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

Pin-Xian Xu[1,2], Ian Woo[1,2], Helen Her[2], David R. Beier[2] and Richard L. Maas[1,2,*]

[1]Howard Hughes Medical Institute, Brigham and Women's Hospital, Boston, MA 02115, USA
[2]Division of Genetics, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

*Author for correspondence (e-mail: maas@rascal.med.harvard.edu)

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 eya* 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 (Fujiwara 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 (Schedl 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* (*ey*) 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 ey* cause an eyeless phenotype (Quiring et al., 1994), and ectopic expression of *ey* 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 by either activating the endogenous *Drosophila ey* 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 *ey*. 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 nasal placodal expression of *Eya1* 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 *Eya1* 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-CATGTGGCGGCCTCCTCG-3′ and 5′-ggaattCTTGATCTTGCG-GTAGCGGAAG-3′. After *Eco*RI digestion, this fragment was cloned into pBluescriptII KS$^+$ (Stratagene) and verified by DNA sequencing. Approximately 1×10^6 clones from a random-primed mouse E11.5 embryonic cDNA library (Clontech) were screened using this PCR fragment. After washing twice with 2× SSC, 0.1% SDS at room temperature and twice with 0.5× 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, *Eya1* and *Eya3* clones were recovered. Longer *Eya1* cDNA clones and several *Eya2* cDNAs were obtained by using a partial *Eya1* 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 *Eya1*, *Eya2* and *Eya3*

For chromosomal mapping by SSCP (single strand conformation polymorphism) analysis, regions of 3′-UTR of *Eya2* and *Eya3* were amplified by PCR using the primers described below and tested for SSCPs between mouse strains (Beier, 1993). Two primer pairs with the sequence 5′-AGAAGTGTCTTCTTCCCTTGGG-3′ (forward, nt 1767-1788) and 5′-TGTCCCTGAAACACAAACTGG-3′ (reverse, nt 1970-1950), and 5′-CCAGCTCGTCTTGTTCTCCTT-3′ (forward, nt 1840-1860) and 5′-ACAAGATGGCGGCATAAGG-3′ (reverse, nt 2065-2047) each identified a polymorphism for *Eya2* 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′-CTCGGTCTCCTTGGCAGTC-3′ (forward, nt 2139-2157) and 5′-AGGCCAGCATCTGACGACT-3′ (reverse, nt 2384-2366), and 5′-GGCATCTCCCATCTTGTAAGC-3′ (forward, nt 2118-2138) and 5′-GCTCTGCAGGAGCACGAG-3′ (reverse, nt 2325-2308) each identified a polymorphism in *Eya3* between C57BL/6J and *M. spretus*, and were used to analyze DNA prepared from the BSS backcross (Rowe et al., 1994). *Eya1* was mapped by Southern analysis of *Pst*I-digested DNA from the BSS cross using a 150 bp fragment of 5′-UTR obtained by PCR from the *Eya1* cDNA. The strain distribution patterns were analyzed using the Map Manager Program (Manley, 1991).

Northern blot analysis

Eya1 (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 (Biotecx 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 0.1× SSC, 0.1% SDS.

Genotype analysis

Genotypes of *Sey*^{Neu}/*Sey*^{Neu} embryos (allele generously provided by Dr J. Favor, Institut fur Saugetiergenetik, Neuherberg) were determined by PCR using genomic DNA from extra-embryonic membranes of E9.5-10.5 embryos. Primers 10.5 (5′-GCATAG-GCAGGTTATTTGCC-3′) and PSTMSE (5′-GGAATTCCTGAG-GAACCAGAGAAGACAGGC-3′) were used at an annealing temperature of 60˚C for 35 cycles to amplify a 220 bp *Pax6* fragment. The *Sey*^{Neu} allele has a single-base-pair change within the *Pax6* gene that gives rise to a novel *Hin*dII site (Hill et al., 1991). After *Hin*dII digestion, the *Sey*^{Neu} 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 digoxygenin-labeled RNA probes were prepared 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 ^{35}S-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 EPY 64T or 160T on a Zeiss Axiophot 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 murine 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 *Eya*1, *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 presomitic 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 (Fig. 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 glossopharyngeal (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

