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Original Article

Chicken *Eyes absent 2* gene: isolation and expression pattern during development

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ABSTRACT In all vertebrates studied (human, mouse, chicken), there are at least three genes related to *Drosophila eyes absent (eya)* gene. The chicken *Eyes absent 2 (Eya2)* cDNA was isolated from 14 day embryonic chicken lenses, and a complete open reading frame encoding a 59 kDa protein was elucidated. The chicken Eya2 protein is moderately conserved and 78-82% identical to the mouse and human Eya2. The *Eya2* gene demonstrated a dynamic expression pattern in different tissues of diverse embryological origin. Expression of *Eya2* was first detected at Hamburger and Hamilton stage 9 in the foregut. At later stages of development, *Eya2* mRNA was detected in neural crest derivatives (dorsal root ganglia, branchial arches and cranial nerve ganglia). In the cranial placodes, expression of *Eya2* was first detected in the nasal pit at stage 13. In the eye, expression of *Eya2* was first convincingly detected in neural retina at stage 24 (day 4). The highest level of *Eya2* mRNA in the lens was detected around day 9. *Eya2* is also expressed in the cornea and iris. Therefore, chicken *Eya2*, as well as mouse *Eya2*, is expressed relatively early in the nasal (but not in the lens) placode and may mediate induction of the nasal placode. Expression of *Eya2* in the wing and limb buds is consistent with its proposed role in the patterning of limb connective tissues.

KEY WORDS: *eye, evolution, Eya2, neural crest, limb*

Introduction

The *Eyes absent (eya)* gene was first identified in *Drosophila* (Bonini *et al.*, 1993). It encodes a novel nuclear protein and is expressed in several embryonic and adult tissues including developing eyes. Null mutation of *eya* leads to embryonic lethality, probably due to defects in head development (Bonini *et al.*, 1993). The mutation *(eya^1)* leading to the *eya* phenotype (absence of the compound eyes) resides in a regulatory element, which is necessary for the expression of *eya* in the eye imaginal disc anterior to the morphogenetic furrow (Bonini *et al.*, 1993). Recent data demonstrated that *eya* is able to direct ectopic eye formation in *Drosophila* (Bonini *et al.*, 1997). Moreover, *eya* is able to act synergistically with either *eyeless* (Bonini *et al.*, 1997), *dachshund* (Chen *et al.*, 1997) or *sine oculis* (Pignoni *et al.*, 1997) to induce ectopic eye formation.

Three homologs of *eya* gene were identified in mammals (Abdelhak *et al.*, 1997b; Duncan *et al.*, 1997; Tomarev, 1997; Xu *et al.*, 1997b; Zimmerman *et al.*, 1997). Mutations in *Eya1* in the human can lead to branchio-oto-renal and branchio-oto syndromes (Abdelhak *et al.*, 1997a,b; Vincent *et al.*, 1997). *Eya2* gene was mapped to chromosome 2 in mouse (Duncan *et al.*, 1997; Xu *et al.*, 1997b; Zimmerman *et al.*, 1997) very close to the known cataract mutation *Lop-4* (West and Fisher, 1986; Duncan *et al.*, 1997). However, it is not clear at present if mutations in *Eya2* are the cause of *Lop-4* phenotype. Comparison of the mammalian and *Drosophila* Eya/eya indicated that the protein might be divided in two domains: the C-terminal conserved domain and PST-rich N-terminal variable domain. The N-termini of Eya1-3 are able to act as transactivation domains *in vitro*, and it was proposed that *Eya* gene products can act as transcriptional activators (Xu *et al.*, 1997a). During mouse development, *Eya1* and *Eya2* are extensively expressed in several tissues and organs including cranial sensory placodes (Duncan *et al.*, 1997; Xu *et al.*, 1997). Expression of *Eya1* and *Eya2* in the prospective lens and nasal ectoderm depends upon expression of *Pax-6* (Xu *et al.*, 1997b).

To gain insight into the cascade of events leading to early eye development in different phyla, we have identified *Eya* genes in the chicken. We demonstrate that there are at least three *Eya* genes in the chicken genome that are expressed in the eye. cDNA containing a complete coding sequence of the chicken Eya2 protein was isolated and characterized. The expression pattern of the chicken *Eya2* gene indicates that this gene may be involved in the specification of nasal placode identity as well as patterning of the wing and limb bud tissues.

