Conservation of *Pax 6* function and upstream activation by *Notch* signaling in eye development of frogs and flies

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Loss of *Pax 6* function leads to an eyeless phenotype in both mammals and insects, and ectopic expression of both the *Drosophila* and the mouse gene leads to the induction of ectopic eyes in *Drosophila*, which suggested to us that *Pax 6* might be a universal master control gene for eye morphogenesis. Here, we report the reciprocal experiment in which the RNAs of the *Drosophila Pax 6* homologs, *eyeless* and *twin of eyeless*, are transferred into a vertebrate embryo; i.e., early *Xenopus* embryos at the 2- and 16-cell stages. In both cases, ectopic eye structures are formed. To understand the genetic program specifying eye morphogenesis, we have analyzed the regulatory mechanisms of *Pax 6* expression that initiates eye development. Previously, we have demonstrated that *Notch* signaling regulates the expression of *eyeless* and *twin of eyeless* in *Drosophila*. Here, we show that in *Xenopus*, activation of *Notch* signaling also induces eye-related gene expression, including *Pax 6*, in isolated animal caps. In *Xenopus* embryos, the activation of *Notch* signaling causes eye duplications and proximal eye defects, which are also induced by overexpression of *eyeless* and *twin of eyeless*. These findings indicate that the gene regulatory cascade is similar in vertebrates and invertebrates.

In the animal kingdom, a large variety of different eye types are found. In vertebrates, the development of the camera-type eye with a single lens depends on coordinated inductive interactions between the presumptive neural retina and the surface ectoderm that will give rise to the lens (1). In contrast to the single lens eye of vertebrates, the development of the compound eye of *Drosophila* is initiated in a small group of epithelial cells in the embryo that form the eye part of eye-antennal imaginal disk after proliferation during larval stages (2). Despite these differences, recent studies have demonstrated that the genetic programs specifying eye development are conserved during evolution (3–5). The striking examples are the *Pax 6* genes, which are key regulators for eye morphogenesis in both animal phyla (6).

The *Pax 6* genes code for transcription factors containing two highly conserved DNA-binding motifs, a paired domain and a paired-type homeodomain (7, 8). Mutations in *Pax 6* result in eye malformations known as *Small eye* in mice (9), Aniridia in humans (10, 11), and *eyeless* in *Drosophila* (12). By targeted expression of *eyeless*, ectopic eyes can be induced on the antennae, the legs, and the wings of the fly (13). Misexpression of *Pax 6* in *Xenopus* also results in the formation of ectopic eye structures (14, 15). To test whether *Pax 6* is a universal master control gene for eye morphogenesis, we have expressed the mouse *Pax 6* gene in *Drosophila* leading to the induction of well developed compound eyes (13). Here, we report a reciprocal experiment in which we express the *Drosophila Pax 6* homologs, *eyeless* and *twin of eyeless* (16), in *Xenopus* embryos and obtain vertebrate eye structures. To understand the genetic hierarchy controlling eye development, we have begun to compare the *Pax 6* downstream target genes in the various animal phyla. A direct target gene for *eyeless*, *sine oculis* (*so*; ref. 17), constitutes a positive feedback loop for *eyeless* with *eyes absent* and *dachshund*

