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RESEARCH

Cloning and Characterization of Two Vertebrate Homologs of the *Drosophila eyes absent* Gene

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The *Drosophila eyes absent* (*eya*) gene plays an essential role in the events that lead to proper development of the fly eye and embryo. Here we report the analysis of two human and two mouse homologs of the fly *eya* gene. Sequence comparison reveals a large domain of ~270 amino acids in the carboxyl terminus of the predicted mammalian proteins that shows 53% identity between the fly sequence and all of the vertebrate homologs. This Eya–homology domain is of novel sequence, with no previously identified motifs. RNA hybridization studies indicate that the mouse genes are expressed during embryogenesis and in select tissues of the adult. Both mouse *Eya* genes are expressed in the eye, suggesting that these genes may function in eye development in vertebrates as *eya* does in the fly. The mouse *Eya2* gene maps to chromosome 2 in the region syntenic with human chromosome 20q13, and the mouse *Eya3* gene maps to chromosome 4 in the region syntenic with human chromosome 1p36. Our findings support the notion that several families of genes (*Pax-6/eyeless*, *Six-3/sine oculis*, and *Eya*) play related and critical roles in the eye for both flies and vertebrates.

[The sequence data described in this paper have been submitted to GenBank under accession nos. U81601–U81604.]

The *Drosophila* eye provides a striking example of how cell–cell interactions, cell autonomous pathways, and hormonal events merge into the generation of an exquisitely organized neural structure (Tomlinson 1989; Wolff and Ready 1993; Heberlein and Moses 1995). The study of genes that function in the fly eye has revealed insight into the developmental mechanisms and genetic interactions of many genes with homologs in vertebrates (Zipursky and Rubin 1994; Bonini and Choi 1995). In particular, the gene regulatory pathway for eye development displays remarkable conservation of molecular features from fly to human. One gene that functions critically in human eye development is the *Aniridia* gene that encodes a *Pax-6* homeobox and paired-box gene (Ton et al. 1991; Glaser et al. 1992; Jordan et al. 1992). Loss of normal gene function in humans leads to eye developmental abnormalities, including loss of the iris in severe cases and cataracts in mild forms (Hanson and van Heyningen 1995). The fly counterpart of *Pax-6* is the *eyeless* gene (Quiring et al. 1994), which when mutated results in loss of the eye. Moreover, expression of *eyeless* in various tissues of the fly can direct cells down an eye developmental pathway, resulting in the formation of ectopic eyes (Halder et al. 1995). The mouse homolog of *eyeless*, *Small eye* (Hogan et al. 1986; Hill et al. 1991), can also direct the eye cell fate when transformed into the fly (Halder et al. 1995), indicating conservation of at least some molecular features of the eye developmental pathway from flies to vertebrates. Given the remarkable conservation of the role of *Pax-6* homologs in eye development from

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flies to vertebrates, the fly is providing an important guide to define new genes in vertebrates that may function in early developmental events of eye formation (Oliver et al. 1995).

The *Drosophila eyes absent* (*eya*) gene is essential for normal eye development (Bonini et al. 1993). With loss of *eya* function, all eye progenitor cells die by programmed cell death early in the differentiation process, resulting in an eyeless adult fly. The role of *eya* is to promote the survival and/or the differentiation of eye progenitor cells at an early step in their development. Although more is known of the role of *eya* in eye development, the *eya* gene is also required in select other tissues of the fly, such as during embryogenesis (Nüsslein-Volhard et al. 1984; Bonini et al. 1993). Select mutations of *eya* specifically affect its role in the eye, suggesting that these mutations define regulatory elements important for gene expression specifically in eye progenitor cells (Leiserson et al. 1994).

The sequence of the fly *eya* gene predicted a protein with no homologs or previously defined motifs indicative of function (Bonini et al. 1993). The protein is present in the nucleus of eye progenitor cells from early stages of development. As one approach to define new genes of relevance to vertebrate eye development, as well as to define critical functional domains within the Eya protein, we sought to define vertebrate homologs of the gene. During the course of these studies, *Eya* homologs were reported in the data banks (Banfi et al. 1996; EST Sequence Database) and by others (Xu et al. 1997). Here we confirm these observations and extend them by providing additional sequence data and detailed chromosomal map localizations of two vertebrate genes that show striking sequence conservation with the predicted fly Eya protein. These homologs are expressed in the mouse eye, suggesting that these genes, like their fly counterpart, may play a role in eye development.

RESULTS

Isolation of Vertebrate Homologs of the Fly *eya* Gene

Previously, we had defined a short region in the carboxyl terminus of the *Drosophila eya* gene that was highly conserved to a gene from the distantly related *Drosophila* species *D. virilis* (N. Bonini and S. Benzer, unpubl.). Subsequently, the Genexpress cDNA Program reported partial sequence of a human brain cDNA that revealed high homology in this same region with the predicted sequence of the *Drosophila* Eya protein. Based on the human sequence, we designed primers to attempt the amplification of a probe for screening mouse and human cDNA libraries (see Methods for details). For these studies we used human genomic DNA and a human retinal cDNA library, and mouse genomic DNA and mouse brain cDNA. Amplification of an anticipated 306-bp product was achieved for both human retinal library and mouse brain cDNA. These amplification products, called H306 from human and M306 from mouse, were then used as probes to screen human retinal, human brain, mouse embryonic, and mouse retinal cDNA libraries to obtain cDNA clones. The M306 probe was also hybridized under high stringency conditions to Southern blots of mouse genomic DNA. By this analysis, M306 detected multiple strongly cross-hybridizing bands in genomic digests of mouse DNA (data not shown).

Sequence Analysis of Human and Mouse Clones Highlights Conserved Features of Eya

From the mouse embryonic cDNA library we isolated two different classes of *eya*-related clones; the longest of each class were sequenced. These clones represent two different homologs of the fly *eya* gene, which we refer to as *Eya2* and *Eya3*; *Eya1* has been identified by others (Xu et al. 1997). These cDNAs recognized a subset of the genomic fragments on mouse genomic Southern blots that were labeled with the M306 probe (data not shown). From the human brain library, we isolated a single class of human cDNA, *EYA2*, that contained an overlapping sequence with the Genexpress cDNA clone and was highly homologous to mouse *Eya2* clones. From the human retinal library, we isolated a second class of human cDNA clones, *EYA3*, with strong identity with the mouse *Eya3* clones. The DNA sequences and predicted amino acid translations of these clones are presented in Figures 1 and 2.

