Mouse *Eya* homologues of the *Drosophila eyes absent* gene require *Pax6* for expression in lens and nasal placode

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**SUMMARY**

We have identified and mapped three members of a new family of vertebrate genes, designated *Eya1*, *Eya2* and *Eya3*, which share high sequence similarity with the *Drosophila eyes absent* (*eya*) gene. Comparison of all three murine *Eya* gene products and that encoded by the *Drosophila eya* gene defines a 271 amino acid carboxyl terminal Eya domain, which has been highly conserved during evolution. *Eya1* and *Eya2*, which are closely related, are extensively expressed in cranial placodes, in the branchial arches and CNS and in complementary or overlapping patterns during organogenesis. *Eya3* is also expressed in the branchial arches and CNS, but lacks cranial placode expression. All three *Eya* genes are expressed in the developing eye. *Eya1* is expressed in developing anterior chamber structures, including the lens placode, the iris and ciliary region and the prospective corneal ectoderm. *Eya1* is also expressed in retinal pigment epithelium and optic nerve. *Eya2* is expressed in neural retina, sclera and optic nerve sheath. Moreover, *Eya1* and *Eya2* expressions in the lens and nasal placode overlap with and depend upon expression of *Pax6*. The high sequence similarity with *Drosophila eya*, the conserved developmental expression of *Eya* genes in the eye and the *Pax6* dependence of *Eya* expression in the lens and nasal placode indicates that these genes likely represent functional homologues of the *Drosophila eye* gene. These results suggest that members of the *Eya* gene family play critical roles downstream of *Pax* genes in specifying placodal identity and support the idea that despite enormous morphological differences, the early development of insect and mammalian eyes is controlled by a conserved regulatory hierarchy.

Key words: cranial placodes, *Eya* genes, *Eya* domain, *eyes absent*, eye and nasal development, organogenesis, *Pax6*, *Small eye*

**INTRODUCTION**

The vertebrate eye originates from primordial tissues derived from a number of sources, including the surface and neural ectoderm, the neural crest and mesodermal mesenchyme. During eye development, a series of reciprocal cellular interactions occur that determine the fate of the prospective eye tissues. The most striking of these is the pulsed succession of signals and responses between the developing lens and neural retina. In particular, induction of the vertebrate lens provides an important paradigm for understanding the mechanism of inductive tissue interactions in early organogenesis (for review, see Grainger, 1996).

*Pax6*, a member of the paired box family of transcription factors, has been identified as a key regulator of eye development in both vertebrates and invertebrates (reviewed in Glaser et al., 1995; Hanson et al., 1995). The mouse *Pax6* gene is expressed throughout eye development (Walther and Gruss, 1991; Grindley et al., 1995), and *Pax6* mutations are responsible for the mouse mutation *Small eye* (*Sey*) and the human ocular defect aniridia (Hill et al., 1991; Ton et al., 1991). In both species, homozygosity for *Pax6* loss of function results in loss of eyes and nasal cavities (Hogan et al., 1986; Glaser et al., 1994). These phenotypes originate from an absence of lens and nasal placode formation, and could result from a failure of inductive interactions between the head surface ectoderm, which gives rise to the placodes, and the underlying neural plate or mesodermal mesenchyme (Hogan et al., 1986).

In vertebrate embryos, *Pax6* is expressed in head surface ectoderm in both the lens- and nose-forming regions prior to placode formation (Walther and Gruss, 1991; Li et al., 1994; Grindley et al., 1995). *Pax6* is subsequently expressed in the placodes themselves and in the developing neural retina. Based upon its expression pattern, it has been suggested that *Pax6* is involved in the early establishment of lens competent regions within the head ectoderm (Li et al., 1994). In fact, recombination experiments between head surface ectoderm and optic vesicle of wild-type and rat *Small eye* (r*Sey*) embryos have shown that *Pax6* function is required in the surface ectoderm but not in the optic vesicle for lens induction (Fujimura et al., 1994). Nonetheless, *Pax6* function in the neuroectoderm is likely to be important for retinal specification since *Pax6* overexpression results in a loss of photoreceptors (Schell et al., 1996). Thus, in vertebrate eye development, *Pax6* appears to
subserve separate ectodermal and neuroectodermal functions involved in patterning the lens and retina, respectively.

In Drosophila, a Pax6 homologue, the eyeless (eya) gene, is initially expressed in eye progenitor cells and subsequently remains strongly expressed during differentiation of the eye imaginal disc anterior to the morphogenetic furrow. Loss-of-function mutations in Drosophila eya cause an eyeless phenotype (Quiring et al., 1994), and ectopic expression of eya in imaginal discs induces ectopic eyes in wings, legs, antennae and halteres (Halder et al., 1995a). Strikingly, the murine Pax6 gene product can also direct the development of ectopic eyes in Drosophila, presumably either by activating the endogenous Drosophila eya gene or by directly activating downstream genes involved in eye development (Halder et al., 1995a). It has been suggested that the Drosophila compound eye and the vertebrate eye evolved from a common ancestor, and that early eye development of mammals and insects is controlled by similar Pax6-regulated genetic cascades (Halder et al., 1995a,b; Zuker, 1995). Pax6 genes have therefore been proposed to be master control genes for eye development throughout metazoa (Halder et al., 1995a).