in *Drosophila* eye development (18–22). A member of the *sine oculis* family, *Six 3*, is a downstream target gene of *Pax 6* in vertebrate eye development (15) and makes a positive feedback loop for *Pax 6* with Rx (23), another homeobox gene (24). However, the Drosophila gene *optix*, which is more closely related to *so* than *Six 3*, is capable of inducing ectopic eyes by an *eyeless*-independent mechanism (25). In planarians, *Pax 6* participates in eye development (26), and the *sine oculis* homolog is essential for eye regeneration (27), suggesting that the basic elements of the genetic pathway are conserved in these prototypic eyes. These observations have suggested that the regulatory network has been adapted to the control of development of different eye types during evolution (28). To understand the genetic program specifying eye development, it is important to study the regulatory mechanisms for *Pax 6* expression, which initiates eye development. Previously, we have demonstrated that *Notch* signaling regulates the expression of *eyeless* and *twin of eyeless* (29); the latter is known to be an upstream regulator for *eyeless* by directly regulating the eye-specific enhancer of the *eyeless* gene in the embryo (16, 30). The *Notch* signaling pathway defines an evolutionarily conserved cell–cell interaction mechanism that throughout development controls the ability of precursor cells to respond to developmental signals (31). Here, we show that in *Xenopus* the activation of *Notch* signaling activates *Pax 6* and other eye-related genes in isolated animal caps, and induces eye duplications as well as proximal eye defects in embryos that are also induced by misexpression of the *Drosophila* homologs of *Pax 6*, *eyeless* and *twin of eyeless*. Furthermore, the misexpression of *eyeless* and *twin of eyeless* results in the formation of ectopic eye structures in *Xenopus* embryos. These results suggest that the regulation of *Pax 6* expression and the function of *Pax 6* in eye development are conserved in vertebrates and invertebrates.

Materials and Methods

Construction of Expression Vectors. *Drosophila eyeless*-pNRRX and *twin of eyeless*-pNRRX were constructed as follows: the ORF of *eyeless* was amplified with primers (5′-CATGTTTACATTG-CAACCAACTCC-3′ and 5′-GCTAGACCCACGGTGAGTA-GAAACA-3′) by using the *eyeless*-pNHT4 plasmid as a template by PCR (12). The ORF of *twin of eyeless* was PCR-amplified with primers (5′-CATGATGCTAACAACTGAACACATAA-3′ and 5′-ATCACTGAAGACGTGGCCA-3′) by using the *twin of eyeless*-pBluescript SK(−) plasmid as a template (16). These fragments were cloned into the *Eco*RV sites of pNRRX; the

pNRRX vector is a modified pBluescript II SK(−) (Stratagene). The fragment of Xglobin 5′-untranslated region and 3′-untranslated region of pSP64T vector (32) was subcloned into the *Pst*I/*Hin*dIII sites of pBluescript II SK(−), and the *Not*I–*Eco*RI–*Eco*RV–*Xho*I linker was inserted into the *Bgl*II site of the Xglobin-untranslated region fragment. *Xenopus* Notch ΔE-pCS2(+) was described before (33). Green fluorescent protein (GFP) (CLONTECH) was subcloned into the *Bgl*II site of pSP64T (32).

Microinjections and Histology. Capped synthetic RNAs of *eyeless*-pNRRX, *twin of eyeless*-pNRRX, *Notch* ΔE-pCS2(+), and GFP-psp64T were *in vitro* transcribed by using T7 or SP6 RNA polymerase (Stratagene). For animal cap assays, *in vitro* synthesized RNAs were injected into both blastomeres of 2-cell stage *Xenopus* embryos. Animal caps were isolated at the late-blastula stage (stage 9) and cultured in Steinberg's solution. For the histological analyses, the synthesized RNAs were injected into one of the blastomeres of 2-cell-stage or 16-cell-stage embryos. Respectively, the embryos were grown to stage 41–42 or stage 48 and examined histologically by using hematoxylin and eosin staining.