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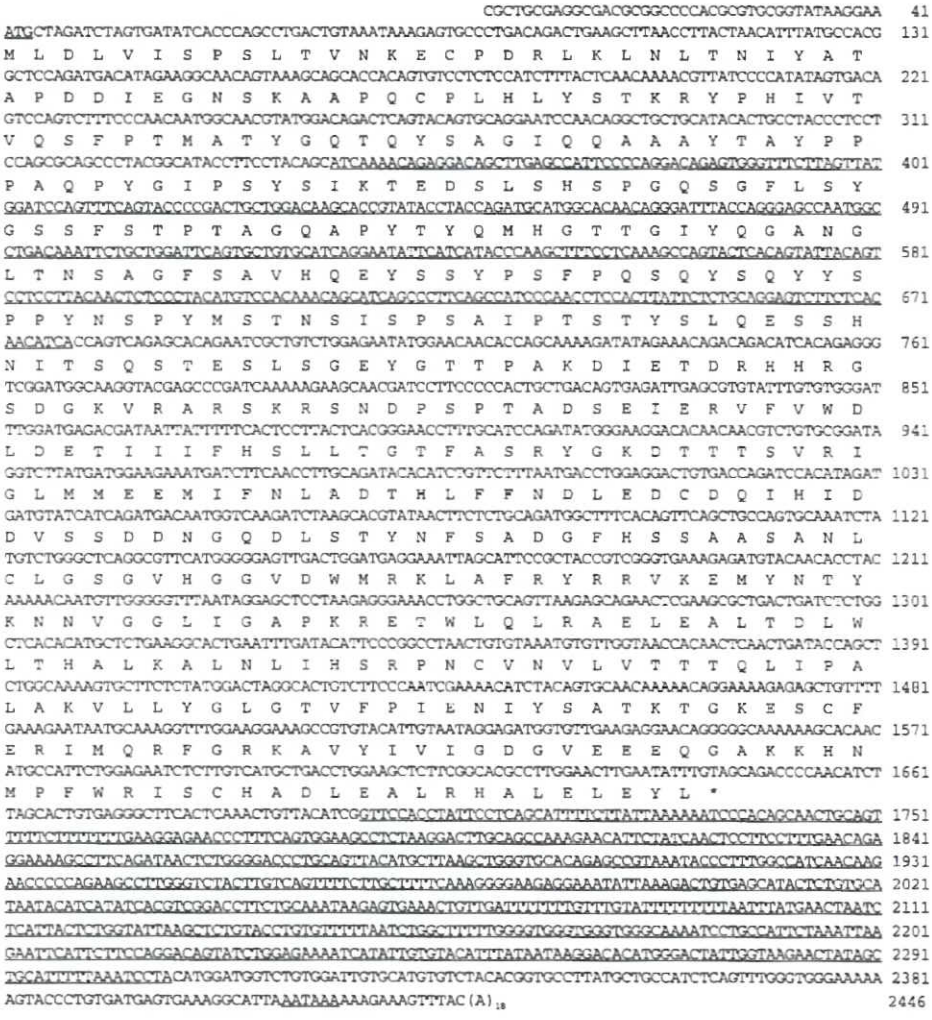

Fig. 1. Nucleotide and deduced amino acid sequence of the chicken *Eya2* cDNA (GenBank accession number AFO31484). *The initiation codon and polyadenylation signal as well as regions used for in situ hybridization are underlined. * indicates the first base of the termination codon (TAG).*

Results

Cloning of the chicken Eya2 cDNA

Eya cDNAs were identified in 14 day embryonic lens cDNA using a PCR approach. A 302bp fragment was generated (see Materials and Methods) and cloned in the pCR-Script vector. Sequencing of individual clones indicated that the isolated PCR product represented a mixture of two sequences corresponding to the *Eya2* and *Eya3* cDNAs identified previously in mammals (Abdelhak *et al.*, 1997b; Duncan *et al.*, 1997; Xu *et al.*, 1997b; Zimmerman *et al.*, 1997). Chicken *Eya1* cDNA was identified in separate experiments using another combination of primers (S.I.T. and R.D. Zinovieva, unpublished). Fragments corresponding to the chicken *Eya2* and *Eya3* genes were used to screen a 14 day embryonic chicken lens cDNA library. The longest cDNA corresponding to the *Eya3* gene was 1732bp long and did not contain information about the N-terminus of Eya3 protein (not shown). The longest cDNA corresponding to the *Eya2* gene that we obtained is 2466bp long (Fig. 1) and contains an open reading frame of 1602bp. The deduced sequence of 534 amino acids has a calculated molecular mass of 59 kDa. Comparison of the sequence obtained with those of human and mouse indicated that they are 82% and 78% identical, respectively (Fig. 2). As expected, the 271 amino acid long C-terminal part of the protein molecule (positions 264-534 in Fig. 2) is the most conserved part of the protein and shows 96-97% identity to vertebrate Eya2 sequences. This C-terminal domain shows 80-84%, 68-69% and 66% identity to the corresponding parts of mouse Eya1 and Eya3 and *Drosophila* eya proteins, respectively. The N-terminus of chicken Eya2 (positions 1-263 in Fig. 2) shows 51-52%, 60-66% and 29-30% identity to the N-terminus of mammalian Eya1, Eya2 and Eya3, respectively. As in other Eya proteins, the chicken Eya2 N-terminal domain is proline-serine-threonine-rich (35%).

To determine how many *Eya2*-related genes are present in the chicken genome, chicken genomic DNA was hybridized with the PCR fragment corresponding to the conserved part of the cDNA (positions 1152-1453 in Fig. 1). The pattern of hybridization (Fig. 3) was consistent with the presence of a single *Eya2* gene in the chicken genome.