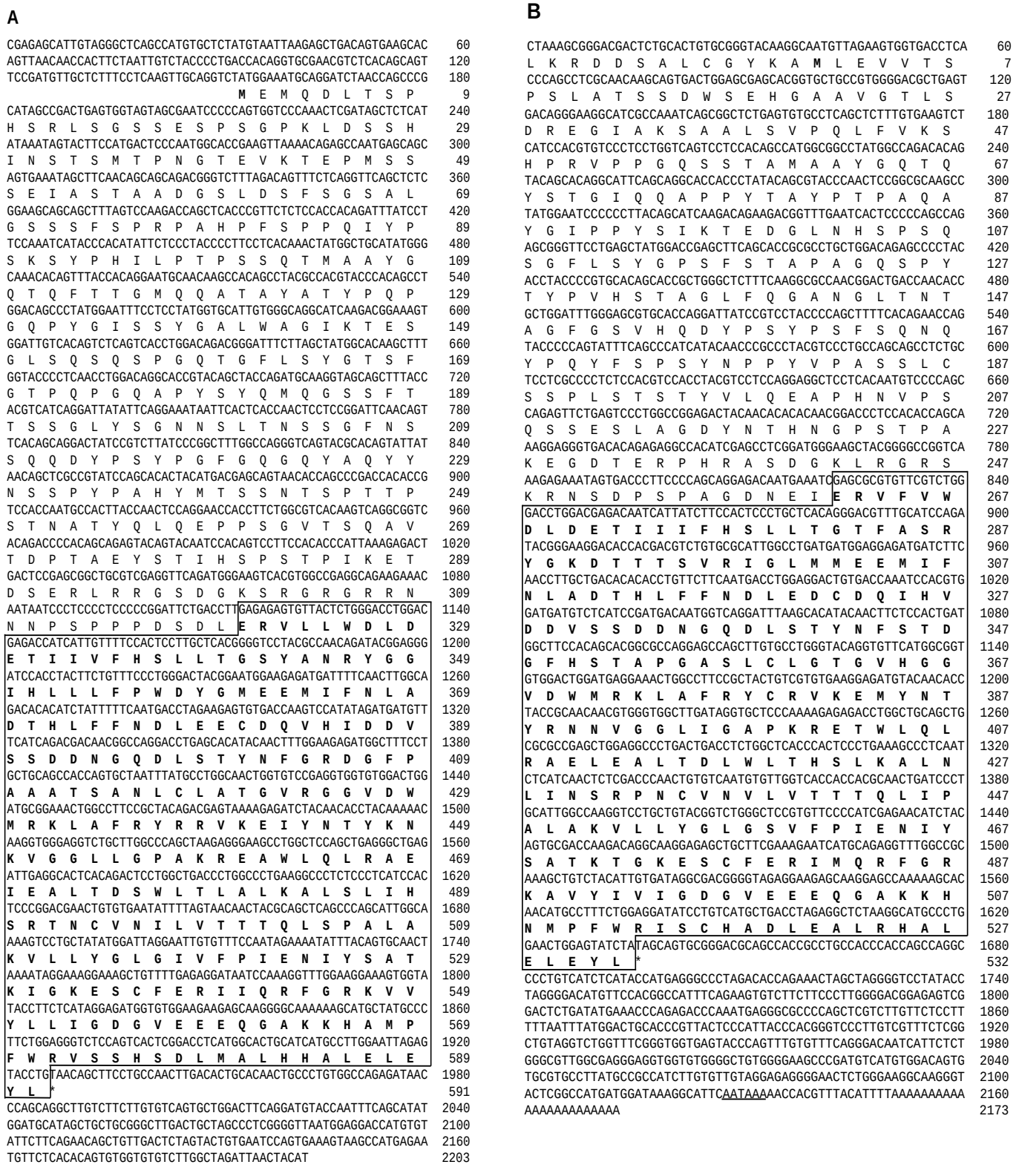

Fig. 1. Nucleotide and predicted amino acid sequences of mouse *Eya1, Eya2* and *Eya3* cDNAs. (A-C) The 271 C-terminal amino acids (boxed and in bold) define the highly conserved Eya domain (see E). The GenBank Accession Numbers are: *Eya1*, U61110; *Eya2*, U61111 and *Eya3*, U61112. The assigned initiation 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*, *Eya2* and *Eya3* transcripts. Sizes are indicated in kb.

C

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AGACGGCTGCAGACACTGCAATTCAGTCCCCTAAGTGTGTTAGGAGATAGTCGTCCTCAT    60
GGAAGAAGAGCAAGACCTACCAGAGCAACCGTCAAGTAAACAACCCAGATGCCAGTGATG   120
AGAAGCCTGAGACATCCAGCCTTGCGTCAAATCTCAGCATGTCAGAGGAAATTATGACAT   180
GCACCGATTACCTCCCTCGCTCATCCAATGATTATACCTCACAAATGTATTCTGCAAACT   240
                          M  I  I  P  H  K  C  I  L  Q  T     11
TCAAGCACAAATGCCAGCCTGATACCCACTTCATCTGCAATTGCCAATATTCCAGCAGCA   300
S  S  T  N  A  S  L  I  P  T  S  S  A  I  A  N  I  P  A  A     31
GCTGTGGCCAGCATCTCAAACCAGGATTATCCCACCTATACTATTCTTGGACAGAATCAG   360
A  V  A  S  I  S  N  Q  D  Y  P  T  Y  T  I  L  G  Q  N  Q     51
TACCAGGCCTGCTACCCCAGTTCCAGCTTTGGAGTCACAGGTCAGACTAACAGTGATGCT   420
Y  Q  A  C  Y  P  S  S  S  F  G  V  T  G  Q  T  N  S  D  A     71
GAGACCACAACATTAGCAGCTACAACATACCAGACGGAGAAGCCTAGTGCTATGGTGCCT   480
E  T  T  T  L  A  A  T  T  Y  Q  T  E  K  P  S  A  M  V  P     91
GCACCAGCCACACAGAGGCTTCCCTCCGACTCCTCTGCAAGCCTACCTTTGTCCCAGACT   540
A  P  A  T  Q  R  L  P  S  D  S  S  A  S  L  P  L  S  Q  T    111
ACACCAAATAAAGATGCTGATGATCAGGCCAGGAAAAACATGACTGTCAAGAACCGGGGC   600
T  P  N  K  D  A  D  D  Q  A  R  K  N  M  T  V  K  N  R  G    131
AAGAGGAAAGCTGATGCCAGCTCTTCCCAGGACAGTGAATTGGAACGGGTATTTCTCTGG   660
K  R  K  A  D  A  S  S  S  Q  D  S  E  L  E  R  V  F  L  W    151
GACTTGGACGAAACCATCATCATCTTTCATTCCCTTCTCACTGGATCCTATGCTCAGAAG   720
D  L  D  E  T  I  I  I  F  H  S  L  L  T  G  S  Y  A  Q  K    171
TATGGAAAGGACCCAACAGTAGTAATTGGCTCAGGTTTAACCATGGAAGAAATGATTTTT   780
Y  G  K  D  P  T  V  V  I  G  S  G  L  T  M  E  E  M  I  F    191
GAAGTGGCTGATACACATCTATTTTTCAATGACTTAGAGGAGTGTGACCAGGTGCATGTG   840
E  V  A  D  T  H  L  F  F  N  D  L  E  E  C  D  Q  V  H  V    211
GAAGATGTGGCTTCTGATGACAATGGCCAGGATTTGAGCAACTACAGTTTCTCCACAGAT   900
E  D  V  A  S  D  D  N  G  Q  D  L  S  N  Y  S  F  S  T  D    231
GGTTTCAGTGGTTCAGGAGGCAGTGGTAGCCACGGTTCATCTGTGGGCGTTCAGGGAGGT   960
G  F  S  G  S  G  G  S  G  S  H  G  S  S  V  G  V  Q  G  G    251
GTGGACTGGATGAGGAAACTGGCCTTTCGCTACCGAAAAGTGAGGGAAATCTATGACAAG  1020
V  D  W  M  R  K  L  A  F  R  Y  R  K  V  R  E  I  Y  D  K    271
CATAAAAGCAATGTGGGTGGCCTCCTCAGCCCCCAGAGGAAGGAAGCACTGCAGAGACTC  1080
H  K  S  N  V  G  G  L  L  S  P  Q  R  K  E  A  L  Q  R  L    291
AGAGCAGAGATCGAGGTGCTGACGGACTCCTGGTTAGGAACTGCGCTCAAGTCCTTGCTT  1140
R  A  E  I  E  V  L  T  D  S  W  L  G  T  A  L  K  S  L  L    311
CTCATCCAGTCTCGAAAGAACTGTGCGAATGTTCTGATCACTACCACGCAGTTGGTTCCA  1200
L  I  Q  S  R  K  N  C  A  N  V  L  I  T  T  T  Q  L  V  P    331
GCCCTGGCCAAGGTTCTCCTGTATGGACTAGGAGAGATATTTCCTATTGAAAACATCTAC  1260
A  L  A  K  V  L  L  Y  G  L  G  E  I  F  P  I  E  N  I  Y    351
AGTGCTACCAAAATCGGTAAGGAGAGCTGCTTTGAGAGAATTGTTTCGAGGTTTGGGAAA  1320
S  A  T  K  I  G  K  E  S  C  F  E  R  I  V  S  R  F  G  K    371
AAAGTCACATATGTAGTGATTGGAGATGGACGAGATGAAGAAATTGCAGCCAAGCAGCAC  1380
K  V  T  Y  V  V  I  G  D  G  R  D  E  E  I  A  A  K  Q  H    391
AACATGCCCTTCTGGAGGATCACAAACCACGGAGATCTGGTGTCCCTGCACCAGGCTTTA  1440
N  M  P  F  W  R  I  T  N  H  G  D  L  V  S  L  H  Q  A  L    411
GAGCTTGACTTCCTCTGAGAACTGGAATGTGGACCCTTCCTTCCTTGTGAGCTTCTCTTT  1500
E  L  D  F  L  *
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GTACAGCAAGAGGTGGCTGGATAGAGCTGCAGACCGGCTGCCGCTACCGTGCTTTAATTT  1740
TTCTGTTTCAATTGAAAAAGGAAGAACAAGAAAAGCCGACGTTGGGCACAGTTGTACTCT  1800
TGCTCGATGACGGACTGACCGGGAGCTGCTCCTGATGTTGTGGATGGAAACTGTCCCCTT  1860
GAGATCGTCTTCTGGTCTCTTAGCTATAGAATGTCTCTCCCAGTGAATGGGTATGTTATT  1920
TTTATAGGTGAGGGTCTGGCCTCTACAGCAGCCTCCCCCACTTTTCTATGAAGAAAGCCG  1980
TGTGTAAAGTTTCCGTGACAGTAGTAATGGAAATATCTAAAATACCTCCGTGACTGACAG  2040
GACAGAGGCTCCAGTGCTCTGCTCCCTGGGGAGGCTCCTTGCCATCTACAAGGCCCTGGT  2100
CACTTATTTTAGACTGTGGCATCTCCCATCTTGTAAGCCTCGGTCTCCTTGGCAGTCCTC  2160
CTTCATCTGTAACTAAATGGCACAACCCTGGAAACTCCTTCCCATAGACAATAAATCTAA  2220
GCCTTTAGCTTGTACTTCAGAAGTTCTGTCCCAGGAGGCTCAGCCTCCAGACTGGAGAGA  2280
AGGGCTCAGATTTCTTAGGACTTTCACCTCGTGCTCCTGCAGAGCAGTACCTGTTCCCAG  2340
CAGATGCTTCCTCTGTGGTCGGCTCAGTCGTCAGATGCTGGCCTCACGTCCTGTCCTCAC  2400
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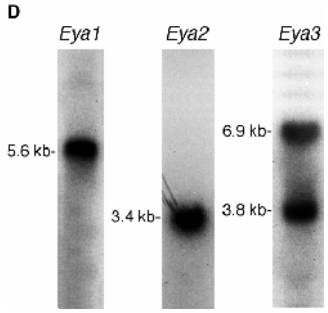