Reverse Transcriptase (RT)-PCR Analysis. Total RNA was extracted with TRIZOL Reagent (GIBCO/BRL) from cultured animal caps. First-strand cDNA was synthesized from 1 μg RNA with an Oligo-(dT) primer by using Superscript II reverse transcriptase (GIBCO/BRL). A negative control excluding enzyme was included for each sample. One-tenth of the cDNA obtained was used for each PCR reaction. Primers and PCR conditions used in this study were as follows: β-crystallin, forward 5′-TGAGTACCCACGTTGGGGATAC-3′ and reverse 5′-AGCCT-GGATACTGATATCCAACC-3′ (29 cycles) (14); EF-1α, forward 5′-TTGCCACACTGCTCACATTGCTTGC-3′ and reverse 5′-ATCCTGCTGCCTTCTTTTCCACTGC-3′ (21 cycles) (34); N-CAM, forward 5′-CACAGTTCCACCAAA-TGC-3′ and reverse 5′-GGAATCAAGCGGTACAGA-3′ (27 cycles) (34); ornitin dehydrocarboxylase, forward 5′-GTCAAT-GATGGAGTGTATGGATC-3′ and reverse 5′-TCCATTC-CGCTCTCCTGAGCAC-3′ (23 cycles) (35); Pax 6, forward 5′-AGTGTCCTCATTCACATCGG-3′ and reverse 5′-GAT-TCTTGGCTGGATCATGG-3′ (25 cycles) (two different length products are amplified by these primers) (36–38); Rx, forward 5′-ATACAGCAGAGAAGAGCTGGC-3′ and reverse 5′-AAGGTTGGAGAGCATGACC-3′ (25 cycles) (these primers fail to distinguish Xrx1 from Xrx2) (24); and Six 3, forward 5′-GCTTCAGCACCAGTCTATCG-3′ and reverse 5′-AAG-GAATCATGCAATGGCC-3′ (25 cycles) (15).

Results

The Two *Pax 6* Homologs of *Drosophila*, *eyeless* and *twin of eyeless*, Induce Eye Structures in *Xenopus* Embryos and Eye-Related Gene Expression in Animal Caps.

In *Drosophila*, expression of *Drosophila Pax 6* homologs, *eyeless* and *twin of eyeless*, and mouse *Pax 6* in imaginal discs result in the formation of ectopic eyes (13, 16), and the misexpression of *Pax 6* in *Xenopus* embryos also results in the formation of ectopic eye structures (14, 15). As a reciprocal experiment, we examined the effects of misexpression of *Drosophila Pax 6* homologs in *Xenopus* embryos. First, we injected *eyeless* and *twin of eyeless* RNA into one of the two blastomeres at the 2-cell stage and allowed the embryos to develop until stage 41 to 42. In addition to duplications of lenses and neural retina cell layers (Fig. 1F), expansion of the retinal pigment epithelium (RPE) from the endogenous eye region was observed in embryos injected with *eyeless* RNA (Fig. 1B and Fig. 2). Misexpression of *twin of eyeless* has almost no effect on expansion of the RPE under these conditions (Table 1). Histological cross sections of the embryos revealed that misexpression of *eyeless* induces the
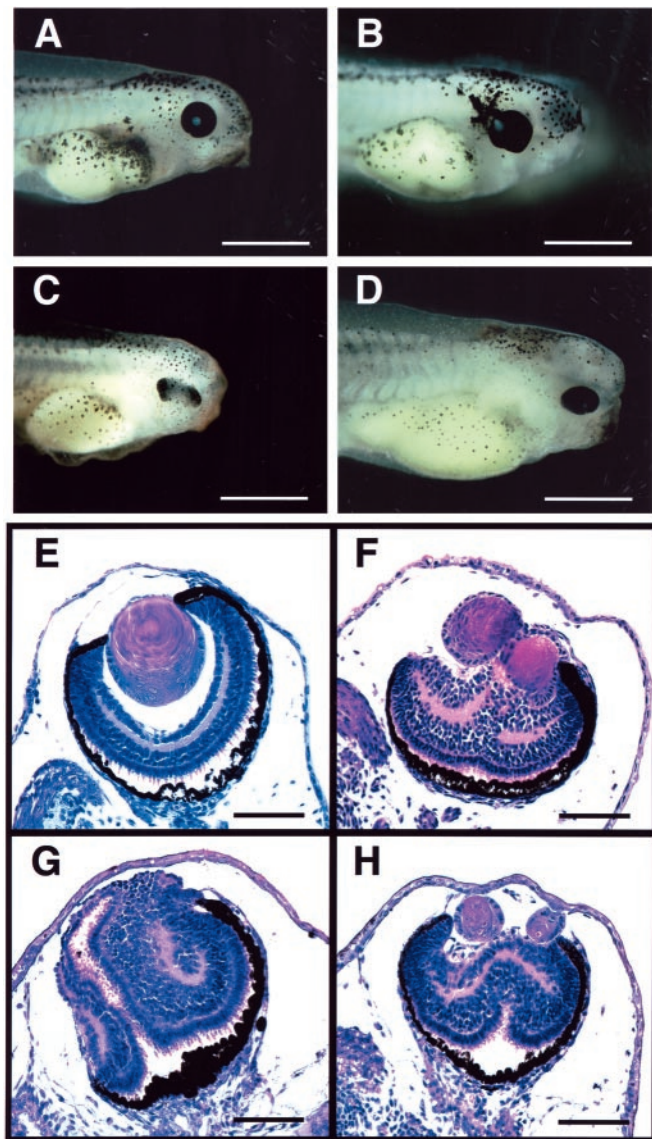