Despite this information, and with the notable exception of various crystallin genes (reviewed in Cvekl and Piatigorsky, 1996), the targets for Pax6 regulation in the developing vertebrate eye are unknown. In Drosophila, genes expressed anterior to the morphogenetic furrow are likely to include direct downstream targets of the Pax6 protein encoded by eya. Two Drosophila genes that affect eye development and are expressed anterior to the morphogenetic furrow are eyes absent (eya) and sine oculis (so). Eya encodes a novel nuclear protein of unknown function, while so encodes a homeoprotein; both are required for eye development (Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O’Tousa, 1994). Loss-of-function mutations in so, eya or ey all result in progenitor cell death anterior to the furrow during the third larval instar and a variably penetrant eyeless or reduced eye phenotype (Ransom, 1979; Bonini et al., 1993). However, unlike ey, neither eya or so can direct ectopic eye formation (Bonini and Choi, 1995). Moreover, ey expression is preserved in eye discs of eya and so mutants (Halder et al., 1995a). Thus, in Drosophila, ey appears to function genetically upstream of eya and so.

As one approach to identifying Pax6 regulatory targets in mammalian eye development, we have sought to identify mouse homologues of genes involved in early Drosophila eye development. In this paper, we describe the isolation, mapping and developmental expression of three murine homologues of the Drosophila eya gene, which we have designated Eya1, Eya2 and Eya3, and we show that the lens and placodal expression of Eyal and Eya2 requires Pax6. The high sequence homology of murine Eya family members with Drosophila eya, their conserved developmental expression in the eye, and the dependence of Eyal and Eya2 expression upon Pax6 indicates that these genes are likely to represent functional homologues of the Drosophila eya gene. Our results support the molecular conservation of early eye development between insects and mammals.

MATERIALS AND METHODS

Isolation of Eya1, Eya2 and Eya3 cDNAs

A 717 bp DNA fragment corresponding to amino acids 372 to 610 of the Drosophila eya gene (Bonini et al., 1993) was generated by PCR amplification of Oregon R genomic DNA using primers 5’-ggaaTTC-CCATGGCGCGCCTCTCT-3’ and 5’-ggaaTCTGATCTTGGCGTGAGCGGAG-3’. After EcoRI digestion, this fragment was cloned into pBluescriptII KS+ (Stratagene) and verified by DNA sequencing. Approximately 1x10⁶ clones from a random-primed mouse E11.5 embryonic cDNA library (Clontech) were screened using this PCR fragment. After washing twice with 2x SSC, 0.1% SDS at room temperature and twice with 0.5x SSC, 0.1% SDS at 50°C, positive clones were subcloned into pBluescriptII KS+, restriction mapped, and sequenced on both strands using Sequenase 2.0 (United States Biochemical). From this screen, Eyal and Eya3 clones were recovered. Longer Eyal cDNA clones and several Eya2 cDNAs were obtained by using a partial Eyal cDNA as a probe. An N-terminal extension of the Eya2 open reading frame (nt 1-300, Fig. 1B) was obtained by PCR from a plasmid cDNA library prepared from mouse postnatal day 0-3 eyes.

Chromosomal mapping of Eyal, Eya2 and Eya3

For chromosomal mapping by SSLP (single strand conformation polymorphism) analysis, regions of 3’-UTR of Eyal and Eya3 were amplified by PCR using the primers described below and tested for SSLPs between mouse strains (Beier, 1993). Two primer pairs with the sequence 5’-GAAGATCTCTTCTCTCCTGG-3’ (forward, nt 1767-1778) and 5’-TGCTCTGCTGAAACACAAACTGG-3’ (reverse, nt 1970-1950), and 5’-CCAGGGCTGTTGGTCTTCCTT-3’ (forward, nt 1840-1860) and 5’-AAAGATGGCGCGCATTAGG-3’ (reverse, nt 2065-2047) each identified a polymorphism for Eyal between C57BL/6J and DBA/2J, and were used to analyze DNA prepared from the BXD recombinant inbred series. Two primer pairs with the sequence 5’-CTCGGTCTCTTCTGGCACGTC-3’ (forward, nt 2139-2157) and 5’-AGGCCAGACATCGACGACT-3’ (reverse, nt 2384-2366), and 5’-GCCATCTCCATCTTGAAGC-3’ (forward, nt 2118-2138) and 5’-GCTCTGAGGAGCCAGG-3’ (reverse, nt 2325-2308) each identified a polymorphism in Eya3 between C57BL/6J and M. spreus, and were used to analyze DNA prepared from the BSS backcross (Rowe et al., 1994). Eyal was mapped by Southern analysis of PstI-digested DNA from the BSS cross using a 150 bp fragment of 5’-UTR obtained by PCR from the Eyal cDNA. The strain distribution patterns were analyzed using the Map Manager Program (Manley, 1991).

Northern blot analysis

Eyal (200 bp, nt 151-350), Eya2 (203 bp, nt 1718-1920) and Eya3 (200 bp, nt 2101-2300) cDNA probe fragments were gel purified and labeled by random priming. Poly(A)+ RNA was prepared from E11.5 CD1 mouse embryos using RNAzol B (Biotech Laboratories) and oligo(dT)₃₀ selection (Qiagen), and 5 μg quantities were electrophoresed in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane. Filters were washed at 65°C in 0.1x SSC, 0.1% SDS.