only in head and branchial arch mesenchyme, and in the brain, limb, eye and all rhombomeres (Figs 3E,F, 4E,F and data not shown). In the E9.5 eye, *Eya3* is expressed in the optic vesicle and perioptic mesenchyme but is absent from the lens placode; by E10.5, *Eya3* is expressed in the lens vesicle and neuroretina (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 craniofacial 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

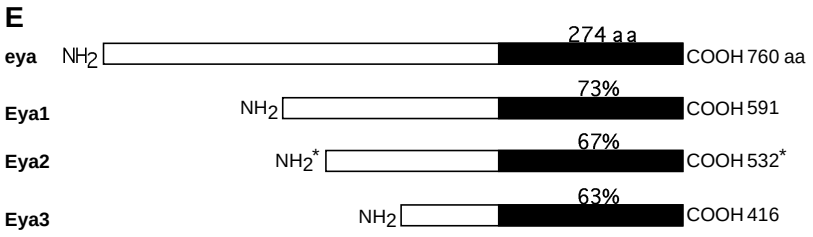
The expression of *Eya1* and *Eya2* in lens and nasal placodes coincides with *Pax6* expression (Fig. 5). *Eya1* begins to be expressed in lens placodal ectoderm at E9.5, after the optic vesicle and overlying surface ectoderm make contact, and then becomes more strongly expressed as the ectoderm thickens (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 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). *Eya* expression in the nasal ectoderm is first detected just before the ectoderm thickens, but after the onset of *Pax6* expression (data not shown). *Pax6* expression thus 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 and floor plate, whereas *Eya2* 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 craniofacial mesenchyme, whereas *Eya2* is strongly expressed in the overlying epithelium (Fig. 6C-F). In



F

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eya   ERVFVWDLDETLIIFHTLLSGSYANRYTKDHSSLMTIAFRMEEMVFNMADTHFFFNEIEECDQVHIDDV
Eya1  ---LL------I-V--S--T-------GGI-LL-FPWDYG----I--L----L---DL-----------
Eya2  -----------I----S--T-TF-S--G--TTTSVR-GLM----I--L----L---DL-D---I-V---
Eya3  ----L------I----S--T----QK-G--PTVVIGSGLT----I-EV----L---DL-------VE--

eya   SSDDNGQDLSAYNFATDGFHTNTPPGAPPNLCLPTGVRGGFDWMRKLAFRYRKIKDIYNSYRGNVGTLL
Eya1  ----------T---GR---PAAAT·S-···-----A------V-----------RV-E---T-KNK--G--
Eya2  ----------T---S-----STA-···-··S---G---H--V----------CRV-EM--T--N---G-I
Eya3  A---------N-S-S----SGSGG·SG··SHGSSV--Q--V-----------VRE--DKHKS---G--