Fig. 1. Embryos injected with *eyeless*, *twin of eyeless*, and an activated form of *Notch* receptor RNA at the 2-cell stage. Embryos were injected with *eyeless* (*ey*), *twin of eyeless* (*toy*), and an activated form of *Xenopus* Notch receptor (*Notch* ΔE) RNA into one blastomere at the 2-cell stage and fixed at stage 41 to 42. Bars indicate 1 mm in *A–D* and 0.1 mm in *E–H*. Cross sections are stained with hematoxylin and eosin. (*A*) Control embryo uninjected. (*B*) Embryo injected with 0.5 ng of *ey* RNA, showing expansion of RPE. (*C*) Embryo injected with 2 ng of *toy* RNA, showing a duplication of the retina. (*D*) Embryo injected with 5 ng of *Notch* ΔE RNA, showing the duplication of the retina (*H*) and the hyperplasia of neural tube. (*E*) Cross section of an eye of a control embryo. (*F*) Cross section of an embryo injected with 0.25 ng of *ey* RNA, showing the duplication of lenses with the increased number of cells in the neural retina. (*G*) Cross section of an embryo injected with 5 ng of *toy* RNA, showing the duplication of the retina with an increase in the number of neural retina cells. (*H*) Cross section of an embryo injected with 5 ng of *Notch* ΔE RNA, showing the duplication of the eye with a duplicated retina and two lenses. The phenotype is similar to that of an embryo injected with 0.25 ng of *ey* RNA in *F*.