Genotype analysis

Genotypes of SeyNeu/SeyNeu embryos (alleles generously provided by Dr J. Favor, Institut fur Saugetiergenetik, Neuberger) were determined by PCR using genomic DNA from extra-embryonic membranes of E9.5-10.5 embryos. Primers 10.5 (5’-GCATAG-GCAGTTATTTTGGCGC-3’) and PSTMSE (5’-GGAAATCTCCGTG- GAACCAGAGAAGCGGCG-3’) were used at an annealing temperature of 60°C for 35 cycles to amplify a 220 bp Pax6 fragment. The SeyNeu allele has a single-base-pair change within the Pax6 gene that gives rise to a novel HindIII site (Hill et al., 1991). After HindIII digestion, the SeyNeu allele yields 140 and 80 bp fragments which were resolved by agarose gel electrophoresis from the uncut wild-type 220 bp PCR product (Quinn et al., 1996).

Whole-mount and tissue section in situ hybridization

Whole-mount in situ hybridization was performed as described.
(Rosen and Beddington, 1993). Sense and antisense digoxigenin-labeled RNA probes were generated from Eya1, Eya2 and Eya3 cDNA inserts in pBluescript II KS+ using a DIG RNA Labeling kit (Boehringer Mannheim). Embryos were fixed in phosphate-buffered saline (PBS) (pH7.3)/0.1% Triton X-100/3.7% formaldehyde and stored in 100% methanol at −20°C. After rehydration, embryos were washed with three changes of detergent mix at room temperature (30 minutes per wash), and then treated with proteinase K (5-10 μg/ml, 10 minutes at room temperature). Hybridization was carried out at 60°C for 16 hours. After high stringency washes and RNase treatment, the embryos were visualized with an alkaline phosphatase-coupled anti-digoxigenin antibody and sectioned using a vibrating microtome.

For tissue section in situ hybridization, embryos were dissected, fixed overnight in 4% paraformaldehyde, dehydrated, embedded in wax and sectioned at 8 μm. High-stringency hybridization, washing and RNase treatment were performed as described (Wilkinson and Green, 1990). T3 or T7 RNA polymerase in vitro transcribed sense or antisense 35S-labeled RNA probes were generated from various pBluescript II KS+ subclones containing different regions of Eya1 and Eya2. The exposure time was 5-10 days at 4°C. Photographs were taken using Kodak EPX 64T or 160T on a Zeiss Axiohot microscope equipped with a dark-field condenser.

RESULTS

Isolation and structural analysis of mouse Eya1, Eya2 and Eya3 cDNAs
cDNAs were obtained corresponding to three distinct Eya genes, designated Eya1, Eya2 and Eya3 (Fig. 1A-C). Overlapping Eya1 and Eya2 cDNAs each spanned 2.2 kb, while the longest Eya3 cDNA spanned 3.5 kb. Northern blot analysis of poly(A)+ RNA prepared from E11.5 mouse embryos revealed Eya1 and Eya2 transcripts of 5.6 and 3.4 kb respectively, and two Eya3 transcripts of 6.9 and 3.8 kb (Fig. 1D). Although the isolated Eya cDNAs are not full length, the Eya1, Eya3 and potentially Eya2 cDNAs contain the complete coding sequences.

The deduced amino acid sequences for the three Eya gene products are shown in Fig. 1A-C. For Eya1 and Eya3, it was possible to unambiguously assign a single initiation codon and to validate the open reading frame. In vitro translation experiments yielded protein products of 65 and 46 kDa respectively, consistent with the predicted sizes for Eya1 (64.5 kDa) and for Eya3 (45.5 kDa) (data not shown). Thus, the mouse Eya1 and Eya3 proteins are respectively 591 and 416 amino acids, smaller than the 760 amino acid Drosophila Eya protein. For Eya2, it was not possible to unambiguously assign an initiation codon because the reading frame in the cDNA remains open N terminal to the ATG. This ATG conforms to the Kozak consensus, however, and would predict a 532 amino acid (58 kDa) gene product.

Analysis of the Eya protein sequences reveals two distinct domains, a non-conserved amino (N-) terminal region differing in length between different Eya proteins, and a highly conserved 271 amino acid carboxyl (C-) terminal region (in Fig. 1A-C (bold), E,F). Although Drosophila Eya shares 43, 46 and 49% respective overall identity with mouse Eya1, Eya2 and Eya3, most of the identity resides in the C-terminal domain. The N-terminal domains of Eya1, Eya2 and Eya3 consist of 41, 35 and 34% proline, serine and threonine residues respectively, and large numbers of alanine, glycine and glutamine residues are also present. However, except for Eya1 and Eya2, which are 47% identical in their N-terminal domains, there is minimal conservation at the primary sequence level between the N termini of the different Eya gene products.

In contrast, when the C termini of the Drosophila and three mammalian Eya gene products are compared, a discrete 271 residue C-terminal domain can be identified based upon a remarkably high degree of sequence conservation (Fig. 1E,F). We have named this highly conserved C-terminal region the Eya domain and the DNA sequence encoding it the Eya box. Within the Eya domain, Eya1, Eya2 and Eya3 share 73, 67 and 63% identity with the Drosophila eya gene product. The striking evolutionary conservation of the Eya domain suggests major functional importance.

Chromosomal mapping of Eya1, Eya2 and Eya3
As shown in Fig. 2, Eya1 maps to mouse chromosome 1 with a LOD likelihood score of 25.0. Eya2 maps to chromosome 2 with a LOD score of 7.8 and was non-recombinant in the 26 BXD substrains with Pmv33, the most distal marker on chromosome 2 mapped in this cross. Eya3 maps to mouse chromosome 4 with a LOD likelihood score of 28.3. No recombinants were found between Eya3 and D4Mit339 in 94 progeny. The chromosomal locations for Eya1, Eya2 and Eya3 correspond to regions of conserved synteny in the human genome.