Northern blot hybridization

We conducted northern blot hybridization experiments to estimate the size of the chicken *Eya2* mRNA and to investigate its pattern of expression in several tissues during chicken development. The same PCR fragment that we have used for Southern hybridization was used for these experiments. The results indi-

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cEya2  MLDLVISPSLTVNKECPDRLKLNLTNIYATAPDDIEGNSKAAPQCPLHLY  50
mEya2  --EV-T----ATSSD......WSEHGAAVGTLS-R--IA-S-ALSVPQ-F  44
hEya2  -VE----------SD-L-K--F-RADAAVWTLS-RQ-IT-S--LRVSQ-F  50

cEya2  STKRYPHIVTVQSFPTMATYGQTQYSAGIQQAAAYTAYPPPAQPYGIPSY 100
mEya2  V.-SH-RVPPG--STA--A-------T-----PP-----T---A----P-  93
hEya2  -.RSCP-VLPR-PSTA--A-------------TP---------A------  99

cEya2  SIKTEDSLSHSPGQSGFLSYGSSFSTPTAGQAPYTYQMHGTTGIYQGANG 150
mEya2  --------N---S--------P----AP---S----PV-S-A-L------ 143
hEya2  --------N-----------------SPT--S-----------F---G-- 149

cEya2  LTNSAGFSAVHQEYSSYPSFPQSQYSQYYSPPYNSPYMSTNSISPSAIPT 200
mEya2  ---T---GS---D-P-----S-N--P--F--S--P--VPAS-LCS-PLS- 193
hEya2  -G-A---GS---D-P---G------P---GSS--P--VPAS--C--PLS- 199

cEya2  STYSLQESSHNITSQSTESLSGEYGT.....TPAKDIETDRHHRGSDGKV 245
mEya2  ---V---AP--VP---S---A-D-NYHNGPS--  EGD-E-P--A----L 243
hEya2  ---V---A---VPN--S---A---N-HNGPS----EGD---P--A----L 249

cEya2  RARSKRSNDPSPTADSEIERVFVWDLDETIIIFHSLLTGTFASRYGKDTT 295
mEya2  -G----NS----AG-N-------------------------------- 293
hEya2  -G-----S----AG-N-------------------------------- 299

cEya2  TSVRIGLMEEMIFNLADTHLFFNDLFDCDQIHIDDVSSDDNGQDLSTYN 345
mEya2  ----------------------------V----------------- 343
hEya2  ----------I-----------------V----------------- 349

cEya2  FSADGFHSSAASANLCLGSGVHGGVDWMRKLAFRYRRVKEMYNTYKNNVG 395
mEya2  --T-----T-PG-S----T-------------------------R---- 393
hEya2  ----------PG---------------------------------- 399

cEya2  GLIGAPKRETWLQLRAELEALTDLWLTHALKALNLIHSRPNCVNVLVTTT 445
mEya2  ----------------------------S--------N---------- 443
hEya2  ----T-------------------------S--------N---------- 449

cEya2  QLIPALAKVLLYGLGTVFPIENIYSATKTGKESCFERIMQRFGRKAVYIV 495
mEya2  ----------S---------------------------------- 493
hEya2  ----------S----------------------------------V- 499

cEya2  IGDGVEEEQGAKKHNMPFWRISCHADLEALRHALELEYL          534
mEya2  ------------------------------------          532
hEya2  ----------------------N-----------             538
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Fig. 2. Sequence comparison of the chicken Eya2 with mouse [mEya2; (Duncan *et al.*, 1997)] and human [hEya2; (Duncan *et al.*, 1997)] sequences. *The amino acid sequence of chicken Eya2 is shown in full; for other proteins, only differing amino acid residues are shown. The conserved 271 amino acid long C-terminal domain is boxed. Hyphens indicate identical residues; dot marks the gaps which were introduced to maximize identity.*

cated that expression of the chicken *Eya2* gene is not lens- or eye-specific. Expression of *Eya2* was detected in lens (Fig. 4A), eyes lacking lens (Fig. 4B), retina (not shown), brain (Fig. 4C) and skeletal muscles (Fig. 4D). No expression of *Eya2* was detected in heart and liver (not shown). In the lens, the highest level of *Eya2* expression was at embryonic day 9 (Fig. 4A) and was comparable to its level of expression in the eye without the lens at embryonic day 5 (Fig. 4D). In all tissues analyzed, expression of *Eya2* declined at later stages of development and could not be detected by northern hybridization after hatching.

The major *Eya2* transcript in all tissues was about 2.6 kb long. The weak hybridization signals of 4 kb and 2 kb may reflect cross hybridization to other members of *Eya* gene family or hybridization to alternatively spliced forms of *Eya2* mRNA.