eya   GPGKREAWLQIRSEIEVATDNWATLALKCLSMISQRENCVNYLVTSTQLAPALAKVLLFGLGGIFNIEN
Eya1  --A-------L-A---AL--S-L-----A--L-HS-T----I---T---S--------Y---IV-P---
Eya2  -AP---T---L-A-L-AL--L-L-HS--A-NL-NS-P--------T---I--------Y---SV-P---
Eya3  S-QR---LQRL-A----L--S-LGT---S-LL-QS-K--A---I-T---V--------Y---E--P---

eya   IYSAHKIGHETCYERIVTRFGRKSTYVVIGDGNEEETAAKAMNFPFWRISAHSDIRALYTALDMGFL*
Eya1  ----T---K-S-F---IQ-----VV-LL----V---QG--KHAM----V-S---LM--HH--ELEY-*
Eya2  ----T-T-K-S-F---MQ-----AV-I-----V---QG--KH-M------C-A-LE--RH--ELEY-*
Eya3  ----T---K-S-F----S---K-V--------RD--I---QH-M-----TN-G-LVS-HQ--ELD--*
```

Fig. 1 continued. (E) Sequence identity of the *Drosophila* and murine *Eya* gene products. Note that the 271 amino acid Eya domains have sustained a 3-residue deletion with respect to the 274 amino acid *Drosophila* Eya domain. The Eya domains are shown in black, along with the percent identity to the *Drosophila* Eya domain. The N terminus and the number of amino acids for Eya2 are indicated with an asterisk to indicate that the initiation codon has not been definitively assigned. Sequence identity of the murine N-terminal domains to the *Drosophila Eya* gene product drops to 20-30%. (F) Amino acid sequence alignment of the Eya domain, defined by the C-terminal 271 amino acids encoded by the murine *Eya* gene products. Residues identical to the *Drosophila* protein are indicated by dashes. Residues conserved between two or more murine genes are indicated in **bold**.

the developing tooth, *Eya1* is expressed in the dental mesenchyme at E12.5, while *Eya2* is expressd 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

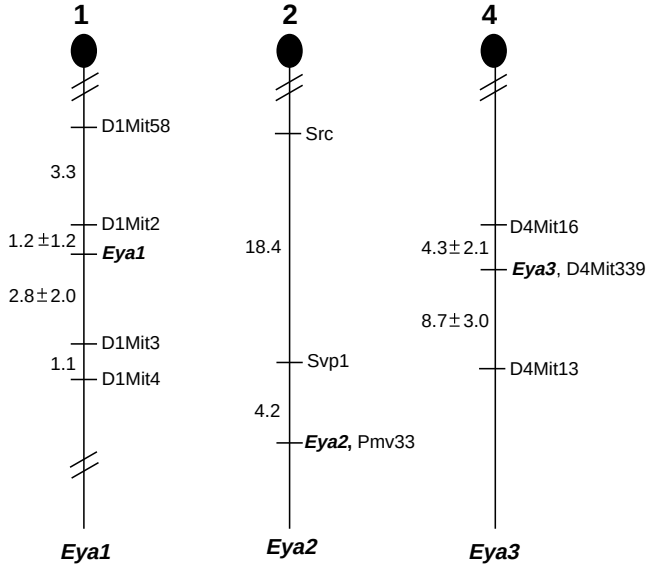

Fig. 2. Chromosomal mapping of *Eya1*, *Eya2* and *Eya3*. Partial linkage maps showing the location of *Eya 1, Eya2* and *Eya3* in relation to linked markers, with recombination frequencies in centimorgans (cM). Human homologues of mouse genes flanking *Eya1* have been mapped to human 8q11.2 (*Oprk1*) and 6q13 (*Col9a1*). A presumptive human *Eya2* orthologue (93% identity over 123 amino acids with mouse *Eya2*) has been mapped as an EST to 20q13.1 (Banfi et al., 1996). Human homologs of mouse genes flanking *Eya3* have been mapped to 1p32-35 (*Lck*) and 1p36.1 (*Pax7*).

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 sclera (Fig. 8D-F). 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 Sey^{Neu}/Sey^{Neu} 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 Sey^{Neu}/Sey^{Neu}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 Sey^{Neu}/Sey^{Neu}embryo (Fig. 10D). *Eya1* expression in the Rathke's pouch is also reduced in Sey^{Neu}/Sey^{Neu}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 Sey^{Neu}/Sey^{Neu} 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 Sey^{Neu}/Sey^{Neu} 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 Sey^{Neu}/Sey^{Neu} embryos (data not shown). Similar results for *Eya1* and *Eya2* expression in Sey^{Neu}/Sey^{Neu} embryos were obtained in six independent experiments involving wild-type and mutant embryos ranging from E9.0-10.0. We conclude that *Pax6* is