Eya1 and Eya2 are expressed in the cranial placodes during placode differentiation
To study whether Eya1, Eya2 and Eya3 expression colocalizes with that of Pax6, Eya expression was analyzed in E8.5-16.5 mouse embryos. Below, we consider the expression patterns for all three Eya genes, then focus in depth on the expression of Eya1 and Eya2, which in many cases is either overlapping or complementary.

At E8.5, Eya1 and Eya2 are already expressed in the pre-somatic mesoderm and head mesenchyme, while Eya3 is expressed in head mesenchyme (data not shown). Subsequently, at E9.5-10.5, Eya1 expression is maintained in head mesenchyme and somites and appears in brain, pharyngeal pouches, nephrogenic cord and branchial arches (Figs 3A,B). Eya2 is similarly expressed in somites and brain, but unlike Eya1, is also expressed in dorsal root ganglia (Fig. 3C,D).

A major defining feature for Eya1 and Eya2 at this stage is their combined expression in all ectodermal cranial placodes and placode derivatives. Both Eya1 and Eya2 are expressed in the epibranchial placodes and their cranial ganglia derivatives, the facio-acoustic (VII-VIII) ganglionic complex and the glossohypophygeal (IX) and vagus (X) ganglia (Figs 3A-D, 4A-D). However, the placodal expression patterns of Eya1 and Eya2 are not identical. Eya1 but not Eya2 is expressed in the otic vesicle, a derivative of the otic placode, and in Rathke’s pouch, the anterior pituitary anlage. Because of its ectodermal origin and capacity for endocrine differentiation, Rathke’s pouch is considered a cranial placode (Verwoerd and van Oostrom, 1979). Conversely, Eya2 is expressed in the trigeminal (V) placode and ganglion, while Eya1 is not. Finally, Eya1 is expressed in both lens and nasal placodes, whereas Eya2 is only expressed in the nasal placode (Fig. 5). We conclude that Eya1 and Eya2 are likely to play critical roles in the induction and differentiation of ectodermal cranial placodes.

In contrast to Eya1 and Eya2, Eya3 at E9.5-10.5 is expressed...
**Fig. 1.** Nucleotide and predicted amino acid sequences of mouse *Eya1*, *Eya2* and *Eya3* cDNAs. (A-C) The 271 C-terminal amino acids (boxed) define the highly conserved Eya domain (see E). The GenBank Accession Numbers are: *Eya1*, U61110; *Eya2*, U61111 and *Eya3*, U61112. The assigned initiation codons are shown in bold. The ATG for *Eya2* is only assigned provisionally because the relevant ORF does not contain a 5' termination codon. For secondary structure predictions, potential nuclear localization signals and other features, see the GenBank Accession entries. (D) Northern blot analysis of *Eya1* and *Eya2* transcripts. Sizes are indicated in kb.
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only in head and branchial arch mesenchyme, and in the brain, limb, eye and all rhomboemeres (Figs 3E,F, 4E,F and data not shown). In the E9.5 eye, Eya3 is expressed in the optic vesicle and periopioc mesenchyme but is absent from the lens placode; by E10.5, Eya3 is expressed in the lens vesicle and neureotina (data not shown). Thus, whereas either Eya1 or Eya2 is strongly expressed in all cranial placodes, Eya3 is not expressed in the placodes at all. Except for the tissues mentioned above, Eya3 expression is restricted to cranial and branchial arch mesenchyme, and in fact appears concentrated in regions underlying or surrounding the cranial placodes (Fig. 4E,F).

**Eya1 and Eya2 are expressed with Pax6 in lens and nasal placodes**

The expression of Eya1 and Eya2 in lens and nasal placodes coincides with Pax6 expression (Fig. 5). Eya1 becomes expressed in lens placodal ectoderm at E9.5, after the optic vesicle and overlying surface ectoderm becomes more strongly expressed as the ectoderm thickens (Fig. 5A). Eya2 expression, in contrast, is not detected in lens placode ectoderm at any time (Fig. 5B). Pax6 expression in head ectoderm can be detected as early as E8.0 (Grindley et al., 1995), significantly earlier than Eya1 expression. Thereafter, Pax6 expression becomes restricted to many parts of the brain, including the ventricles (Fig. 5A). Eya2 expression, in contrast, is not detected in lens placode ectoderm at any time (Fig. 5B). Pax6 expression in head ectoderm can be detected as early as E8.0 (Grindley et al., 1995), significantly earlier than Eya1 expression. Thereafter, Pax6 expression becomes restricted to the lens and nasal placode forming regions (Fig. 5C). Similar to the co-expression of Eya1 and Pax6 in the lens placode, both Eya1 and Eya2 are strongly co-expressed with Pax6 in the nasal placode (Fig. 5D-F). Eya1 expression in nasal placodes is restricted to the lens and nasal placode forming regions, whereas Pax6 expression overlaps with but precedes that of Eya1 and Eya2 in the lens and nasal placodes.