Whole-mount in situ hybridization with Eya2

To investigate the expression pattern of *Eya2* at earlier stages of embryonic chicken development and to obtain more detailed information on the localization of the *Eya2* mRNA in the embryo, we conducted *in situ* hybridization experiments. Embryos of Hamburger and Hamilton (Hamburger and Hamilton, 1951) stages 7 (-) to 28 (day 6) were hybridized with *Eya2* sense and antisense probes. Embryos from stages 7 (-) to 8 showed no significant *Eya2* hybridization signals. Hybridization to *Eya2* mRNA was first observed in the foregut at stage 9 (Fig. 5A). At the time of anterior neuropore closure (Stage 11), *Eya2* was expressed in the midbrain and pharyngeal pouch (Fig. 5C). Expression in the optic vesicle at this stage of development was not consistent. At stage 13, expression of *Eya2* was first detected in the nasal pit in addition to its expression in the midbrain and pharyngeal pouches (not shown). Expression in the nasal pit persisted at later stages of development (stage 21, embryonic day 3.5, Fig. 6A). At stage 21, *Eya2* was strongly expressed in pharyngeal arches III, IV and VI, dorsal root ganglia, dermomyotome and the tip of the tail (Fig. 6A,C). At this stage of development, *Eya2* was also expressed in the mesenchymal condensation at the base of wing and limb buds (Fig. 6C). These mesenchymal cells differentiate into the muscle cells and become intermingled with lateral dermomyotome cells migrating to the limb bud and forming limb muscle (Fig. 7B). The muscle fate of the *Eya2*-expressing cells in the wing and limb buds was confirmed by immunostaining with antibodies against chicken *Pax-7* (Kawakami *et al.*, 1997). At stage 27 (embryonic day 5), expression of *Eya2* was still observed in pharyngeal arches IV and VI, dorsal root ganglia and dermomyotome (Fig. 7A,B). Expression in dorsal root ganglia and dermomyotome could be clearly seen after sectioning embryos after whole-mount hybridization (Fig. 8).

To examine expression of *Eya2* in the eye at later developmental stages, we conducted *in situ* hybridization with paraffin sections. We used those stages of development which gave the highest hybridization signals in northern hybridization experiments (see Fig. 4). At stage 27, expression of *Eya2* was detected in undifferentiated neural retina preferentially in the prospective ganglion and amacrine cells (Fig. 9) and in the cranial nerve ganglia (V, VII-VIII, and IX-X, not shown). At stage 35 (embryonic day 9), *Eya2* was expressed in corneal epithelium, stroma and endothelium (Fig. 10A-C), lens epithelium and nuclear fibers (Fig. 10A,D), and

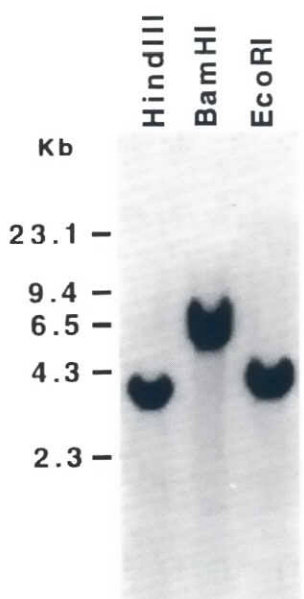

Fig. 3. Southern blot hybridization of the chicken *Eya2* gene. *Size markers are shown on the left.*

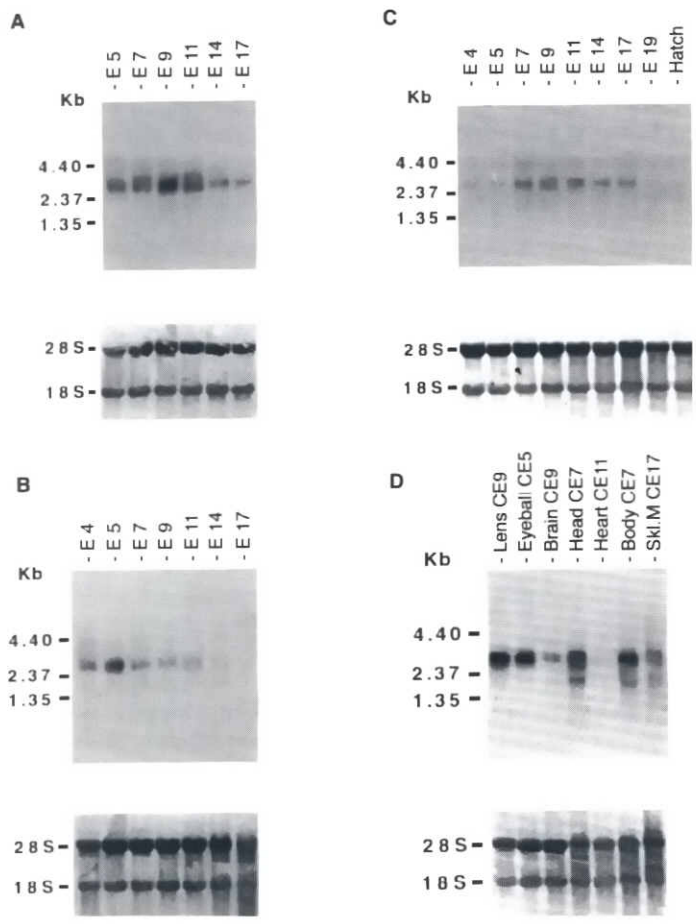

Fig. 4. Expression of the chicken *Eya2* gene in different tissues. *Ten μg of total RNA isolated from lens* **(A)**, *eyeball lacking lens* **(B)**, *and brain* **(C)** *were loaded on the gel.* **(D)** *includes samples of RNA from tissues and stages of development giving the highest hybridization signals. Upper boxes show hybridization, and lower boxes are filters stained with methylene blue after hybridization. The stages of development are indicated at the top of each lane. Positions of markers are shown on the left.*

mesenchyme of iris (Fig. 10C). No expression was detected in the retina (Fig. 10E). *Eya2* expression overlapped with *Pax-6* expression in some part of the eye (corneal and lens epithelium, see Fig. 11D). In general, the level of *Pax-6* expression, which we have used as a control, was much higher than the level of *Eya2* expression by both northern and *in situ* hybridization.