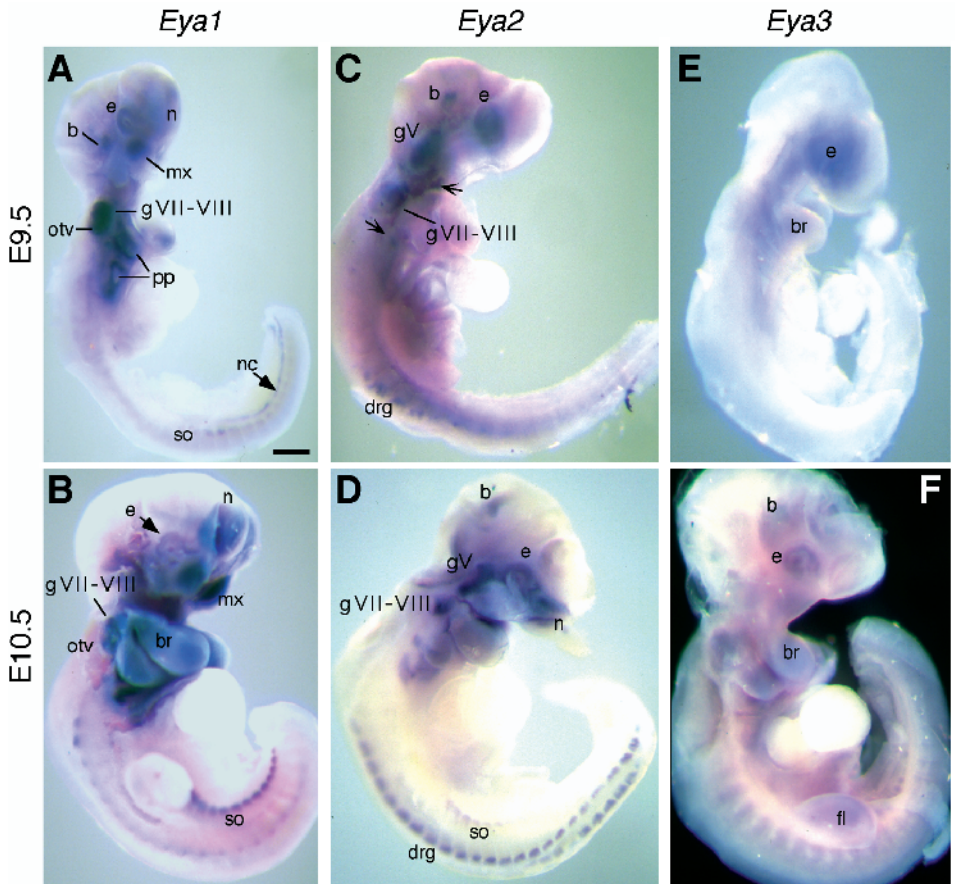

Fig. 3. *Eya1*, *Eya2* and *Eya3* expression in E9.5-10.5 mouse embryos. All three genes are expressed in the eye (e). Both *Eya1* and *Eya2* are expressed in the facioacoustic ganglionic complex (gVII-VIII), the epibranchial placodes (arrows in C; not shown for *Eya1*), the nasal placodes (n) and somites (so). *Eya1* and *Eya2* are also differentially expressed with *Eya1* in branchial arch mesenchyme (br), the pharyngeal pouches (pp) and otic vesicle (otv), and *Eya2* in branchial arch ectoderm, the trigeminal (gV) and dorsal root ganglia (drg). *Eya3* is expressed in the head and branchial arch mesenchyme (br), forelimb (fl), hindlimb and eye (e). Other abbreviations: b, brain; mx, maxillary component of the first branchial arch; nc, nephrogenic cord. Scale bar, 200 μm.

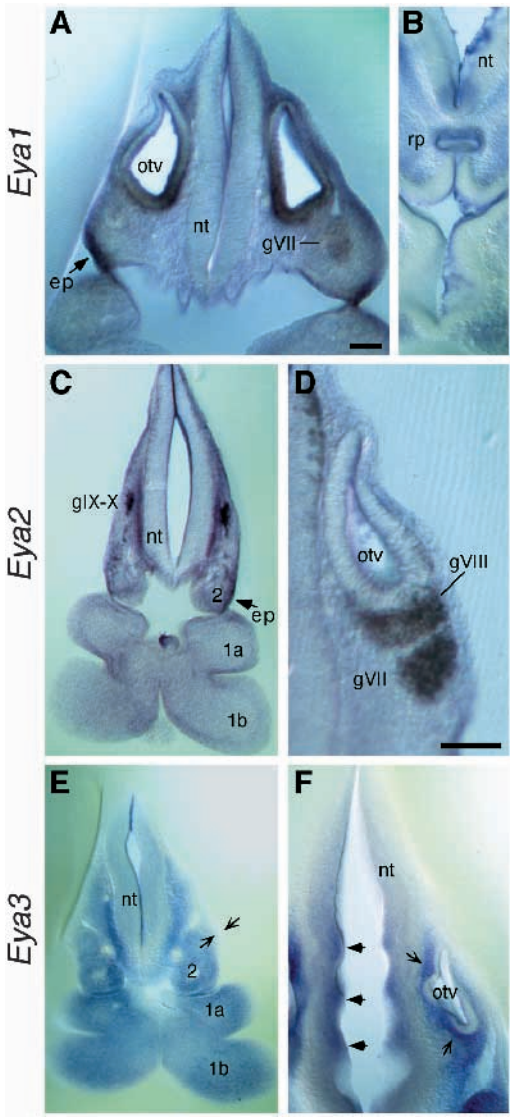

Fig. 4. *Eya1* and *Eya2* are expressed in cranial placodes and ganglia, while *Eya3* is expressed in mesenchyme. (A) *Eya1* expression in the otic vesicle (otv), epibranchial placode (ep, arrow) and facial ganglion (gVII) at E9.5, and (B) in Rathke's pouch (rp) at E10.5. (C) *Eya2* expression in the glossopharyngeal and vagus ganglionic complex (gIX-X), epibranchial placode (ep, arrow), and (D) in the facioacoustic ganglionic complex (gVII, gVIII) at E9.5. (E) *Eya3* expression in head and branchial arch mesenchyme at E9.5, and (F) in rhombomeres (arrowheads), and in mesenchyme surrounding the otic vesicle (arrows) at E10.5. Note that *Eya3* is excluded from craniofacial ectoderm (arrows in E) and largely excluded from the otic vesicle (F). Abbreviations: nt, neural tube; 1a, maxillary component of first branchial arch; 1b, mandibular component of first branchial arch; 2, second branchial arch. Dorsal is up. Scale bar, 200 μm.

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,

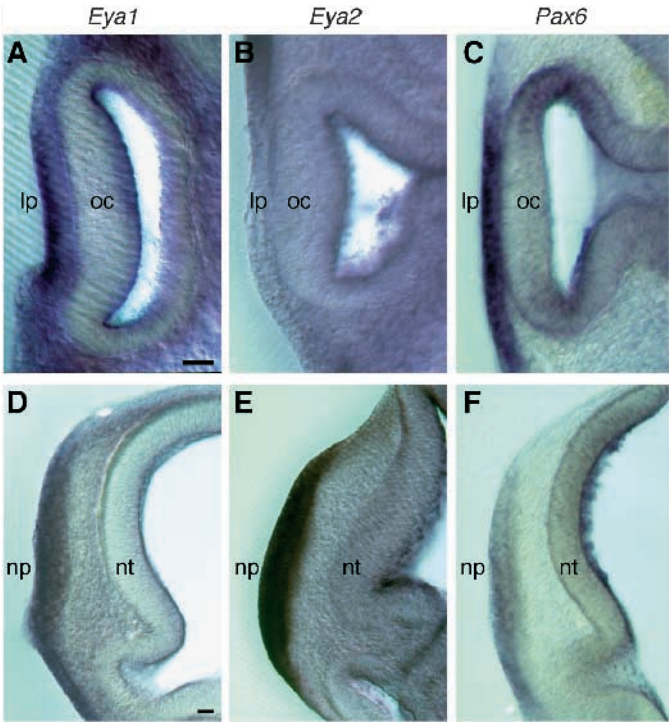

Fig. 5. *Eya1*, *Eya2* and *Pax6* are expressed in lens and nasal placodes. *Eya1* (A) and *Pax6* (C) are expressed in the lens placode ectoderm, while *Eya2* (B) is not. (B,C) The prospective lens ectoderm is at an early stage of placode morphogenesis, defined by contact between the optic vesicle and surface ectoderm without ectodermal thickening; (A) the prospective lens ectoderm is at a later stage of placode morphogenesis. *Eya1* is expressed later than *Pax6* and is only weakly expressed in the lens ectoderm at the early stage (data not shown). (D-F) *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.