The longest human *EYA2* clone is not likely full length and contains a partial open reading frame (ORF) of 244 amino acids that is 96% identical at the predicted protein level with mouse *Eya2* clones (Fig. 2C). *EYA3* clones are 95% identical at the predicted amino acid level over the carboxy-terminal 407 amino acids with the mouse *Eya3* class (Fig. 2D). These and other data (see Fig. 7, below; Discussion; Banfi et al. 1996) indicate that clones of the *EYA2* class are likely to be the human homologs of the mouse *Eya2* clones, whereas human *EYA3* cDNAs are the homologs of mouse *Eya3* clones. The amino-terminal-most sequence of the predicted protein corresponding to *EYA3* differs from the corresponding region of the protein encoded by the longest mouse *Eya3* clone that we have (Fig. 2D). These and

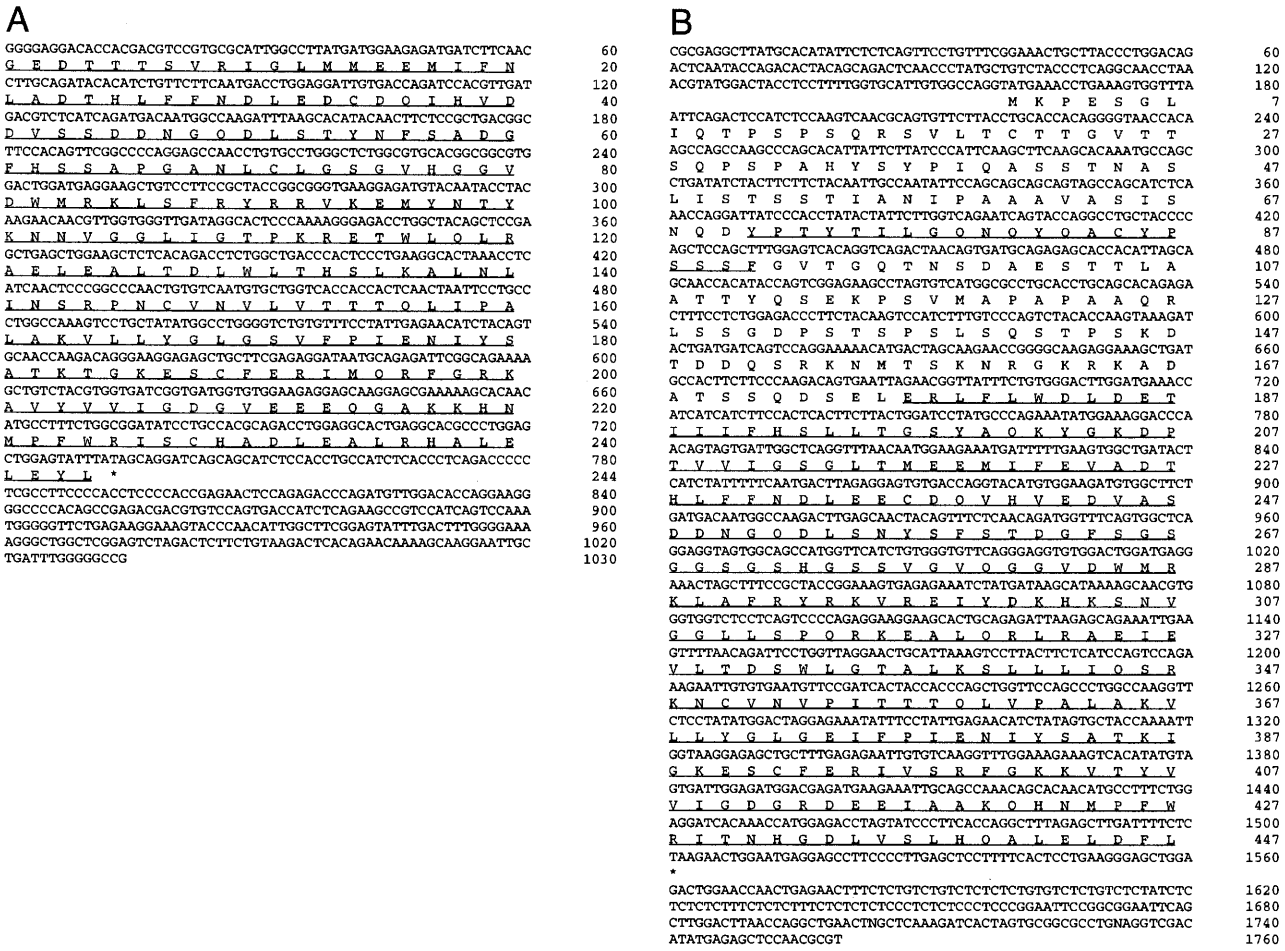

Figure 1 Sequence of human homologs of *eya*. DNA and predicted amino acid sequences of human *eya* homologs, *EYA2* (*A*) and *EYA3* (*B*). The *EYA2* clone has an incomplete ORF that stops within the large conserved domain ED1 (Eya homology domain 1). The *EYA3* clone is translated from the first upstream methionine. The conserved domains ED1 and ED2 (Eya homology domain 2) are underlined in the predicted protein sequences; ED1 is the large carboxy-terminal domain. GenBank accession nos. are U81601 for *EYA2* and U81602 for *EYA3*.

other data (below) suggest that there may be alternative splicing at the 5′ end of the *EYA3/Eya3* class of clones.

The longest mouse *Eya2* clone predicts a 52-kD protein of 473 amino acids, pI 5.5, assuming that the methionine at nucleotide position 166 is the initiation codon (Fig. 2A). The sequence around this potential translation initiation site (CAGCCATGG) shows strong consensus to the Kozak initiation sequence (CCA/GCCAUGG; Kozak 1981, 1984). The sequence upstream of this methionine, however, remains in-frame; possibly, there is an initiation methionine farther upstream. The largest ORF in the mouse *Eya3* clone predicts a 510-amino-acid protein of 56 kD, pI 4.9, assuming that the first methionine is the initiation site (Fig. 2B). The sequence around

Figure 2 (*A,B*) Sequence of mouse homologs of *eya*. DNA and predicted amino acid sequences of mouse homologs *Eya2* (*A*) and *Eya3* (*B*). Both are translated from the farthest upstream in-frame methionine. *Eya2* remains in-frame 5′ to the indicated start site, and therefore may predict a protein that is longer than indicated from the clones in hand. The conserved domains ED1 and ED2 are underlined in the predicted protein sequences; the ED1 domain is the large carboxy-terminal domain. GenBank accession nos. are U81603 for *Eya2* and U81604 for *Eya3*. (*C,D*) Comparison between human and mouse *Eya* homologs. Comparison of the amino acid sequences of the proteins predicted from human *EYA2* and mouse *Eya2* (*C*) and from human *EYA3* and mouse *Eya3* (*D*). The human and mouse *Eya2* sequences are 96% identical over the regions shown; the human and mouse *Eya3* clones are 95% identical over the carboxy-terminal 407 amino acids but diverge at their amino termini. Amino acid identities are boxed in black; similarities are shaded.