Discussion

Eya2 belongs to a family of *Eya* genes consisting of at least three members in all vertebrates studied. In the chicken, partial sequences for *Eya1* (S.I.T. and R.D. Zinovieva, unpublished) and *Eya3* (N.M. and S.I.T., unpublished) and full coding sequence of *Eya2* (present work) are known. Chicken Eya2, as all other Eya proteins, contains conserved C-terminal and more variable N-terminal domains. Although there is no significant similarity in the sequence of the N-terminal domains of *Drosophila* eya and vertebrate Eya, mouse *Eya2* is able to complement mutations in the *eya* gene in *Drosophila* (Bonini *et al.*, 1997). Similarly, vertebrate and squid Pax-6 possess 90-95% identity to *Drosophila* eyeless in the region of the paired- and homeodomains but not in other regions of the protein molecule. Despite these differences, both the vertebrate (Halder *et al.*, 1995) and squid (Tomarev *et al.*, 1997) proteins can induce ectopic eyes in *Drosophila*. The conserved C-terminal domain is probably critical for the function of Eya/eya proteins. In *Drosophila*, this domain is essential for physical interactions of eya with sine oculis (Pignoni *et al.*, 1997) or duchshund (Chen *et al.*, 1997). The ability of *eya* to induce ectopic eyes in *Drosophila* (Bonini *et al.*, 1997) and act synergistically with *eyeless* (Bonini *et al.*, 1997), *dachshund* (Chen *et al.*, 1997) or *sine oculis* (Pignoni *et al.*, 1997), as well as the ability of the PST-rich N-terminal domain of Eya proteins to transactivate transcription *in vitro* (Xu *et al.*, 1997a) and in yeast cells, suggests that Eya2 is directly involved in gene regulation, most likely as a coactivator of transcription (Chen *et al.*, 1997). We do not know at present if Eya2 is able to interact with DNA directly or whether it interacts with other DNA-binding proteins and changes their specificity of action.

The expression pattern of chicken *Eya2* is similar in many respects to that of its mouse homolog (Duncan *et al.*, 1997; Xu *et al.*, 1997b). It was suggested that members of the mouse *Eya* gene family play critical roles downstream of *Pax* genes in specifying cranial placode identity (Xu *et al.*, 1997b). Early expression (stage 13) of the chicken *Eya2* in the nasal pit is consistent with it having

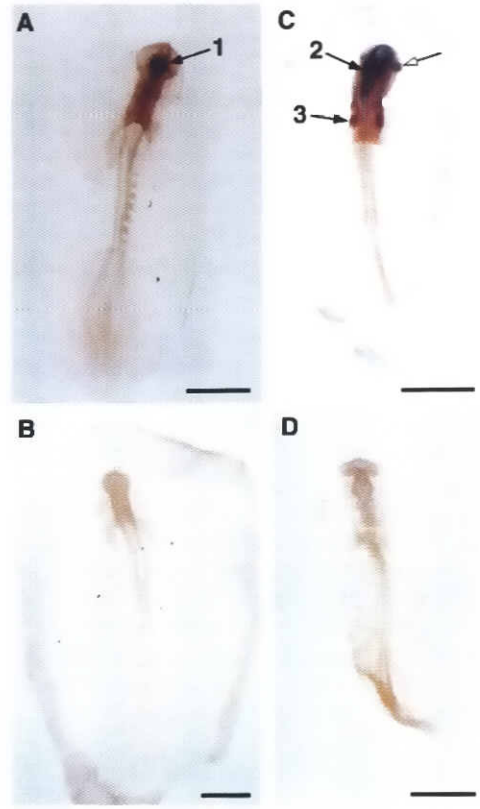

Fig. 5. Expression of chicken *Eya2* as detected by whole-mount *in situ* hybridization. **(A)** *Stage 9; antisense probe. Arrow (1) indicates expression in the foregut.* **(B)** *Stage 9; sense probe.* **(C)** *Stage 11-; antisense probe; arrows indicate expression in the midbrain (2), pharyngeal pouch (3) and optic vesicle (white).* **(D)** *Stage 10+; sense probe. Bar, 1 mm.*