ectopic formation of lenses, RPE, and neural retina in the body trunk (Fig. 2B) and developmental defects including lack of lens, ectopic RPE formation, and hyperproliferation of the neural retina in the region of the original eye (Fig. 2C). Next, we injected *eyeless* and *twin of eyeless* RNA into one animal blastomere at the 16-cell stage and allowed the embryos to develop
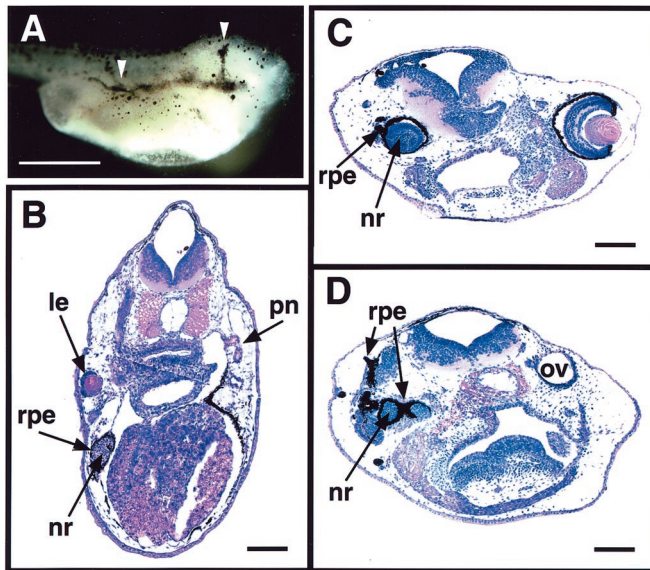
Fig. 2. Ectopic eye structures formed in embryos injected with *eyeless* RNA at the 2-cell stage. *Xenopus* embryos were injected with various amounts of *eyeless* RNA into one of the two blastomeres at the 2-cell stage and fixed at stage 41 to 42. Bars indicate 1 mm in *A* and 0.1 mm in *B–D*. (*A*) Embryo injected with 2 ng of *ey* RNA. Arrows indicate the extension of the RPE toward the midline and along the body trunk. (*B*) Cross section of an embryo injected with 0.25 ng of *ey* RNA, showing the ectopic formation of a lens, RPE, and neural retina in the trunk region at the pronephros level. (*C*) cross section of an embryo injected with 0.5 ng of *ey* RNA, showing a developmental defect in the region of the original eye with the lack of a lens, the ectopic formation of RPE, and the hyperproliferation in the neural retina. (*D*) More posterior cross section of the same embryo as in C, showing the ectopic formation of RPE and neural retina in the region of the original otic vesicle. le, lens; nr, neural retina; ov, otic vesicle; pn, pronephros.

until stage 48. As shown in Fig. 3 *A–C*, embryos injected either with *eyeless* or *twin of eyeless* RNA at the 16-cell stage show ectopic formation of RPE. The frequency of ectopic RPE formation of *eyeless*-injected embryos was much higher than that of *twin of eyeless*-injected embryos (Table 2), and *eyeless* was

Table 1. Summary of the eye phenotypes of embryos that are injected with RNA for *eyeless*, *twin of eyeless*, and an activated form of Notch receptor at the 2-cell stage

	Normal	Duplication	Eye expansion	Other	Dead	Total
ey						
100 pg	91% (29)	0% (0)	0% (0)	9% (3)	0% (0)	32
250 pg	44% (14)	44% (14)	6% (2)	6% (2)	0% (0)	32
500 pg	23% (22)	23% (22)	39% (37)	12% (11)	2% (2)	94
1 ng	0% (0)	2% (1)	91% (58)	6% (4)	2% (1)	64
toy						
100 pg	100% (32)	0% (0)	0% (0)	0% (0)	0% (0)	32
500 pg	87% (82)	2% (2)	0% (0)	4% (4)	6% (6)	94
1 ng	31% (10)	50% (16)	0% (0)	13% (4)	6% (2)	32
2 ng	6% (2)	81% (26)	3% (1)	0% (0)	9% (3)	32
Notch E						
1 ng	30% (11)	22% (8)	0% (0)	43% (16)	5% (2)	37
5 ng	38% (26)	26% (18)	0% (0)	29% (20)	6% (4)	68