**Eya1 and Eya2 are expressed in overlapping or complementary patterns during CNS and craniofacial development and organogenesis**

Within the CNS, high levels of Eya1 and Eya2 transcripts could be detected in many parts of the brain, including the ventricular zone (VZ) of the developing forebrain and hindbrain at E11.5-12.5 (Fig. 6A,B,E,F). In the developing spinal cord, Eya1 is weakly expressed in the dorsal neural tube but absent from the floor plate (Fig. 6C,D). In the craniofacial region, Eya1 and Eya2 show complementary expression patterns. At E11.5-12.5, Eya1 is strongly expressed in the dorsal neural tube but absent from the floor plate (Fig. 6C,D). In the craniofacial region, Eya1 and Eya2 show complementary expression patterns. At E11.5-12.5, Eya1 is strongly expressed in the craniofacial mesenchyme, whereas Eya2 is strongly expressed in the overlying epithelium (Fig. 6C,F). In
the developing tooth, Eya1 is expressed in the dental mesenchyme at E12.5, while Eya2 is expressed in oral ectoderm including the dental lamina at E11.5 and the developing tooth bud at E12.5 (Fig. 6C,D). From E12.5 to E14.5, both genes also show complementary expression in the whisker follicle (Fig. 6E,F). Eya1 transcripts are distributed in the condensed mesenchyme surrounding the developing whisker follicle, whereas Eya2 transcripts are abundant in the ectodermal component of the follicle (Fig. 7A,B).

Expression of both Eya1 and Eya2 is also detected throughout organogenesis in overlapping or complementary patterns. At E11.5-14.5, high levels of expression of the Eya genes were detected in the prevertebrae. Eya1 transcripts were first detected in the precartilage primordium and later strongly in the condensed mesenchymal blastema of the prevertebrae, whereas Eya2 transcripts are localized in the mesenchyme outside the blastema in the region fated to become intervertebral disc, and in the future intercostal muscles (Fig. 7C,D). Eya1 and Eya2 also show differential expression in the gut. Gut mesenchyme expresses Eya1 strongly, while Eya2 is expressed in the endoderm; both genes are expressed in an asymmetric, dorsoventrally graded fashion (Fig. 7E,F). Both genes are also strongly expressed in the developing kidney and genital tubercle (Fig. 6E,F). In the developing limb bud, both Eya1 and Eya2 are expressed in myogenic and connective tissue progenitors (data not shown). The expression of Eya1 and Eya2 during organogenesis, often in adjacent tissue layers, suggests a general function in inductive tissue interactions.

**Eya1 and Eya2 are differentially expressed in the developing eye and nose**

Eya1 and Eya2 are differentially expressed during eye and nasal development in a highly dynamic fashion. Subsequent to lens placode invagination, Eya1 expression is maintained in the lens vesicle and optic stalk, and appears in the outer layer and at the peripheral margin of the bilayered optic cup (Fig. 8A,B). The outer layer of the optic cup will differentiate into retinal pigment epithelium while the periphery will differentiate into the iris and ciliary body regions. Only low levels of Eya1 expression were detected in the neural retina. In the lens, beginning at E12.5, Eya1 transcripts become progressively stronger in the anterior...
epithelial layer and fainter in the lens fiber cells (Fig. 8B,C). Later on at E16.5, Eya1 expression is observed in the surface ectoderm destined to form cornea (data not shown).

In contrast to the expression of Eya1 in anterior ocular structures, Eya2 expression is restricted to posterior parts of the developing eye. These include the neural retina and prospective lens and nasal development. In the neural retina, Eya2 expression was first detected at E11.5 in retinal progenitor cells in the central retina (Fig. 8D). From E12.5-14.5, the expression of Eya2 becomes restricted to the inner nuclear cell layer of the retina, and is specifically excluded from the peripheral neural retina where Eya1 is expressed (Fig. 8E,F). The complementary nature of Eya1 and Eya2 expression extends to additional ocular structures. Whereas Eya1 is strongly expressed in the optic nerve, Eya2 is excluded from the nerve and is expressed in the surrounding optic nerve sheath; both genes also appear to be differentially expressed in extraocular muscles (data not shown). The expression of Eya1 and Eya2 suggests that these genes function in multiple steps of ocular development.

Eya1 and Eya2 are also expressed during nasal development. Subsequent to nasal placode formation, expression of both genes in the developing olfactory epithelium continues during the formation of the nasal pits and the vomeronasal (Jacobson’s) organ, the latter a derivative of the olfactory placode (Fig. 9A-F). At E14.5, Eya2 expression becomes noticeably weak in the anterior region of the olfactory epithelium whereas Eya1 expression remains uniform (Fig. 9C,D). By E16.5, Eya1 and Eya2 show complementary expression within the olfactory epithelium (Fig. 9E,F). Eya1 is strongly and uniformly expressed throughout the apical epithelial layer whereas Eya2 expression is absent. In contrast, Eya2 is strongly expressed in the basal epithelial layer where Eya1 expression is either weak or absent. Eya1 and Eya2 appear to play general but distinct roles in patterning the olfactory epithelium.