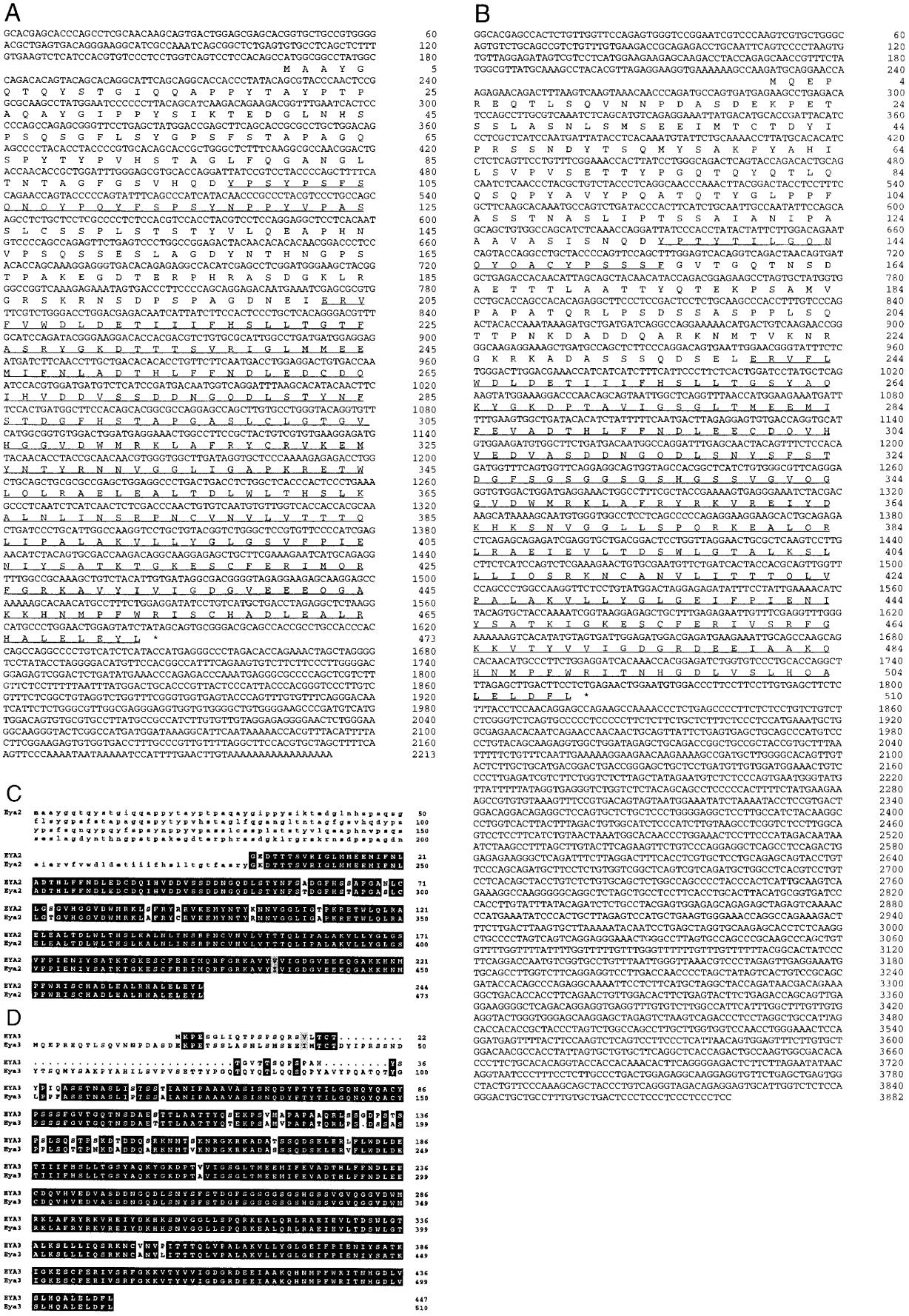

Figure 2 (*See facing page for legend.*)

this potential translation site CCAAGATGC shows consensus to the Kozak initiation sequence. The longest human *EYA3* clone, which differs from mouse *Eya3* clones at its most amino-terminal portion (Figs. 1B and 2C), predicts a protein of 447 amino acids of 49 kD, with a pI of 5.5.

Comparison of the predicted protein sequences of the vertebrate clones to the predicted fly Eya protein reveals several domains of homology (Fig. 3). The largest domain is the carboxy-terminal portion of the protein, referred to as ED1 (for Eya homology domain 1), which shows 53% identity over 271 amino acids of all the vertebrate and fly clones. Comparison of the vertebrate clones individually to the fly sequence in this carboxy-terminal region shows EYA2 to be 64% identical and the mouse Eya2 class to be 67% identical, whereas the human EYA3 and mouse Eya3 classes are 62% identical to the predicted fly protein sequence. Within the amino-terminal domain, the sequences show little conservation. A short region of weak homology, ED2 (Eya homology domain 2), occurs between the vertebrate and fly sequences in the amino terminus. In this domain, a run of spaced tyrosine residues is conserved (Fig. 3C); the homology in this region to the Eya2 homolog is greater and longer (10/28 amino acids for 36% identity) than with the EYA3/Eya3 homologs (5/21 amino acids for 24% identity). Because of the low level of homology, the significance of this region is unclear. In addition, the fly sequence has a weak PEST protein degradation sequence; in a similar location, the mouse Eya2 sequence has a region that may serve as a weak PEST site (Fig. 3A). The fly sequence has a run of basic charge between the ED1 and ED2 domains (see Fig. 3A; Bonini et al. 1993). Although this sequence is not strongly conserved, a short cluster of basic charge occurs in a similar location in both Eya2 and the EYA3/Eya3 homologs (Fig. 3A). The fly Eya protein sequence has a consensus nuclear localization signal at the amino terminus, and the protein is nuclear by immunocytochemistry (Bonini et al. 1993). However, none of the vertebrate sequences show a motif indicative of a nuclear localization sequence (Chelsky et al. 1989; Dingwell and Laskey 1991); possibly, the cluster of conserved basic charge noted above, or a second region of basic charge within the ED1 conserved domain (in the fly, amino acids 600–629) serves this purpose.

The Mouse *Eya* Homologs Are Expressed in the Eye

We addressed expression pattern of the vertebrate homologs using the mouse clones to probe Northern blots of poly(A)$^+$ RNA isolated from various mouse tissues, including the eye. Unique fragments in the 3′-untranslated regions for the respective genes were used as probes. Both *Eya2* and *Eya3* were strongly expressed in poly(A)$^+$ RNA isolated from adult mouse eye (Figs. 4A,D). For *Eya2*, a similar sized transcript of 2.4 kb was expressed in the eye as in other tissues of the animal. The *Eya3* probe, however, detected multiple transcripts in the eye—one strongly expressed transcript of 5.5 kb and three additional transcripts of 4.4, 2.5, and 1.9 kb.

In other tissues and during development the *Eya2* gene showed a more restricted pattern of expression compared to the *Eya3* gene. During embryonic development, the *Eya2* gene was first strongly expressed at 11 days and then showed a gradual reduction in expression to 17 days (Fig. 4B). In contrast, the *Eya3* gene was expressed at roughly similar levels during all stages of mouse embryonic development, with only the largest 5.5-kb transcript be-

Figure 3 (*A*) Conserved domains of *eya* homologs of human, mouse, and fly. Schematic representation of domains of homology of the fly, mouse, and human clones. Select additional features are indicated, including the consensus nuclear localization signal (NLS) of the fly sequence and a domain enriched in amino acids of basic charge (++). Percent amino acid identity with the fly sequence is indicated for ED1 and ED2 for each homolog. In the fly sequence of the type I cDNA, the NLS runs from amino acid 18 to 23, the *opa* repeat from amino acid 40 to 62, and ED2 from amino acid 326 to 354, potential PEST sequence from amino acid 373 to 388, basic domain 1 from amino acid 449 to 471, ED1 from amino acid 487 to 760. The human EYA2 sequence is incomplete at the 5′ end, and the incomplete ORF is indicated with an asterisk (*). In mouse Eya2, ED2 runs from amino acid 98 to 125, the potential PEST sequence from amino acid 144 to 161, the short charge cluster from amino acid 183 to 190, and ED1 from amino acid 203 to 473. In human EYA3, ED2 runs from amino acid 71 to 91, short cluster of basic charge from amino acid 159 to 165, and ED1 from amino acid 177 to 447. In mouse Eya3, ED2 runs from amino acid 135 to 155, the basic charge cluster from amino acid 222 to 228, and ED1 from amino acid 240 to 510. (*B*,*C*) Sequence lineups of ED1 (*B*) and ED2 (*C*) of fly, human, and mouse homologs. Amino acid identities are boxed in black; conservative changes are shaded. The consensus sequences for ED1 were derived in relation to the fly sequence. Amino acid similarities were defined with the default symbol comparison table based on the Dayhoff PAM-250 matrix. By this program, the following amino acids are considered similar: F ,Y; L, M; I, V; E, D.

