C interacts with the Eya-conserved Domain, with the EF2 subdomain perhaps being of special importance. No selective interaction by these studies was revealed with the third domain of Eya, although this domain is not only highly conserved in vertebrates, but also highly conserved in the nematode C. elegans. C. elegans appears to have a dac homologue as well as an eya homologue (GenBank accession no. U80953); it will be of interest to address whether the nematode Dac
homologue interacts with the C. elegans Eya homologue, or if the Eya-Dac interaction is not evolutionarily conserved. One point mutation found within EF3 appears critical for the function of the Eya Domain: a G (glutamate) mutation at residue 723 in \( \text{eya}^{E3} \). This mutation appears to be more severe than those with premature termination within the Eya Domain, perhaps indicating that this mutation generates an unstable form of the protein subject to degradation. Overall, interactions between the mutant forms of the Eya Domain and the EF subdomains with the So and Dac proteins were considerably weaker than with the wild-type Eya Domain. This may suggest that, despite some evidence for selective interactions, both Eya Domain mutations—either due to a requirement for additional protein domains or to a three-dimensional structure conferred by the smaller region in the context of the entire Eya domain—may disrupt interactions to some degree with both protein partners in vivo. This might contribute to the weaker activity of these Eya mutant forms in vivo in restoring eye development to \( \text{eya} \) mutants.

**Eya Domain interactions with So and Dac in vivo:**

We extended our analysis of the mutations in the Eya Domain to the situation in vivo by generating transgenics expressing the selective point mutants that disrupted interactions with So and Dac in the yeast two-hybrid system. Although this failed to provide evidence in support of a special functional relevance of the Dac interaction—both mutant forms appeared to interact similarly in ectopic eye formation upon coexpression with Dac—we did find evidence supporting the importance of the So interaction. These data indicated that the \( \text{eya}^{E11} \) mutant form showed a diminished ability to synergize with So upon coexpression. This supports the hypothesis that the \( \text{eya}^{E11} \) mutation within the Eya Domain disrupts interactions in vivo with so (and/or possibly with other Six homologues) that are critical for the function in eye formation. The \( \text{Eya}^{E7} \) mutant form, which shows a disrupted Dac interaction, still supported ectopic eye formation, although at decreased penetrance compared to normal Eya. Dac null mutations frequently show some degree of eye development (Mardon et al. 1994), suggesting that dac may be partially redundant in eye formation. Therefore, even if interaction with Dac in vivo were disrupted by the \( \text{eya}^{E7} \) mutation, eye formation might still occur due to compensation by such mechanisms. Nevertheless, this \( \text{eya}^{E7} \) allele also showed a dominant reduced-eye phenotype when coexpressed with so—a new property not observed with the wild-type Eya protein. The \( \text{eya}^{E7} \) mutation may generate a protein with some dominant-negative property in eye formation. Our data that So and Dac may interact, in part, differentially within the conserved Domain of Eya supports the idea that the three proteins have the potential to interact in a single complex in vivo (c.f. Desplan 1997). Such an hypothesis, however, is complicated by other data indicating that the molecular activity of Eya-So coexpression in eye formation is at least in part distinct from that of Dac or Eya-Dac coexpression: whereas Dac, and Eya coexpression with Dac, activate an eye enhancer, Eya alone or Eya with So fail to activate enhancer activity, despite ectopic eye formation (Bui et al. 2000).

The Eya protein of Drosophila has defined a new set of genes in vertebrates that are expressed in the developing and adult eye, as well as in other tissues (Duncan et al. 1997; Xu et al. 1997a,b; Zimmer man et al. 1997; Borsani et al. 1999). Human mutations in Eya1 show developmental defects in organ formation (Abdelhak et al. 1997a,b; Kumar et al. 1998) also reflected in mouse knock-out mutations of Eya1 (Xu et al. 1999). In humans, a number of mutations have been found within the Eya Domain of the Eya1 gene. Our data on detailed analysis of fly mutations within the Eya Domain indicate that these may not be null mutations or, depending upon the site, may selectively disrupt interactions with proteins such as Six homologues, Dac homologues, or other yet-to-be-defined partners. Six homologues are critical for many aspects of differentiation, mutation of which can lead to severe developmental defects such as holoprosencephaly (Wallis et al. 1999). Given the potentially conserved nature of So/Six interactions with Eya proteins, aspects of the developmental defects due to Six homologue mutations may result from disrupted function of Six-Eya complexes critical for various tissues and at different developmental times. Continued study of \( \text{eya} \) in Drosophila is therefore likely to yield information of critical importance to a molecular understanding of the role of the Eya family of proteins and its partner proteins in fundamental aspects of development.

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**LITERATURE CITED**

Abdelhak, S., V. Kalatzis, R. Heilig, S. Compain, D. Samson et al., 1997a Clustering of mutations responsible for branchio-oto-renal (BOR) syndrome in the \( \text{eyes} \) absent homologous region (\( \text{eyaHR} \)) of EYA1. Hum. Mol. Genet. 6: 2247–2255.


Oliver, G., A. Mailhos, R. Wehr, N. G. Copeland and N. A. Jenkins et al., 1995 Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development 121: 4045–4055.


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