Eya1 and Eya2 expression in lens and nasal placodal ectoderm requires Pax6

To determine if Eya1 and Eya2 expression in the prospective nasal and lens placodal ectoderm requires Pax6, Eya1 and Eya2 expression was analyzed in SeyNeu/SeyNeu embryos at E9.5. At E9.5, overt morphologic differences between wild-type and mutant embryos are not yet apparent. Eya1 is expressed in wild-type prospective lens and nasal placodal ectoderm at this stage, when the lens and nasal placodes are just beginning to form. In SeyNeu/SeyNeu embryos, Eya1 expression in both the lens and nasal placodal ectoderm is markedly reduced (Fig. 10A-F). In contrast, the level of Eya1 expression in the perinasal mesenchyme appears to be increased in SeyNeu/SeyNeu embryo (Fig. 10D). Eya1 expression in the Rathke’s pouch is also reduced in SeyNeu/SeyNeu embryo at E10.0 (data not shown). In wild-type embryos, Eya2 expression at E9.5 is strongly detected in prospective nasal but not lens placodal ectoderm. In contrast, in SeyNeu/SeyNeu embryos, Eya2 expression in the prospective nasal ectoderm is undetectable (Fig. 10G-J), similar to Eya1. Eya2 expression in the perioptic and perinasal mesenchyme is not detectable in wild-type embryos; however, in SeyNeu/SeyNeu embryo, Eya2 is ectopically expressed in the perioptic and perinasal mesenchyme (Fig. 10G-J). Eya1 and 2 expression in other embryonic regions remains well preserved in SeyNeu/SeyNeu embryos (data not shown). Similar results for Eya1 and Eya2 expression in SeyNeu/SeyNeu embryos were obtained in six independent experiments involving wild-type and mutant embryos ranging from E9.0-10.0. We conclude that Pax6 is
required for *Eya* expression in lens and nasal placodal ectoderm.

**DISCUSSION**

*Eya1* and *Eya2* are widely expressed in cranial placodes and at sites of inductive tissue interactions during organogenesis, often in complementary or overlapping patterns. These features suggest major roles for *Eya* genes in the development of vertebrate sensory systems and organs. In addition, *Eya1* and *Eya2* require *Pax6* for their expression in lens and nasal placode ectoderm, while *Eya2* is not. The prospective lens ectoderm is at an early stage of placode morphogenesis, defined by contact between the optic vesicle and surface ectoderm without ectodermal thickening; *Eya1* is expressed later than *Pax6* and is only weakly expressed in the lens ectoderm at the early stage (data not shown). *Eya1, Eya2* and *Pax6* are all expressed in the nasal placode at E9.5. At this time, the nasal placode has already thickened, anticipating the equivalent stage in the contiguous lens placode which lags behind by 6-12 hours. Abbreviations: lp, lens placode; np, nasal placode; nt, neural tube; oc, optic cup. Orientation: ventral is up. Scale bar: 50 μm.

The *Eya* genes may mediate induction of the cranial placodes

The cranial placodes arise as thickenings in head ectoderm adjacent to the neural tube, and comprise the anlagen of the vertebrate lens, nose, ear, anterior pituitary, precursors of the cranial sensory ganglia and, in fishes, the lateral line organ (reviewed in Verwoerd and van Oostrom, 1979; Nieuwkoop et al., 1985; Webb and Noden, 1993). The lens placode excepted, the cranial placodes differentiate into neuronal or endocrine cells, which comprise the respective sensory and endocrine organs and peripheral nervous system. The trigeminal, epi-
branchial and otic placodes provide mitotic neuroblasts, which delaminate from the ectoderm and coalesce with neural crest to form the sensory ganglia for the trigeminal (V), the glosopharyngeal (IX), vagus (X) and facial (VII), and the acoustic (VIII) nerves, respectively (D’Amico-Martel and Noden, 1983; Webb and Noden, 1993). Together, Eya1 and Eya2 are expressed in all cranial placodes, while Eya3 expression appears concentrated in craniofacial mesenchyme surrounding the placodes. Eya1 and Eya2 expression in head surface ectoderm precedes and then coincides with the first morphologic stages of placode formation, and is maintained in placode derived structures up to and including E16.5, the latest stage examined for these structures (data not shown). Thus, Eya genes are likely to play a central role in mediating both the induction and differentiation of cranial placodes.

Previous studies on placodal development in amphibian embryos have suggested that the cranial sensory placodes may be induced by similar mechanisms, beginning with very early inductive events during mid-gastrula stages. Although differences exist between placodes with respect to ease of inducibility and onset and duration of ectodermal competence, one model suggests that initially a common placodal state is activated in a large region of head ectoderm (Jacobson, 1966; Nieuwkoop et al., 1985; Grainger, 1996; Gallagher et al., 1996). Subsequently, during neural tube formation, interactions with particular regions of the developing brain lead to the formation of different placodes in their appropriate location and association with neural tissue. The cranial placodes are thus formed by a series of inductors, with forebrain completing induction of the nasal placode, optic vesicle completing induction of the lens placode and hindbrain completing induction of the otic placode.

One molecule potentially involved in placode induction could be FGF3, which is expressed in the hindbrain in rhombomeres r5 and r6 adjacent to the otic placode. FGF3 knockout mice exhibit normal otic vesicle development, but the adjacent epibranchial placode-derived VII/VIII cranial ganglia are reduced or absent (Mansour et al., 1993). In addition, experimental inhibition of FGF3 mRNA interferes with formation of the nodose (X) placode (Qin and Kirby, 1995). Eya1 and Pax2 are differentially expressed in the portion of the otic vesicle flanking the hindbrain, suggesting that their expression could depend upon
a hindbrain derived signal (Nornes et al., 1990). Similarly, recent results suggest that an optic vesicle derived signal regulated by the LIM homeobox gene \textit{Lhx2} acts to maintain \textit{Pax6} expression in lens placode ectoderm (F. D. Porter, personal communication). Thus, \textit{Eya} genes may function along with \textit{Pax} genes in a molecular pathway within the ectoderm which is activated or maintained in response to neuroectoderm derived signals.