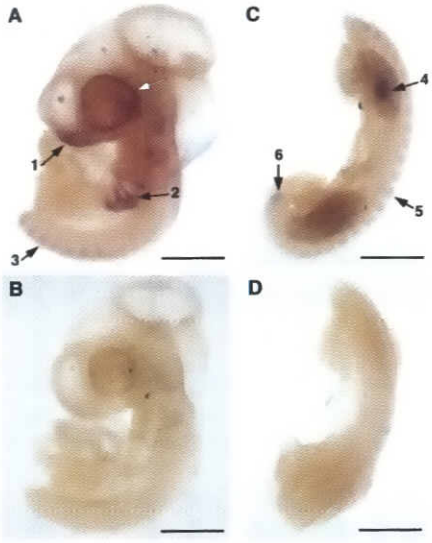

Fig. 6. Expression of chicken *Eya2* at stage 21 (embryonic day 3.5) embryos. *Embryos were cut in two halves [***(A,B)** *anterior part;* **(C,D)** *posterior part] and hybridized as whole-mount with antisense (A,C) and sense (B,D) probe. Expression of Eya2 was detected in nasal pit (1), branchial arches III, IV and VI (2), dorsal root ganglia (3,5), limb buds (4), and the tip of the tail (6). White arrow points to the eye. Bar, 1 mm.*

a role in the development of the nasal placode. However, we could not convincingly detect expression of *Eya2* in the optic vesicle and the lens placode. This implies that chicken *Eya2* is probably not involved in the early inductive events of eye development. Experiments with mice indicate that *Eya1* might be involved in the specification of the lens placode (Xu *et al.*, 1997b). This remains to be tested in the chicken. Later in development (stages 24-27) expression of *Eya2* was detected in the retina and subsequently in the lens, cornea and mesenchyme of iris (Figs. 9,10). Expression in the retina and lens was much weaker than expression of *Pax-6* by both northern and *in situ* hybridization. Expression of *Eya2* in the corneal stroma and iris mesenchyme, which are derived from cranial neural crest, is consistent with the observation that *Eya2* is expressed in several neural crest derivatives (dorsal root ganglia, branchial arches). Relatively late expression of *Eya2* in the eye suggests that it is necessary for differentiation and function of several eye tissues. Other *Pax* genes, *Pax-3* or *Pax-7*, might be involved in regulation of *Eya2* expression in dermomyotome and developing wing and limb muscles. *Pax-3* and *Pax-7* are expressed in these structures, and it was proposed that *Pax-3* and *Pax-7* might be involved in specification of migratory limb muscle precursors. It was demonstrated recently that *Eya* genes, and *Eya2* in particular, might be necessary for patterning of tendons of the limb in mice (Xu *et al.*, 1997a). This suggests that in the chicken *Eya2* might be also involved in the patterning of the limb and wing tendon tissues. Development of the limb buds in vertebrates shows many similarities to that of the branchial arches (see Grigoriou *et al.*, 1998 for references). *Eya2* expression in limb buds and branchial arches III, IV and VI, which participate in the formation of the thymus, parathyroid glands and cardiac outflow septation,

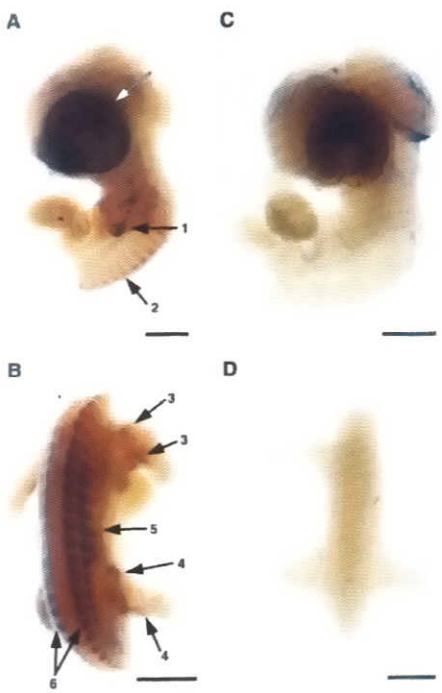

Fig. 7. Expression of chicken *Eya2* at stage 27 (embryonic day 5) embryos. *Embryos were cut in two halves [***(A,C)** *anterior part;* **(B,D)** *posterior part] and hybridized as whole-mount. (A,B) antisense probe; (C,D) sense probe. Expression of Eya2 was detected in the branchial arches IV and VI (1), dorsal root ganglia (2,6), muscles of the wing (3) and limb (4), and in dermomyotome (5). White arrow points to the eye. Bar, 1 mm.*

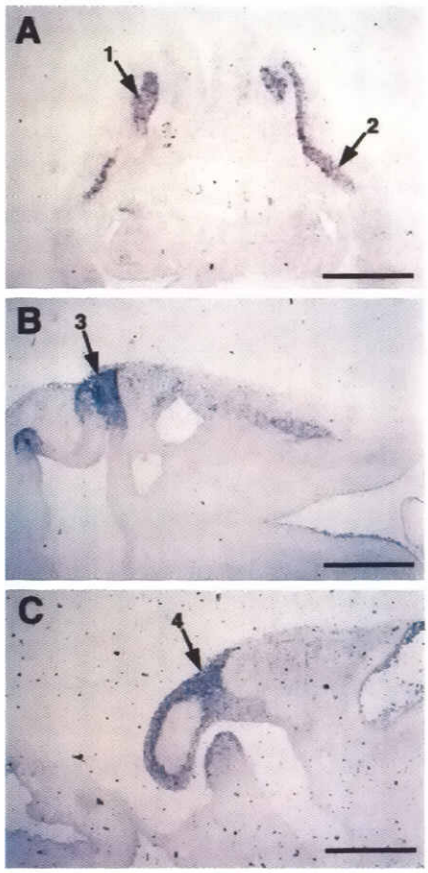

Fig. 8. Expression of chicken *Eya2* at stage 27 embryos. *Transverse 10 μm sections through the different parts of the chicken embryos hybridized as whole-mount. Arrows point to dorsal root ganglia (1), dermomyotome (2), and neural crest-derived mesenchyme of the branchial arches (3,4). Bar, 0.2 mm.*