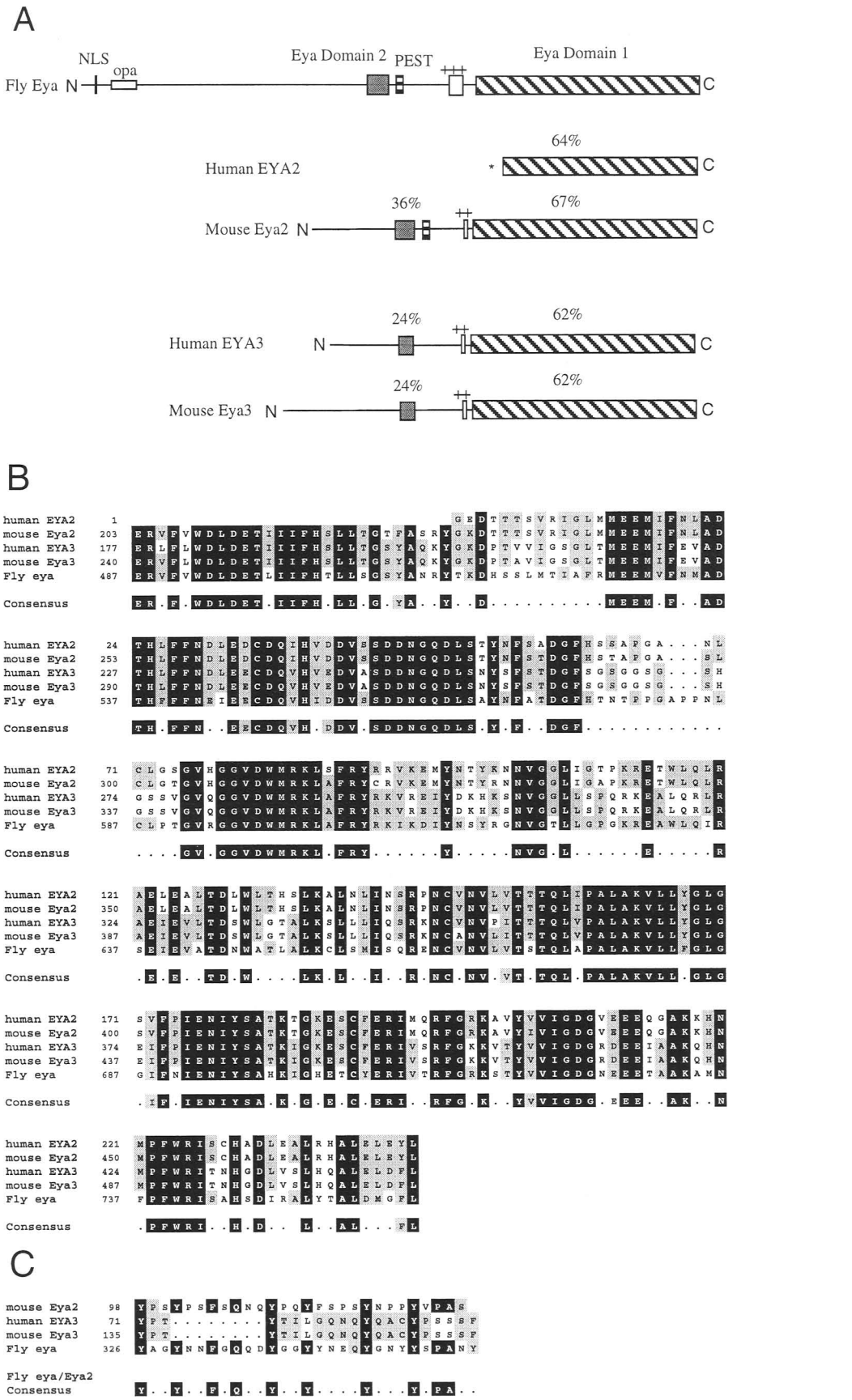

Figure 3 *(See facing page for legend.)*

ing expressed to appreciable levels (Fig. 4E). In adult tissues, the 5.5-kb transcript of the *Eya3* gene was expressed in all tissues tested except spleen, whereas *Eya2* expression was detectable only in lung (Fig. 4 C,F). We also determined that *Eya2* was expressed in thymus and uterus (data not shown).

Chromosomal Mapping of the Genes

A chromosomal location of the mouse *Eya2* gene was originally established in the Jackson Laboratory Backcross DNA Panel—a community genetic mapping resource (Rowe et al. 1994). Segregation analysis of backcross progeny placed the *Eya2* gene to the distal portion of mouse chromosome 2. No recombinants were detected between *Eya2* and the *Iapt15* (intercisternal A particle tumor-specific-15) gene in 91 animals typed in common, whereas the same cross placed the *Eya2* gene 6.3 cM proximal to the *Gnas* gene (upper 95% confidence limit). We compared the map location of *Eya2* with composite mouse linkage maps that report the map location of many uncloned mouse mutations (provided from the mouse genome database, a computerized database maintained at the Jackson Laboratory). *Eya2* maps in a region of the composite map that contains the eye mutation *blind-sterile* [*bs* (Varnum 1983)]. The *bs* locus has been mapped 1.7 ± 1.2 cM proximal to *Emv13/Emv15* loci (Spence et al. 1992). Because the *Emv13/Emv15* loci were mapped previously in another large interspecific backcross, typed for >2200 loci (Copeland and Jenkins 1991), *Eya2* and *Eya3* were mapped in this backcross. The mapping results confirmed that *Eya2* is located in the distal region of chromosome 2 and showed that the *Eya3* gene maps to the distal region of chromosome 4 (Fig. 5). Moreover, this set of mapping data determined that *bs* is not a candidate for a mutation in *Eya2*, as *bs* has been located proximal to the ectopic murine provirus-15 (*Emv15*) gene by interspecific backcross mapping (Spence et al. 1992) while *Eya2* has been mapped distal to this marker. No recombinants were detected between the *Eya3* gene and the *Fgr* genetic locus in 83 animals typed in common, suggesting that these loci are within 4.3 cM of each other (upper 95% confidence limit). There are no known eye-specific mutations that map in this chromosomal region.

We performed fluorescence in situ hybridization (FISH) with a P1 probe containing the human *EYA3* gene and demonstrated that the *EYA3* gene hybridized to the terminal band of chromosome 1 (1p36) in all cells examined (Fig. 6). This is the region suggested from the mouse *Eya3* mapping localization, based on synteny between mouse and human chromosomes (Fig. 5). The human *EYA2* gene has been localized to human chromosome 20 (20p13.1; Banfi et al. 1996), which is the region suggested by the mapping of the mouse *Eya2* gene (Fig. 5).