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, supporting the molecular conservation of the insect and mammalian eye-forming regulatory hierarchies. Below, we consider the possible functions of *Eya* genes in cranial placode induction and in eye morphogenesis.

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 glossopharyngeal (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). *Eya 1* and *Pax2* are differentially expressed in the portion of the otic vesicle flanking the hindbrain, suggesting that their expression could depend upon

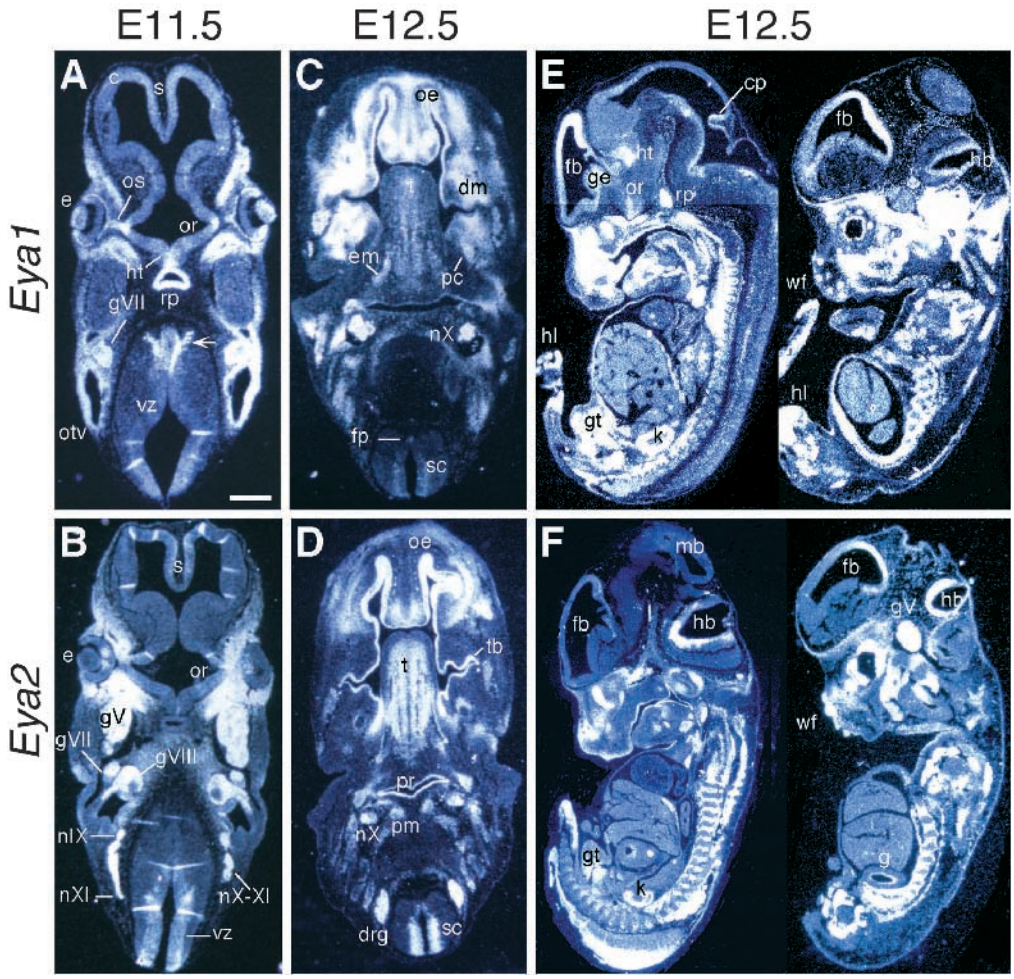

Fig. 6. Expression of *Eya* genes in the developing CNS and craniofacial region and during organogenesis. Transverse (A,C) and parasagittal (E) sections show *Eya1* expression in cerebral cortex (c), septum (s), ventricular zone (VZ) of the forebrain (fb), hindbrain (hb) and in motor neurons in the hindbrain (arrow in A), weakly in spinal cord (sc) and floor plate (fp), in the ganglionic eminence (ge), choroid plexus (cp), hypothalamus (ht), optic stalk (os), optic recess (or), eye (e), Rathke's pouch (rp), facial ganglion (gVII), vagus nerve (nX) and otic vesicle (otv) at E11.5-12.5. Expression is also seen in the intrinsic and extrinsic (em) muscles of the tongue (t), dental (dm) and craniofacial mesenchyme, and in the precartilage tissues (pc) and the olfactory epithelium (oe) at E11.5-12.5. Transverse (B,D) and parasagittal (F) sections show *Eya2* expression in the septum (s), the ventricular zone (vz) of forebrain (fb) and hindbrain (hb), the spinal cord (sc), optic recess (or) and eye (e), in the trigeminal ganglion (gV), facioacoustic ganglia (gVII-VIII), glossopharyngeal nerve (nIX), vagus nerve (nX), cranial accessory nerve (nXI), and in intrinsic muscles of the tongue (t), tooth bud (tb), pharyngeal region (pr) and dorsal root ganglion (drg) at E11.5-12.5. *Eya2* expression is also seen in the prevertebral premuscle mass (pm) and in the olfactory epithelium (oe). Both *Eya1* and *Eya2* are expressed in vibrissal (whisker) follicle (wf), prevertebrae, gut (g), kidney (k), genital tubercle (gt) and limb bud. Data not shown: *Eya2* is strongly expressed in the developing thymus and proximal bronchial epithelium, whereas *Eya1* is expressed in distal bronchial epithelium. No expression of *Eya1* or *Eya2* was detected in the developing heart or liver. Other abbreviations: hl, hindlimb; mb, midbrain. Orientation in A-D: ventral is up. Scale bar, 400 μm in (A-D) and 1000 μm in (E,F).

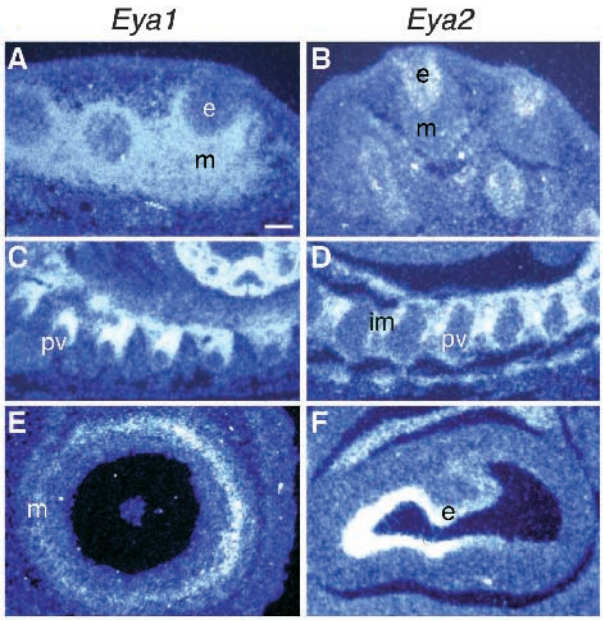

Fig. 7. Complementary expression of *Eya1* and *Eya2* during organogenesis. *Eya1* (A) and *Eya2* (B) expression in the E14.5 vibrissal follicle. *Eya1* is expressed in follicular mesenchyme (m), while *Eya2* is expressed in follicular epithelium (e). *Eya1* (C) and *Eya2* (D) are expressed in the anlage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