Embryos were injected with *eyeless* (*ey*), *twin of eyeless* (*toy*), and an activated form of *Xenopus* Notch receptor (*Notch*) RNA into one blastomere at the 2-cell stage and allowed to develop to stage 41 to 42. Typical examples of the duplication of eyes and eye expansion are presented in Figs. 1 *F* and 2 *A*, respectively. The other phenotypes are mainly caused by head defects.
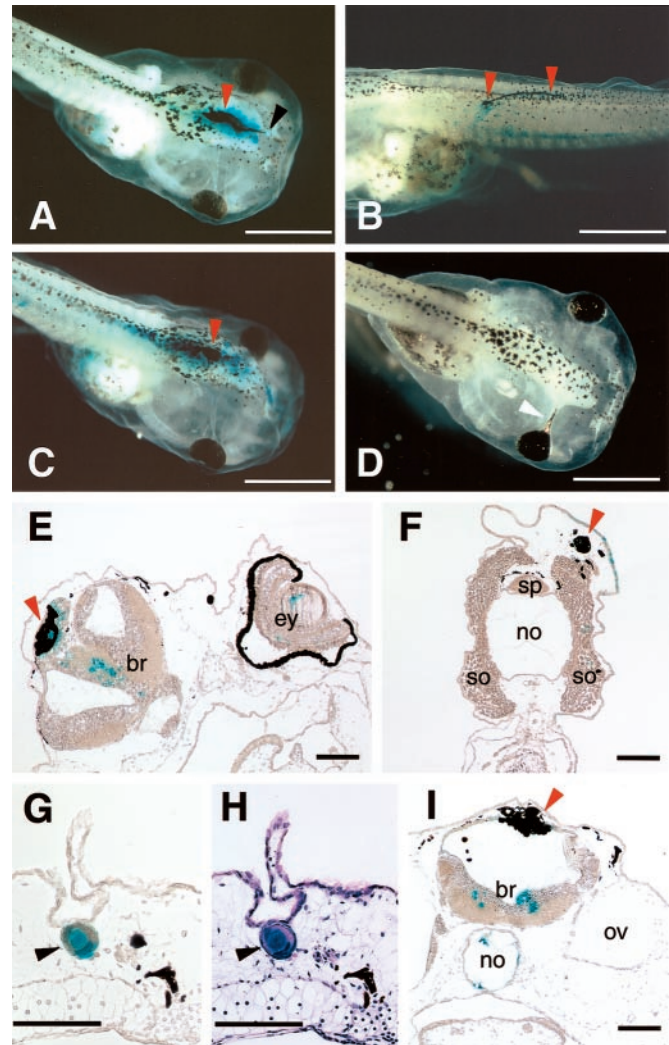

Fig. 3. Embryos injected with *eyeless*, *twin of eyeless*, and an activated form of *Notch* receptor RNA at the 16-cell stage. Embryos were injected with *eyeless*, *twin of eyeless*, and an activated form of *Xenopus* Notch receptor (*Notch* ΔE) RNA into one of the animal pole blastomeres at the 16-cell stage and fixed at stage 48. In total, 0.25 ng of nuclear localized β-galactosidase RNA was coinjected and detected by X-gal staining. Bars indicate 1 mm in *A–D* and 0.1 mm in *E–I*. (*A*) Embryo injected with 0.25 ng of *ey* RNA. Arrows indicate the ectopic formation of a lens in black and RPE in red. (*B*) Embryo injected with 0.25 ng of *ey* RNA. Arrows indicate the ectopic formation of RPE. (*C*) Embryo injected with 0.5 ng of *toy* RNA. Arrow indicates the ectopic formation of RPE. (*D*) Embryo injected with 0.5 ng of *Notch* ΔE RNA. Arrow indicates the proximal eye defect. (*E*) Cross section of the same embryo as in *A*. The ectopic RPE (red arrow) colocalizes with nuclear β-galactosidase. (*F*) Cross section of same embryo as in *B*. The ectopic RPE (red arrow) colocalizes with nuclear β-galactosidase. (*G*) Cross section of the ectopic lens of embryo (*A*). The ectopic lens (black arrow) colocalizes with nuclear β-galactosidase. (*H*) Cross section shown in *G* stained with hematoxylin and eosin. (*I*) Cross section of same embryo as in *C*. The ectopic RPE (red arrow) colocalizes with nuclear β-galac-tosidase. br, brain; ey, eye; no, notochord; ov, otic vesicle; so, somite; sp, spinal cord.

found to induce ectopic lenses, whereas *twin of eyeless* failed to do so (Fig. 3*A*). The coinjection analyses of a lineage tracer RNA encoding nuclear β-galactosidase together with that encoding *eyeless* and *twin of eyeless* revealed that the ectopic formation of RPE and lens are cell-autonomous effects caused by *eyeless* and *twin of eyeless* (Fig. 3 *E–I*). These results indicate that the two *Drosophila* homologs of *Pax 6*, *eyeless* and *twin of eyeless*, induce eye structures in *Xenopus* embryos in a cell-autonomous manner.