DISCUSSION

Eya Gene Family

We report the isolation and initial characterization of two vertebrate genes related in sequence to the *Drosophila eya* gene. This analysis has revealed striking features of the *eya* protein sequence that are conserved in vertebrates. The largest domain of homology, ED1, spans ~270 amino acids and covers the carboxy-terminal portion of the predicted proteins. The degree of homology over this long stretch of amino acids—53% over all vertebrate and fly protein sequences analyzed here—suggests that this domain is of special importance to the function of the gene products. Within this region are short amino acid runs of exceptionally high conservation (see consensus sequence, Fig. 3B); however, there are no previously defined motifs that speak to biochemical function. Rather, this domain defines a new domain of conservation. A second, smaller amino-terminal domain, ED2, of ~30 amino acids shows conservation for fly and Eya2 of a spaced run of tyrosine amino acids. Other features common to the vertebrate and fly gene products include a region of enriched basic charge just amino-terminal to the large ED1 domain, and, in the Eya2 homolog, a possible PEST protein degradation sequence.

Overall, the vertebrate genes predict proteins with the most homologous domain limited to the carboxy-terminal portion of the protein. The amino-terminal region of the fly sequence, which contains numerous runs of repeated amino acids, may not be critical to function of the protein, with the exception of the short regions of homology noted. Alternatively, the amino terminus may contain important biological information that is not recognized as a conserved sequence, or is not conserved in the vertebrate homologs identified to date. In the latter case, we might anticipate the eventual identification of proteins with greater homology to the amino terminus. Notably the fly sequence has an *opa* triplet repeat at the amino terminus that is not present in any of the vertebrate sequences defined so far. Triplet repeats tend to be associated with genes important to nervous system function and development; expansion of such triplet repeats is associated with a number of human neurodegen-

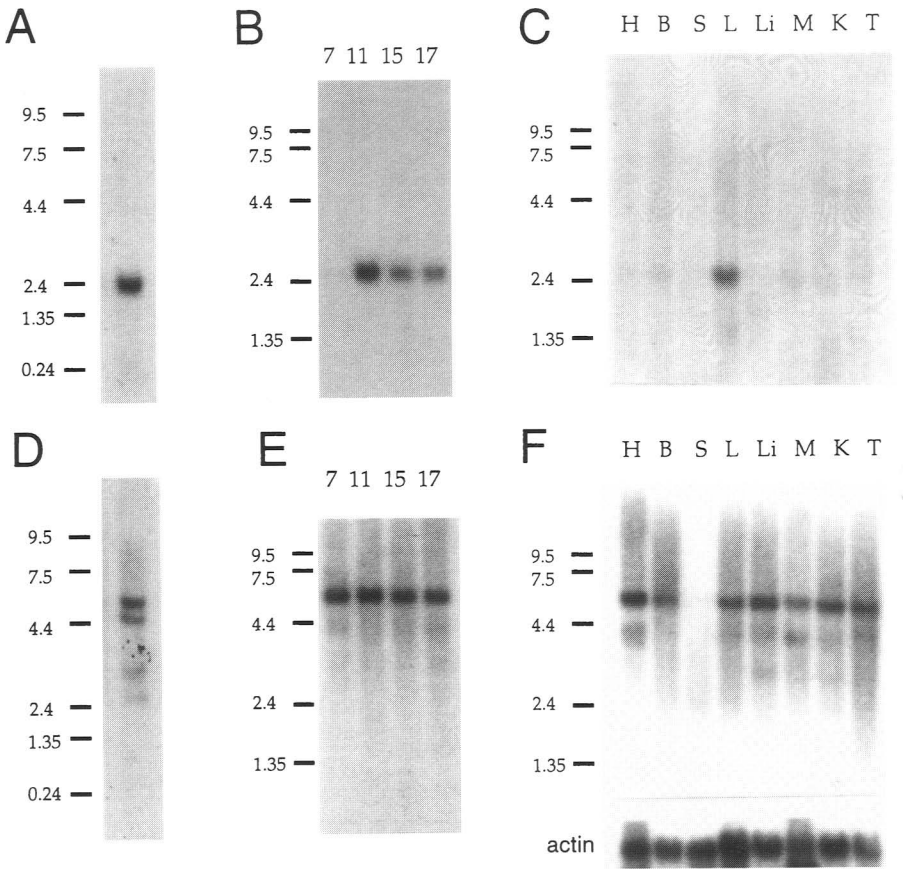

Figure 4 Expression by Northern analysis of the mouse *Eya2* and *Eya3* genes. Expression of *Eya2* (*A*) and *Eya3* (*D*) genes is shown in poly(A)$^+$ RNA isolated from mouse adult eye. Expression of the *Eya2* (*B*) and *Eya3* (*E*) genes is shown at 7, 11, 15, and 17 days of mouse embryonic development. Expression of the *Eya2* (*C*) and *Eya3* (*F*) genes is shown in adult tissues; the panel beneath *F* is the same blot hybridized with a β-actin cDNA probe. Lanes are labeled as follows: (H) Heart; (B) brain; (S) spleen; (L) lung; (Li) liver; (M) skeletal muscle; (K) kidney; (T) testis.

erative diseases (Ashley and Warren 1995; Karlin and Burge 1996). As mutation of the fly gene leads to cell death, at least in the eye (Bonini et al. 1993), it will be of interest to determine whether there may be an additional vertebrate *eya* homolog that contains this repeat or whether there are alternative splice products of the currently defined genes that contain a triplet repeat. The mouse *Eya3* gene, for which we have defined a number of alternatively spliced products expressed in the eye in particular, is such a candidate. The mouse *Eya3* and human *EYA3* genes, although highly similar over most of the predicted amino acid sequence, diverge significantly at their amino termini. Alternative splicing likely accounts for this variation, with the *Eya3* and *EYA3* clones that we have defined representing different splice variants. Consistent with this, another mouse *Eya3* clone identified (Xu et al. 1997) displays alternative splicing at the 5′ end compared to our *Eya3* clones. The isolation of additional splice forms, as well as analysis of genomic clones, will aid in defining the intron/exon structures of the vertebrate genes. In addition, analysis of mutations in the fly that alter specific subfunctions of the gene (Bonini et al. 1993; Leiserson et al. 1994) may reveal roles of the amino-terminal domain and potential regulatory elements, in addition to defining amino acids within the highly conserved domains that are critical for function.

The two mouse *Eya* genes show striking differences in expression during development and in adult tissue. The *Eya2* gene is expressed in a rather restricted manner, being expressed strongly in select adult tissues and with a temporal pattern developmentally, whereas the *Eya3* gene is expressed in a more widespread manner. This indicates that the genes are likely to be under distinct regulatory control, both during development and in the adult animal. These data are supported by tissue in situ analysis of the genes during mouse development, which also demonstrate widespread expression of the mouse *Eya1* gene (Xu et al. 1997). All genes, however, are expressed in eye tissue during development (Xu et al. 1997), and at least *Eya2* and *Eya3* in the adult eye, with the *Eya3* gene showing appreciable levels of expression of multiple transcripts (see Fig. 4). During development, the genes show complex tissue expression, especially in the nervous system, some of which is in overlapping tissues and other in adjacent tissues (Xu et al. 1997).