It should be noted, however, that \textit{Eya} genes could function at multiple steps during placode induction. For example like \textit{Eya1}, \textit{Eya3} is expressed during otic vesicle induction, but unlike \textit{Eya1}, \textit{Eya3} is not expressed in the otic vesicle. Instead, \textit{Eya3} is expressed in the adjacent hindbrain rhombomeres and in the mesenchyme surrounding the otic vesicle. In potentially analogous fashion, \textit{Eya1} is expressed in both the lens placode and the subjacent perioptic mesenchyme, and both tissue components are believed to interact during lens induction. Thus, while our results suggest a critical ectodermal function for \textit{Eya} genes in mediating placode induction, they also support a broader function in regulating the general exchange of inductive signals between tissue layers during placode induction and organogenesis.

\textbf{Eye development depends upon similar \textit{Pax6} regulated pathways in mammals and insects}

In the \textit{Drosophila} eye imaginal disc, \textit{ey} controls a genetic hierarchy involving \textit{eya} and \textit{so} that is required for eye formation. In vertebrate eye development, \textit{Pax6} function is required in head surface ectoderm for lens formation. To determine if the genetic hierarchy regulated by the insect and mammalian \textit{Pax6} genes is conserved at the molecular level, we examined \textit{Eya} expression in the prospective lens and nasal ectoderm in wild-type and \textit{Sey/Sey} mutant embryos. We show that, in wild-type embryos, \textit{Pax6} expression precedes that of \textit{Eya1} in prospective lens placodal ectoderm. However, in contrast to wild-type embryos, \textit{Eya1} and \textit{Eya2} expression in lens or nasal placodal ectoderm of \textit{Sey Neu/Sey Neu} embryos

\textbf{Fig. 7.} Complementary expression of \textit{Eya1} and \textit{Eya2} during organogenesis. \textit{Eya1} (A) and \textit{Eya2} (B) expression in the E14.5 vibrissal follicle. \textit{Eya1} is expressed in follicular mesenchyme (m), while \textit{Eya2} is expressed in follicular epithelium (e). \textit{Eya1} (C) and \textit{Eya2} (D) are expressed in the anlage of the anterior vertebral body anlage (pv) and the future intercostal muscles (im), respectively. \textit{Eya1} (E) and \textit{Eya2} (F) are expressed in the gastric mesenchyme (m) and endoderm (e), respectively at E13.5; dorsal is to the left. Scale bar, 50 \textmu m.

\textbf{Fig. 8.} \textit{Eya1} and \textit{2} are expressed in complementary patterns in the developing eye. (A–C) \textit{Eya1} at E11.5-14.5 is expressed throughout the lens (l) and in the peripheral retinal margin which is destined to become the iris and ciliary body (arrows), in pigmented retina (pr) and optic stalk (os), and only weakly in the neur retina (nr). At E12.5-14.5, \textit{Eya1} expression in the lens becomes stronger in the anterior epithelium (e) and weaker in the lens fiber cells (lf). (D–F) \textit{Eya2} at E11.5-14.5 is expressed in perioptic mesenchyme and in migrating retinal progenitor cells. By E12.5-14.5, \textit{Eya2} expression strongly localizes to the inner nuclear layer (inl) of the retina but is excluded from the pigmented layer. \textit{Eya2} expression is also observed in the sclera (s). Other abbreviations: onl, outer nuclear layer; se, surface ectoderm. Orientation: transverse sections, nasal aspect at top. Scale bar, 50 \textmu m.
Eya1, Eya2 and Eya3 genes in the mouse embryo

cannot be detected. The marked reduction of Eya1 and Eya2 expression in the prospective lens or nasal ectoderm in Sey Neu/Sey Neu embryos establishes that directly or indirectly, Pax6 is required for Eya expression in placodal ectoderm. In Drosophila eye development, so appears to function downstream of ey. Similar to Eya and Pax genes, three mouse so homologues, Six1-Six3, are also expressed in the nasal, otic and trigeminal placodes and in Rathke’s pouch (Oliver et al., 1995a,b); Six3 expression is also detected in lens placode (S. Wawersik, P-X. Xu and R. Maas, unpublished data). In addition, Eya1 and Eya2 strikingly co-localize in mid-gestation mouse embryos with the expression of Six genes in brain, dorsal root ganglia, somites, kidney, limb, tendons and in various mesenchymes, suggesting that these genes function together in multiple developmental contexts. In Drosophila imaginal disc development, both so and eya reside downstream of ey, but eya is epistatic to so (Cheyette et al., 1994). Although Six3 expression is maintained in some contexts in Sey/Sey mouse embryos (Oliver et al., 1995b), the striking similarity in Eya and Six gene expression during embryogenesis leads us to propose that different combinations of Pax, Eya and Six genes act within a hierarchical pathway similar to that employed in the Drosophila eye imaginal disc to specify individual cranial placode identities in vertebrate head ectoderm. Consistent with this, ectopic expression of the murine Six3 gene in the Japanese medakafish, Oryzias latipes, transforms the otic placode into a lens placode resulting in a well formed but ectopic lens (J. Wittbrodt et al., personal communication).