a hindbrain derived signal (Nornes et al., 1990). Similarly, recent results suggest that an optic vesicle derived signal regulated by the LIM homeobox gene *Lhx2* acts to maintain *Pax6* expression in lens placode ectoderm (F. D. Porter, personal communication). Thus, *Eya* genes may function along with *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 *Eya* genes could function at multiple steps during placode induction. For example like *Eya1*, *Eya3* is expressed during otic vesicle induction, but unlike *Eya1*, *Eya3* is not expressed in the otic vesicle. Instead, *Eya3* is expressed in the adjacent hindbrain rhombomeres and in the mesenchyme surrounding the otic vesicle. In potentially analogous fashion, *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 *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.

Eye development depends upon similar *Pax6* regulated pathways in mammals and insects

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

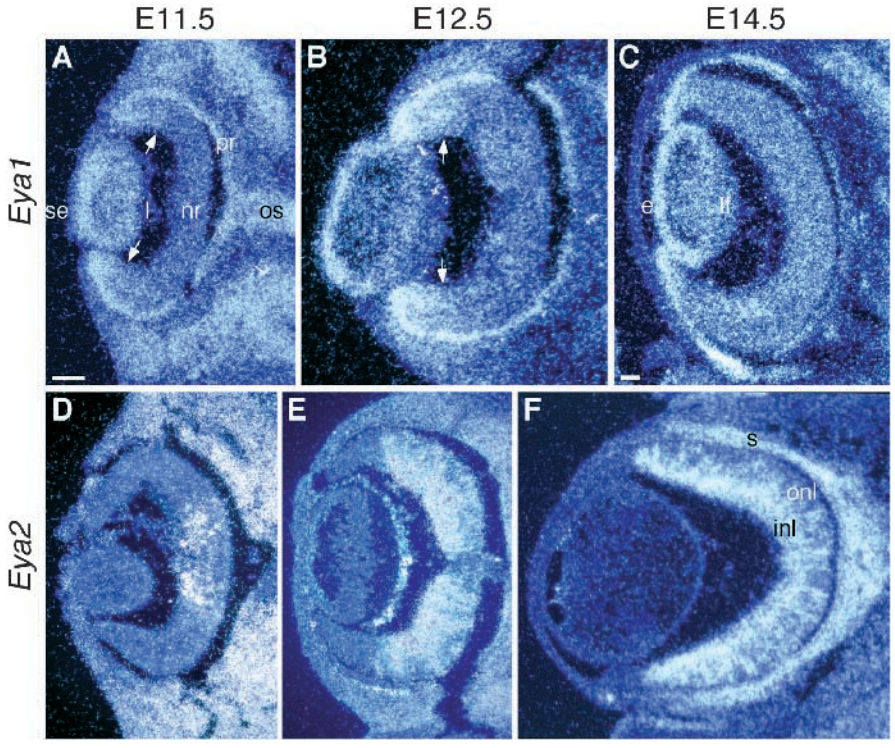

Fig. 8. *Eya1* and *2* are expressed in complementary patterns in the developing eye. (A-C) *Eya1* at E11.5-14.5 is expressed throughout the lens (l) and in the peripheral retinal margin which is destined to become the iris and ciliary body (arrows), in pigmented retina (pr) and optic stalk (os), and only weakly in the neuroretina (nr). At E12.5-14.5, *Eya1* expression in the lens becomes stronger in the anterior epithelium (e) and weaker in the lens fiber cells (lf). (D-F) *Eya2* at E11.5-14.5 is expressed in perioptic mesenchyme and in migrating retinal progenitor cells. By E12.5-14.5, *Eya2* expression strongly localizes to the inner nuclear layer (inl) of the retina but is excluded from the pigmented layer. *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 μm.

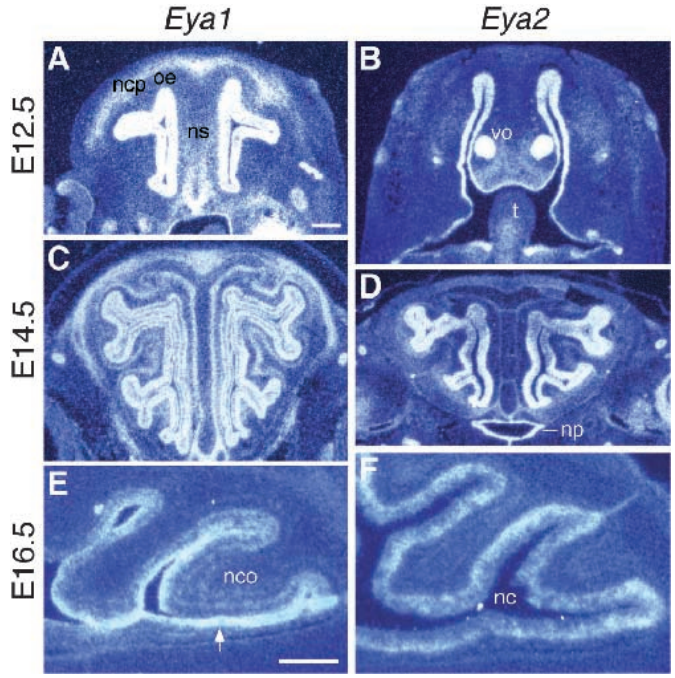

Fig. 9. Expression of *Eya1* and *2* in the developing nose. (A,C,E) *Eya1* is strongly expressed in the olfactory epithelium (oe), nasal septum (ns) and nasal capsule (ncp). (B,D,F) *Eya2* is strongly expressed in the olfactory epithelium and in nasopharyneal ectoderm (np). The vomeronasal (Jacobsen's) 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.

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-

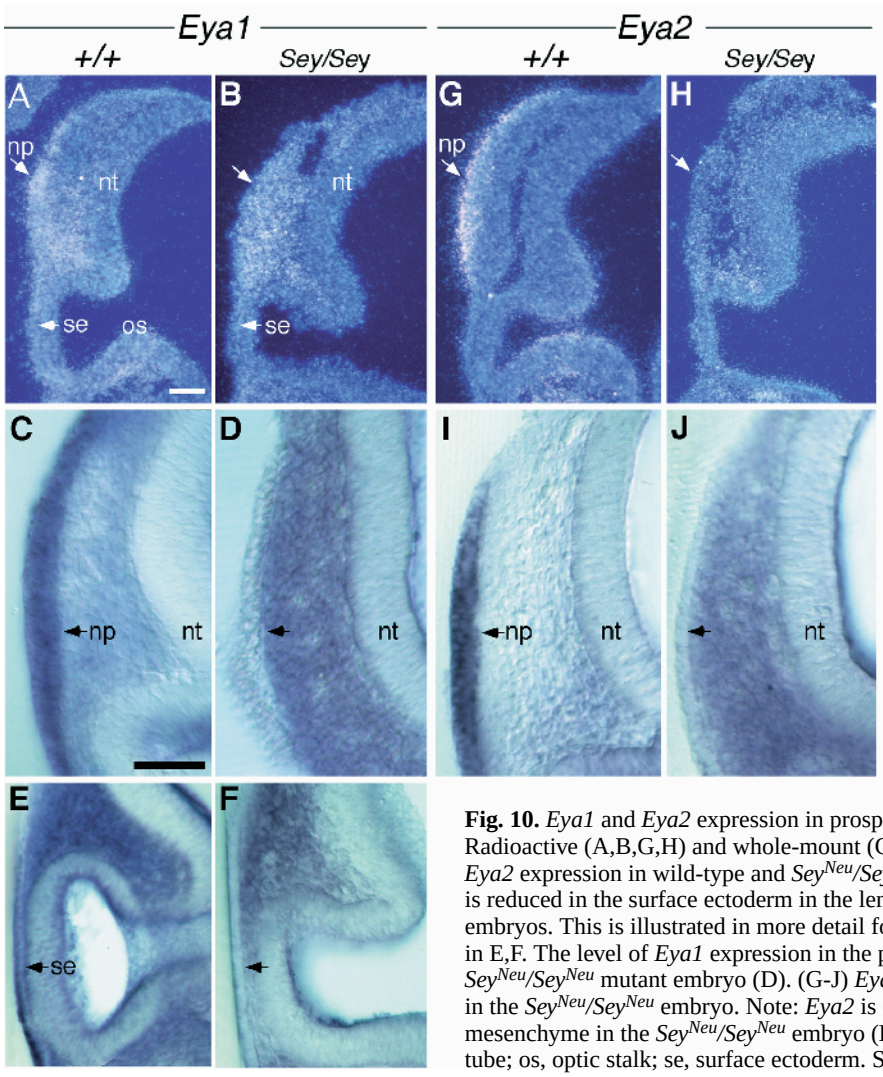