The distal region of mouse chromosome 2 is syntenic with the long arm of human chromosome 20, whereas the distal part of chromosome 4 is syntenic with human chromosome 1 (see Figs. 5 and 6). The placement of *Eya2* between the markers adeno-

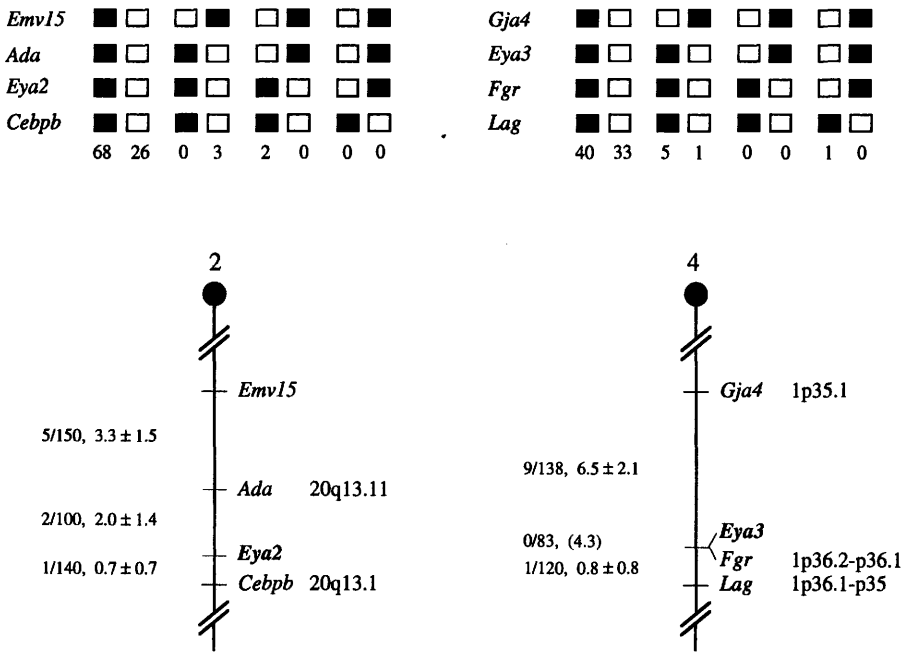

Figure 5 Chromosomal map positions of mouse *Eya2* and *Eya3* in the Frederick interspecific backcross. Partial chromosome linkage maps showing the mouse chromosomal location of *Eya2* and *Eya3* as determined by interspecific backcross analysis. The segregation patterns of the two *Eya* genes and flanking genes are shown at the *top*. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. (■) The presence of a C57BL/6J allele; (□) the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed beneath each column. Although 99 mice were analyzed for every marker shown in the segregation analysis of *Eya2*, up to 150 mice were typed for some pairs of markers. Similarly, although 80 mice were analyzed for every marker shown in the segregation analysis of *Eya3*, up to 138 animals were typed for some pairs of markers. Partial chromosome linkage maps of chromosomes 2 and 4, indicating the location of *Eya2* and *Eya3* in relation to linked genes, are shown at the *bottom*. Recombination distances between loci (cM) are indicated to the *left* of the chromosome (± S.E.); the positions of loci in human chromosomes are shown to the *right*. Where no recombinants were found between loci, the upper 95% confidence limit of the recombination distance is given in parentheses. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base; http://gdbwww.gdb.org/), a computerized database of human linkage information maintained by the William H. Welch Medical Library of the Johns Hopkins University (Baltimore, MD).

sine deaminase (*Ada*) and CCAAT/enhancer binding protein β (*Cebpb*) suggests that the human *Eya2* gene resides on human chromosome 20q13.1; this has been shown to be the case using a human clone of the *Eya2* class (Banfi et al. 1996). Similarly, the tight linkage between *Eya3* and *Fgr* suggested that human *Eya3* lies on human chromosome 1p36.2–p36.1. FISH analysis presented in this paper confirms the localization to the terminal portion of human chromosome 1. Several interesting genetic disorders have been located to these two chromosomal regions, although localization to the *EYA2/Eya2* and *EYA3/Eya3* genes has not yet been demonstrated. Although the mouse *Eya2* homolog maps near the mouse eye mutation *bs*, and *bs* has been speculated to be a mutation in the *Eya2* gene (Banfi et al. 1996), our mapping data rule out this possibility.

Evolution of *Eya*

Based on the large and highly conserved ED1 domain, we have constructed a similarity tree defining the relatedness of the *eya* homologs currently identified (Fig. 7). The tree suggests that two gene duplication events occurred in vertebrates, one leading to the separation of the *Eya3* branch from the *Eya1* and *Eya2* branch, and a second duplication event leading to the *Eya1* and *Eya2* genes. Furthermore, existence of a human homolog of the mouse *Eya1* gene is predicted. The mammalian genes apparently duplicated about the time of the fly/mammalian divergence, as all vertebrate genes show a relatively similar degree of divergence from the fly gene. At this point in time, other *Drosophila* genes related in sequence to Eya have not been defined. One interpretation would be that multiple fly *eya* genes do not exist and that the divergence observed in vertebrates occurred subsequent to the fly/vertebrate split. It will be of interest to determine the extent to which functional properties assigned to the fly gene be assigned to members of the vertebrate *Eya* family, or whether the proteins have diverged, at least in part, in function. Our results also suggest the possibility that fly *eya* may be the founding member of an even larger class of vertebrate homologs, as Southern blot studies with the M306 probe are indicative of additional related genes.

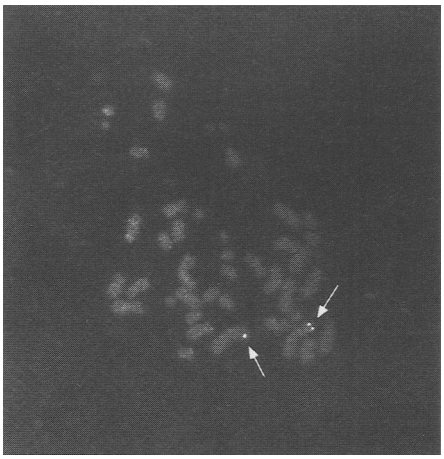

Figure 6 Localization of *EYA3* to human chromosome 1. FISH of a P1 probe containing *EYA3* on a metaphase spread from a normal individual. Signal is present on chromosome 1p36 on both homologs (arrows). Localization was also performed by double-labeling with the P1 probe and a chromosome 1-specific α-satellite probe (data not shown).

The biochemical mechanism of action of the fly Eya product has not yet been defined, although the protein is known to be nuclear. Whereas the fly protein has a consensus nuclear localization sequence, none of the vertebrate clones shows conservation of this sequence. A fundamental feature of nuclear localization sequences is a basic charge (Dingwell and Laskey 1991), so possibly the region of basic charge amino-terminal to the ED1 domain, or other sites of basic charge within the predicted proteins, subserves this purpose. Alternatively, the proteins may be carried into the nucleus through association with other proteins that have more conventional nuclear localization signals. Divergence of the proteins at the amino-terminus may therefore indicate functions related to subcellular localization, among other possibilities. Nevertheless, defining whether the vertebrate proteins are localized to the nucleus may be crucial for addressing potential conservation of biological function of the genes. Moreover, although the vertebrate proteins show striking homology to the fly protein, especially in the carboxyl terminus, it will be of interest to address whether the vertebrate homologs are similar enough in sequence to substitute biologically for their fly counterpart. Such a test will address the degree to which the biological pathway of *eya* gene activity is conserved from flies to vertebrates, potentially addressing fundamental conservation of gene order and function in the *Pax-6/eyeless* pathway of eye development. The identification of interacting proteins of known function, as well as analysis of the role of the *eya* family of genes in the eye and other tissues of flies and vertebrates, will lead toward an understanding of the biological role of these genes.