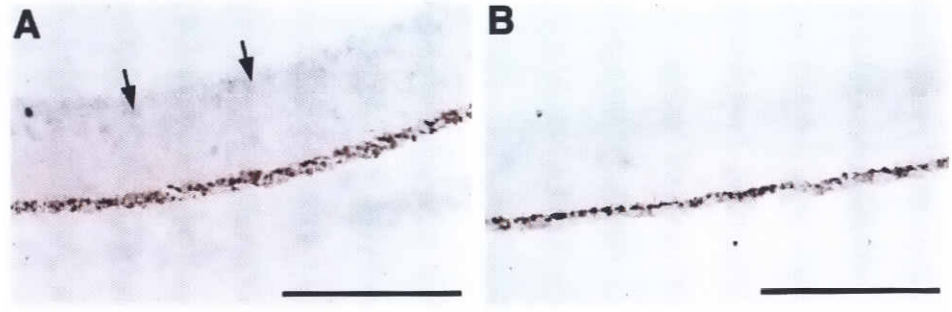

Fig. 9. Expression of chicken *Eya2* in the retina of stage 27 (embryonic day 5) embryos. (A) *Antisense probe;* **(B)** *sense probe. Arrows point to the weak hybridization signals in prospective ganglion and amacrine cell layers. Bar, 25 μm.*

adds another common element to the list of factors involved in the development of both limb buds and branchial arches.

Several genes were identified in *Drosophila* as being critical for early eye development. They include *eyeless* (Quiring *et al.*, 1994) *eyes absent* (Bonini *et al.*, 1993), *sine oculis* (Cheyette *et al.*, 1994) and *duchshund* (Mardon *et al.*, 1994). None of these genes is eye-specific and all of them are expressed outside the eye at different developmental stages. In *Drosophila*, different combinations of these genes are able to produce larger ectopic compound eyes and in additional locations compared with any of these genes alone (Bonini *et al.*, 1997; Chen *et al.*, 1997; Pignoni *et al.*, 1997). In vertebrates, each of these genes is a member of larger gene family. Understanding the function and various interactions of these important regulatory proteins remains as a challenge to future research.

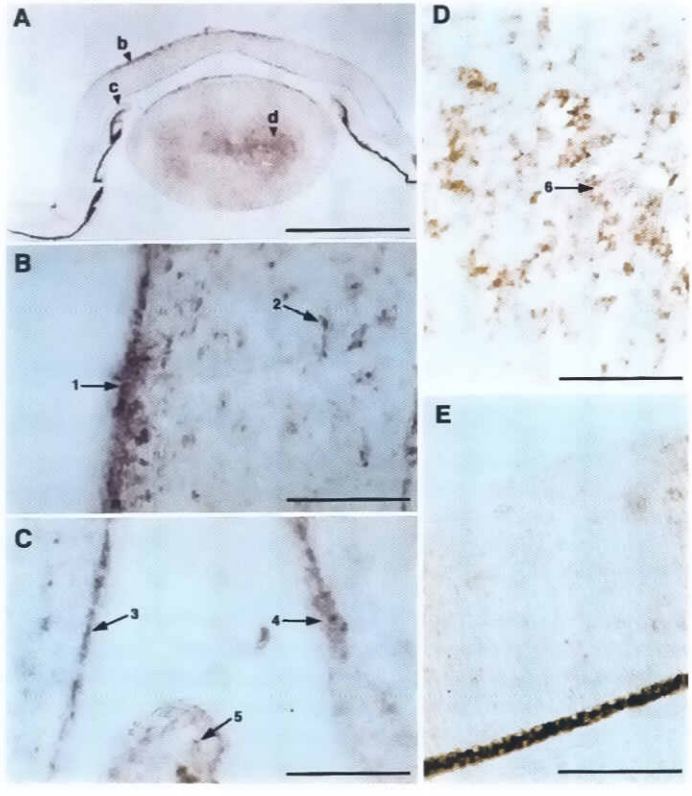

Fig. 10. Expression of chicken *Eya2* in the eye of stage 35 (embryonic day 9) embryos. *Lower* **(A)** *and higher* **(B,C,D,E)** *magnification of different parts of the eye. Abbreviations: b, cornea; c,iris; d, lens; 1, corneal epithelium; 2, corneal stroma; 3, corneal endothelium; 4, lens epithelium; 5, mesenchyme of iris; 6, nuclear lens fibers area. E shows the retina area. Bar, 1 mm in A and 0.1 mm in B-E.*