Fig. 10. *Eya1* and *Eya2* expression in prospective lens and nasal placodes requires *Pax6*. Radioactive (A,B,G,H) and whole-mount (C-F,I,J) in situ hybridization analyses of *Eya1* and *Eya2* expression in wild-type and Sey^{Neu}/Sey^{Neu} mutant E9.5 embryos. (A,B) *Eya1* expression is reduced in the surface ectoderm in the lens and nose forming region in Sey^{Neu}/Sey^{Neu} 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 *Pax1* and *Eya1* in the pharyngeal pouches, *Pax2* and *Eya1* in the optic nerve, otic vesicle and kidney and *Pax3* and *Eya1* and *Eya2* in the somites (Wallin et al., 1996; Dressler et al., 1990; Nornes et al., 1990; Goulding et al., 1994). For the regulation of *Eya* expression by Pax6 and possibly other Pax proteins to be direct would require that Pax proteins bind either distinct or a common DNA recognition sequence in the *Eya* genes. The latter hypothesis is plausible, since Pax2, Pax3, Pax5, Pax6 and *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 *Pax6-Eya* regulatory hierarchy involves additional factor(s), such as *dachshund* (*dac*), which is also involved in the *ey* controlled pathway in *Drosophila* (Bonini and Choi, 1995). *dac* is expressed in the eye imaginal disc, similar to *eya* and *so*, and encodes a novel nuclear protein required for early eye development (Mardon et al., 1994). Ectopic expression of *dac* can also direct ectopic eye formation (Shen and Mardon, 1977). Identification of vertebrate homologues of *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 *Pax* and *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 *Pax6*, *Eya2* and *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, *Drosophila* mutations in *ey*, *eya* and *so* each result in cell autonomous apoptosis in the unpatterned epithelium anterior to the morphogenetic furrow (Bonini et al., 1993; Ransom, 1979). While the function of *Eya2* in retinal patterning is unknown, some retinal functions of *Pax6* are also executed cell autonomously (Quinn et al., 1996). These considerations suggest that *Eya2* may execute some functions of *Pax6* in retinal specification.

Although the *Drosophila eya* gene product encodes a nuclear protein of unknown function, there are some clues to its function. The N-terminal regions of the murine and *Drosophila* Eya proteins are highly divergent, but resemble the proline-serine-threonine (PST) transactivation domains found in other transcription factors. Despite their sequence divergence, the Eya N termini could have retained a conserved molecular function. For example, the PST domain of Pax6 can function as a transactivation domain (Glaser et al., 1994) and, although the corresponding PST domain in Eyeless is highly divergent, both can be inferred to function equivalently in vivo (Halder et al., 1995a). The N-terminal PST domains of Eya may also encode a transactivation function. Although the *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 Eya protein sequence suggests that the highly conserved Eya domain could mediate such molecular interactions. This hypothesis can now be subjected to experimental test.

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