Evolutionary Pathway of Eye Development

The fly *eya* gene has a critical role in development of the eye, with selective loss of gene function in the eye leading to complete loss of this structure (Bonini et al. 1993). Striking conservation of fly and verte-

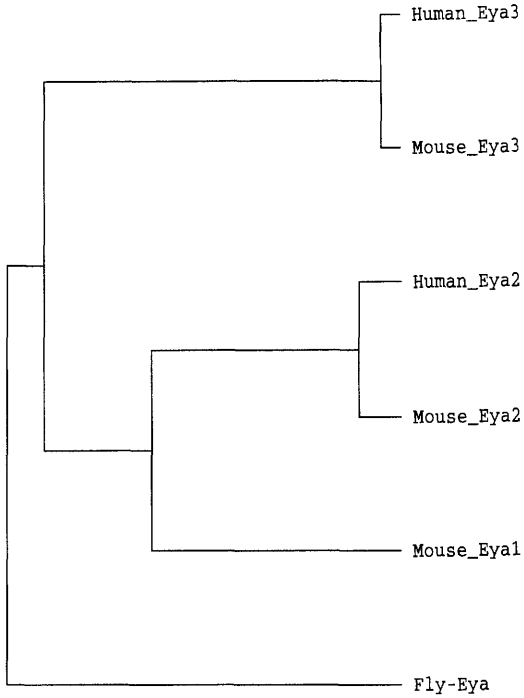

Figure 7 Similarity tree of *eya* family homologs. The tree was constructed from sequence alignment with the PILEUP Program (GCG; University of Wisconsin). Only the 271 amino acid ED1 domain, which is 274 amino acids in the fly, were used for the alignment. For the human EYA2 homolog, which is incomplete for the amino-terminal 27 amino acids of this domain, we completed that part of the sequence using the homologous region of the mouse Eya2 homolog: we reasoned that the human *EYA3* and mouse *Eya3* genes predicted proteins are 100% identical over this sequence region; therefore, the human *EYA2* and mouse *Eya2* genes are likely to be identical as well. However, using the incomplete sequence of human EYA2 for construction of the tree results in a similarity tree that is not significantly altered. The mouse Eya1 sequence is from GenBank accession no. U61110. A human *EYA1* gene has not yet been characterized; however, the tree predicts that it exists.

brate genes expressed and functioning in the eye is seen with the *eyeless/Pax-6* paired-box and homeobox gene (Quiring et al. 1994; Halder et al. 1995), and the *Six-3* homeobox gene, defined by the fly mutant *sine oculis* (Cheyette et al. 1994; Serikaku and O'Tousa 1994; Oliver et al. 1995). The *Eya2* and *Eya3* genes are both expressed in the mouse eye in the adult (see Fig. 4), and other studies have shown that all three mouse *Eya* genes are expressed in the developing eye (Xu et al. 1997). Thus, we anticipate that the vertebrate *Eya* genes will function in eye development, as their counterpart does in the fly.

These three genes—*eyeless*, *sine oculis*, and *eya*—share the property of being essential early in *Drosophila* eye development, with expression prior to the first signs of neural differentiation (Bonini et al. 1993; Cheyette et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994). This raises the possibility that a fundamental set of genes (*eyeless/Pax-6*, *sine oculis/Six-3*, and *Eya* among them) functions across a remarkably large evolutionary distance in eye differentiation. It has been proposed recently that the eye of both vertebrates and invertebrates, despite dramatic structural differences, evolved from a common ancestor with a primitive eye (Halder et al. 1995; Gehring 1996). The alternative and longstanding hypothesis is that the fly and vertebrate eye evolved independently (see Dickinson and Seger 1996). Without necessarily distinguishing between these two hypotheses, in this report we add weight to the argument that the fly and vertebrate eye arose because of the use of a common set of genes. With respect to this issue, both *Pax-6* and *Six-3* genes contain motifs indicating that they function as transcription factors, and, at least in the fly, the Eya protein shows nuclear localization. Will potential targets of these genes be conserved from flies to vertebrates? For the *Eya* gene family, some aspects of fly *eya* gene regulation are remarkably specific to expression and function of the gene in the eye (Leiserson et al. 1994); it will be of interest to determine whether related regulatory elements are found in the *Eya* vertebrate genes. Toward this end, studies in the mouse indicate that expression of *Eya* genes is reduced in *Pax-6* mutant mice (Xu et al. 1997).

A related and important issue is that these genes have functions in addition to eye development, as mutants have phenotypes in addition to loss of the eye and gene expression is not limited to the eye (Bonini et al. 1993; Cheyette et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994). For example, mutations of *eya* in the fly can be embryonic lethal, as well as show adult female sterility in select combinations of alleles (Nüsslein-Volhard et al. 1984; Bonini et al. 1993). Expression of the vertebrate *Eya* genes in tissues other than the eye was thus anticipated and confirmed by our study. *Pax-6* is present in organisms including *Caenorhabditis elegans*, which have no photoreceptor cells (Chisholm and Horvitz 1995; Zhang and Emmons 1995). These observations therefore raise the questions, is there a fundamental function of these genes in animal patterning, and how does their function lead to the differentiation of an eye in select cells of some animals, but not in other cells nor in other animals? This property of selective eye formation is true even for the *Pax-6/eyeless* gene, which can direct ectopic eye formation when aberrantly expressed.

One hypothesis is that the pathway involving these genes arose specifically for eye development. An alternative—and we would argue, more likely—hypothesis, however, is that these genes may be part of a genetic network that underlies a more fundamental signaling process than eye development itself. During evolution, a genetic circuit using these genes is likely to have arisen in a primitive ancestor of both flies and vertebrates—the gene network itself being more ancient than eye development. Subsequently, the entire circuit became co-opted into eye development at least once. In theory, such a hypothesis does not distinguish between the vertebrate and fly eyes having evolved either from a common ancestor or independently, as the genetic circuit could have been co-opted into eye development multiple times. In view of these issues, it will be of interest to define in detail the genetic interactions among these genes, whether a circuit involving these genes is present in tissues other than the eye, and whether circuits involving these genes are present in all other organisms with eyes, and in those like *C. elegans*, which have no eyes.