Materials and Methods

RNA isolation and cloning of the chicken eya homolog

RNA was isolated from the indicated chicken tissues by the acidic guanidinium thiocyanate-phenol-chloroform extraction method (RNazol B, Tel-Test, Friendswood, TX). Poly(A)$^+$RNA was purified from total RNA isolated from the lenses of 14 day embryos (Dynabeads mRNA purification kit; Dynal Inc., Oslo, Norway). This poly(A)$^+$ RNA was used for lens cDNA library construction, as described elsewhere (Duncan *et al.*, 1995). cDNA obtained in the course of library construction was also used as a template for PCR. Primers used for PCR were designed following the amino acid sequences of *Drosophila* eya (Bonini *et al.*, 1993) and human Eya2. They were 5'-GACTGGATGAGGAAACTAGCTTT(C/T)CG(G/C)TA-3' and 5'-GCACTATAGAT(A/G)TT(C/T)TC(A/G/T)AT-3' (positions 1152-1180 and 1434-1453 in Fig. 1). Samples underwent 30 cycles of amplification (94°C 2 min, 55°C 1 min 30 sec, 72°C 1 min) and a final incubation at 72°C for 5 min. The amplification products were purified by gel electrophoresis, cloned into pCR-Script (Strategene, La Jolla, CA) and sequenced (Sequenase Version 2.0 sequencing kit; Amersham). The cDNA insert encoding a portion of *Eya2* was used as a probe to screen a 14 day embryonic chicken lens cDNA library as described (Duncan *et al.*, 1995).

Southern and Northern hybridization

About 10 μg of chicken DNA was digested with the indicated restriction enzymes, separated by electrophoresis in a 0.8% agarose gel, transferred to Duralon UV filters (Stratagene), crosslinked by ultraviolet irradiation (Stratalinker, Stratagene), and hybridized in QuikHyb (Stratagene) with a [^{32}P]-labeled PCR fragment (positions 1152-1453 in Fig. 1). Conditions for hybridization were as recommended by manufacturer.

Ten μg of total RNA from each of the indicated tissues were separated by electrophoresis on 1.2% agarose, 2.2 M formaldehyde gel, transferred to a Duralon UV membrane, crosslinked and hybridized as above. Filters were stained with 0.02% methylene blue after autoradiography for normalization of the amount of loaded RNA (Herrin and Schmidt, 1988).

Probe preparation and in situ hybridization

Riboprobes were prepared from both strands of the chicken *Eya2* cDNA as described (Li *et al.*, 1994). Sense and antisense riboprobes corresponding to two regions of the *Eya2* cDNA (positions 345-678 and 1698-2308 in Fig. 1) were prepared. Both probes gave identical patterns of hybridization; the second probe was used in most experiments. *Pax-6* probes were prepared as in Li *et al.* (1994). Whole-mount *in situ* hybridization was performed as described (Li *et al.*, 1994) with slight modifications. Variable concentrations (2-20 μg/ml) of proteinase K were used according to the size of hybridized tissue. Following *in situ* hybridization, embryos were either viewed as whole-mounts or were imbedded in JB-4 glycol methacrylate resin (Polysciences Inc.) and sectioned with a glass knife. *In situ* hybridization to paraffin sections

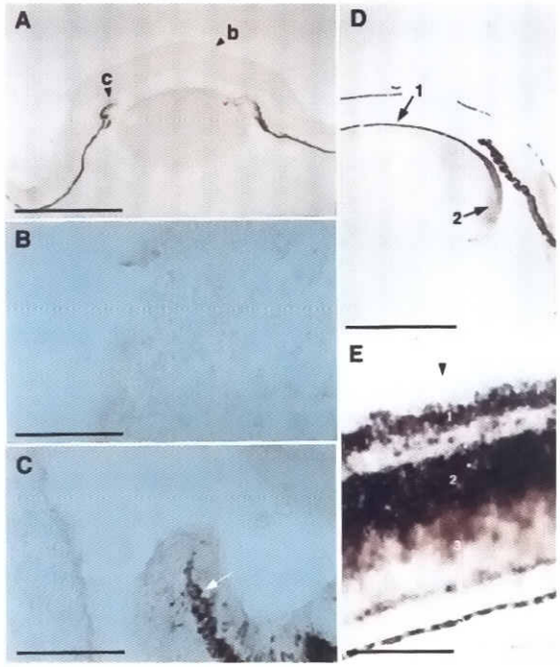

Fig. 11. Expression of chicken *Pax-6* in the eye of stage 35 embryos (D,E). **(A-C)** *show control hybridization with Eya2 sense probe.* **(D)** *1, lens epithelium; 2, bow region of the lens;* **(E)** *Presumptive ganglion (1), amacrine (2), bipolar (3) and horizontal (4) cell layers, 5, retinal pigmented epithelium; (A) b, corneal epithelium; c, iris. Bar, 1 mm in A and D and 0.1 mm in B,C, and E.*

was conducted as described (Belecky-Adams *et al.*, 1997).

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