It is worth considering the molecular implications of a Pax-Eya regulatory hierarchy. Besides the cranial placodes and developing eye, Eya and Pax genes are co-

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**Fig. 9.** Expression of Eya1 and 2 in the developing nose. (A,C,E) Eya1 is strongly expressed in the olfactory epithelium (oe), nasal septum (ns) and nasal capsule (ncp). (B,D,F) Eya2 is strongly expressed in the olfactory epithelium and in nasopharyngeal ectoderm (np). The vomeronasal (Jacobsen’s) organ (vo) expresses both genes. (E,F) At E16.5, Eya1 and Eya2 show complementary expression in the olfactory epithelium. Eya1 is strongly expressed in the apical epithelial layer (arrow) where Eya2 is not expressed, while Eya2 is expressed in the basal epithelial layer where Eya1 is weak or absent. Other abbreviations: t, tongue; nco, nasal conchae. Orientation: ventral is up. Scale bar, 200 μm.

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**Fig. 10.** Eya1 and Eya2 expression in prospective lens and nasal placodes requires Pax6. Radioactive (A,B,G,H) and whole-mount (C-F,I,J) in situ hybridization analyses of Eya1 and Eya2 expression in wild-type and Sey Neu/Sey Neu mutant E9.5 embryos. (A,B) Eya1 expression is reduced in the surface ectoderm in the lens and nose forming region in Sey Neu/Sey Neu mutant embryos. This is illustrated in more detail for the nasal ectoderm in C,D and the lens ectoderm in E,F. The level of Eya1 expression in the perinasal mesenchyme appears to be increased in Sey Neu/Sey Neu mutant embryo (D). (G-J) Eya2 expression is not detectable in the nasal ectoderm in the Sey Neu/Sey Neu embryo. Note: Eya2 is ectopically expressed in the perioptic and perinasal mesenchyme in the Sey Neu/Sey Neu embryo (H,J). Abbreviations: np, nasal placode; nt, neural tube; os, optic stalk; se, surface ectoderm. Scale bar, 50 μm.
expressed in many contexts, with \textit{Pax1} and \textit{Eya1} in the pharyngeal pouches, \textit{Pax2} and \textit{Eya1} in the optic nerve, otic vesicle and kidney and \textit{Pax3} and \textit{Eya1} and \textit{Eya2} in the somites (Wallin et al., 1996; Dressler et al., 1990; Nornes et al., 1990; Goulding et al., 1994). For the regulation of \textit{Eya} expression by \textit{Pax6} and possibly other \textit{Pax} proteins to be direct would require that \textit{Pax} proteins bind either distinct or a common DNA recognition sequence in the \textit{Eya} genes. The latter hypothesis is plausible, since \textit{Pax2}, \textit{Pax3}, \textit{Pax5}, \textit{Pax6} and \textit{Drosophila} Paired can all bind to a similar DNA recognition sequence via the N-terminal subregions of their paired domains (Epstein et al., 1994, 1996; Chalepakis and Gruss, 1995; Czerny and Busslinger, 1995; Xu et al., 1995). It also could be possible that \textit{Pax6-Eya} regulatory hierarchy involves additional factor(s), such as \textit{dachshund} (\textit{dac}), which is also involved in the \textit{ey} controlled pathway in \textit{Drosophila} (Bonini and Choi, 1995). \textit{dac} is expressed in the eye imaginal disc, similar to \textit{eya} and \textit{so}, and encodes a novel nuclear protein required for early eye development (Mardon et al., 1994). Ectopic expression of \textit{dac} can also direct ectopic eye formation (Shen and Mardon, 1977). Identification of vertebrate homologues of \textit{dac} will further strengthen the idea that the early development of mammalian and insect eyes is under the control of similar genetic cascades.

The existence of a conserved molecular pathway involving \textit{Pax} and \textit{Eya} genes could be taken to suggest that vertebrate cranial placodes and insect imaginal discs, both ectodermal tissues, are phylogenetically related. Nonetheless, evolutionary considerations suggest that the retina may be more closely related to the eye imaginal disc than the lens. Development of both the vertebrate retina and the insect eye disc results in the genesis of rhodopsin-based photoreceptor cells, and the determination of cell fate in each relies similarly upon cell-cell interactions and intercellular factors. The overlapping expression of \textit{Pax6}, \textit{Eya2} and \textit{Six3} in retinal cell progenitors suggests that a regulatory hierarchy similar to that in prospective lens ectoderm may also be utilized in retinal specification. In addition, \textit{Drosophila} mutations in \textit{ey}, \textit{eya} and \textit{so} each result in cell autonomous apoptosis in the unpatterned epithelium anterior to the morphogenetic furrow (Bonini et al., 1993; Ransom, 1979). While the apoptosis in the unpatterned epithelium anterior to the morphogenetic furrow in the \textit{Drosophila} embryo leads to the specification of the entire eye, the \textit{Eya} N termini could have retained a conserved molecular function. For example, the \textit{Pax6} domain of \textit{Pax6} can function as a transcriptional domain (Glaser et al., 1994) and, although the corresponding \textit{PST} domain in \textit{Eyesless} is highly divergent, both can be inferred to function equivalently in vivo (Halder et al., 1995a). The N-terminal \textit{PST} domains of \textit{Eyesless} may also encode a transactivation function. Although the \textit{Eya} gene products do not possess a known DNA-binding motif, they could interact either with DNA or with a DNA-binding protein to activate transcription. Analysis of the \textit{Eya} protein sequence suggests that the highly conserved \textit{Eya} domain could mediate such molecular interactions. This hypothesis can now be subjected to experimental test.

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