METHODS

Isolation of cDNA Clones

Clone 1ce06 (GenBank accession no. Z39529) was obtained from the Genexpress cDNA Program, Laboratoire Genethon, Evry, France. Primers were designed to the ends of the sequence available of this clone: a forward primer 5′-GACTGGATGAGGAAACTAGCTTTC-3′, corresponding to amino acids DWMRKLAF, and a reverse primer 5′-GGTAGCACTATAGATGTTCTCAATAGG-3′, corresponding to the amino acids PIENIYSAT, which fall within a conserved domain of *EYA*. The primers were used to amplify a predicted 306-bp product from mouse brain cDNA made by RT–PCR from mouse brain mRNA of stage E9.5 and to amplify a 306-bp product from a human retinal library (Clontech). Amplification was performed by standard protocols using an annealing temperature of 55°C, extension at 72°C, for 35 cycles. The amplification product M306 was used to probe a mouse

brain cDNA library (Stratagene), and a mouse retinal cDNA library (American Type Culture Collection). The human product H306 was used to probe human retinal and human brain libraries (Clontech). Positive clones were amplified from phage and subcloned into pGEM-T (Promega), tested for cross-hybridization, mapped, and sequenced by automated sequencing using sequential primers. The mouse *Eya2* sequence is a composite of sequence derived from clones M27 and M30; the mouse *Eya3* sequence from clone M25; the human *EYA2* sequence from clones H13 and H12; and the human *EYA3* sequence from clones 1ce06 and HB1. The H13 and 1ce06 clones were fusion cDNAs with part of the sequence highly homologous to *eya* and part of the sequence completely divergent; the divergent parts of the sequences were removed in this analysis. For Southern hybridization, mouse genomic DNA of strain C57BL/6J was digested with *Bam*HI, *Pst*I, *Sal*I, and *Xba*I, run on a 7% agarose gel, and transferred to nitrocellulose following standard protocols. Blots were probed with radioactively labeled M306, *Eya2* and *Eya3* clones at 5×10^6 cpm/ml, and exposed by PhosphorImager analysis.

Sequence Analysis

Sequence comparisons between fly and vertebrate ORFs were performed using the GCG software package, version 8 (Genetics Computer Group), with the program PILEUP, presented with Microsoft Excel highlighting of sequence identity and alignment (Haygood 1993). Amino acid similarities were defined with the default symbol comparison table based on the Dayhoff PAM-250 matrix. By this program, the following amino acids are considered similar: F ,Y; L, M; I, V; E, D. The ED2 domain was identified with the sequence alignment program MACAW (National Center for Biotechnology Information). The phylogenetic tree was generated using programs available from the GCG software package, from the PILEUP alignment.

Northern Blot Analysis

Northern blots with poly(A)$^+$ RNA from various adult mouse tissues and different developmental stages of the mouse (2 μg per lane on formaldehyde/1.2% agarose gels) were purchased from Clontech. For mouse adult eye RNA, total RNA was isolated from adult female mouse eyes of the CF1 strain using Trizol reagent (GIBCO-BRL). Total RNA was stored as a precipitate until use in 100% ethanol at −70°C. Poly(A)$^+$ RNA was isolated using oligo(dT) columns (Clontech) and stored as an ethanol precipitate until use. RNA (2 μg per lane) was electrophoresed, using a formaldehyde/1% agarose gel in 1× TAE buffer containing 0.6% formaldehyde, and transferred to positive charged nylon following standard procedures (Sambrook et al. 1989). For all probing, blots were prehybridized for 30 min at 68°C and hybridized for 1 hr at the same temperature in ExpressHyb solution (Clontech). The probes used were 3′ untranslated regions of clone M27 for the *Eya2* gene and of clone M25 for the *Eya3*, and a human β-actin cDNA probe supplied by Clontech. Probes were labeled with [α-^{32}P] dCTP using random hexamers following standard procedures (Sambrook et al. 1989). The blots were washed for 40 min in 2× SSC, 0.05% SDS, at room temperature followed by 40–60 min in 0.1× SSC, 0.1% SDS, at 50°C. The blots were then exposed to film at −70°C. Hybridization of Northern blots with a β-actin cDNA probe indicated equal amounts of poly(A)$^+$ RNA (2 μg) were loaded per lane.

Interspecific Mouse Backcross Mapping

To establish the chromosomal location of the *Eya2* locus, we performed a segregation analysis in the Jackson Laboratory Backcross DNA BSS Panel (C57BL/6JEi × SPRET/Ei) × SPRET/Ei, which has been typed previously for a large number of loci by restriction fragment length polymorphism (RFLP), motif-primed PCR polymorphisms, and microsatellites (Rowe et al. 1994). The map location was determined by the analysis of a polymorphism detected by restriction enzyme digestion of the 306-bp PCR product amplified with the forward and reverse primers (above), which are within the highly conserved region of the gene. This amplicon was subsequently digested by *Hae*III and subjected to single-strand conformation polymorphism (SSCP) analysis. PCR conditions were as described (Bucan et al. 1995), except that an 8% nondenaturing acrylamide gel was used. Detailed mapping data are available at BC Panel Mapping Resource page (http://www.jax.org/resources/documents/cmdata). Genes linked to *Eya2* are *Pltp* (phospholipid transfer protein; Le Boeuf et al. 1996), *Iapt15* (Lueders and Frankel 1994), and *Gnas* (guanine nucleotide binding protein; Piltz et al. 1992; Wilkie et al. 1993).

Eya2 and *Eya3* were then mapped relative to an additional set of described loci (Copeland and Jenkins 1991). Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins 1991). A total of 205 N_2 mice were used to map the *Eya2* and *Eya3* loci. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins et al. 1982). All blots were prepared with Hybond-N+ nylon membrane (Amersham). The probes, a 0.5-kb *Eco*RV/*Xho*I fragment containing the 3′-untranslated sequences of the mouse M27 clone encoding the *Eya2* gene, and a 1.3-kb *Pst*I/*Xho*I fragment containing the 3′-untranslated sequences from the mouse M25 clone encoding the *Eya3* gene, were labeled with [α-^{32}P]dCTP using a random priming kit (Stratagene); washing was done to a final stringency of 1.0× SSCP, 0.1% SDS, 65°C. The *Eya2* probe detected an *Eco*RV fragment larger than 23 kb in C57BL/6J DNA and a 15-kb fragment in *Eco*RV-digested *M. spretus* DNA. The presence or absence of the 15-kb *Eco*RV *M. spretus*-specific fragment was followed in backcross mice. The *Eya3* probe detected 5.5- and 4.3-kb major fragments in *Pvu*II-digested C57BL/6J DNA and 2.4- and 0.9-kb major fragments in *Pvu*II-digested *M. spretus* DNA. The 2.4- and 0.9-kb *M. spretus* fragments cosegregated and their presence or absence was followed in backcross mice.

A description of probes and RFLPs for loci linked to *Eya2* and *Eya3*, including *Emv15*, *Ada*, the *Cebpb*, the gap junction protein *Gja4*, Gardner–Rasheed feline sarcoma viral oncogene homolog (*Fgr*), and the leukemia-associated phosphoprotein (*Lag*) have been reported previously (Haefliger et al. 1992; Storm et al. 1994; Chen et al. 1995; Jenkins et al. 1995). Recombination distances and gene orders were determined using MapManager (Manley 1993).

Mapping to the Human Chromosomes by FISH

Screening of a human P1 library (DuPont Merck Pharmaceu-

tical Company Human Foreskin Fibroblast P1 Library 1) was performed with PCR primers to the *eya* gene. DNA from the P1 clone was prepared and labeled with biotin-16-dUTP and hybridized to metaphase spreads of a normal individual as described (Lawrence et al. 1990).

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