Table 2. Summary of the phenotypes of embryos that are injected with RNA for *eyeless*, *twin of eyeless*, and an activated form of Notch receptor at the 16-cell stage

	Normal	Ectopic RPE	Eye defect	Other	Dead	Total
ey						
100 pg	86% (49)	2% (1)	2% (1)	0% (0)	11% (6)	57
125 pg	68% (40)	15% (9)	14% (8)	2% (1)	2% (1)	59
250 pg	25% (14)	25% (14)	38% (21)	5% (3)	13% (7)	56
toy						
100 pg	91% (48)	0% (0)	0% (0)	2% (1)	8% (4)	53
500 pg	80% (90)	3% (3)	9% (10)	1% (1)	9% (10)	113
notch_E						
500 pg	83% (45)	0% (0)	11% (6)	4% (2)	2% (1)	54
2 ng	86% (96)	0% (0)	8% (9)	3% (3)	5% (5)	111

Embryos were injected with *eyeless* (*ey*), *twin of eyeless* (*toy*), and an activated form of *Xenopus* Notch receptor (*Notch*) RNA into one animal pole blastomere at the 16-cell stage and allowed to develop to stage 48. Typical examples of the ectopic RPE and the proximal eye defect are presented in Fig. 3 *A* and *D*, respectively. The other phenotypes are mainly caused by head defects.

To determine whether *eyeless* and *twin of eyeless* induce the expression of genes implicated in early eye development, we injected RNA for *eyeless* and *twin of eyeless* into both blastomeres of 2-cell stage embryos and analyzed the expression of eye-related genes, N-CAM (neural cell adhesion molecule), *Six 3*, endogenous *Pax 6*, Rx, and β-crystallin in the animal cap ectoderm (Fig. 4*A*). The misexpression of *eyeless* induced the expression of N-CAM, *Six 3*, endogenous *Pax 6*, Rx, and β-crystallin in the animal caps. *Twin of eyeless* requires five times more RNA than *eyeless* to induce the expression of N-CAM, *Six3*, *Pax6*, and Rx, and the expression of β-crystallin was not induced in the animal cap by the injection of *twin of eyeless* RNA even at high doses, which is in accordance with the phenotypical analyses (Tables 1 and 2; Fig. 3). No expression of N-CAM, *Six 3*, *Pax 6*, Rx, and β-crystallin was observed in animal caps isolated from uninjected embryos, and a very weak or no expression of these genes was found in the animal caps isolated from control embryos injected with GFP RNA. These results indicate that the two *Drosophila* homologs of *Pax 6*, *eyeless* and *twin of eyeless*, are sufficient to induce eye-related gene expression including endogenous *Pax 6* in the isolated animal caps of *Xenopus* embryos.

Induction of Eye Duplications and Proximal Eye Defects by Activation of *Notch* Signaling. To determine the role of *Notch* signaling in vertebrate eye development, we injected a constitutively activated form of *Xenopus* Notch receptor (*Notch* ΔE) RNA into one of the two blastomeres at the 2-cell stage and allowed the embryos to develop until stage 41 to 42. As shown in Fig. 1 *D* and *H*, the activation of *Notch* signaling induced duplications of lenses with a massive increase in the number of neural retina cells, in addition to the hyperplasia of the neural tube that has been reported before (33). These duplications are similar to ones observed in embryos injected with *eyeless* and *twin of eyeless* RNA described above (Fig. 1 *F* and *G*). Sometimes, the formation of the lens is inhibited under these conditions (Fig. 1*G*). These results are summarized in Table 1. Chow *et al.* (15) have found that embryos injected into one of the animal blastomeres at the 16-cell stage with *Xenopus* Pax 6 also displayed eye-related phenotypes, proximal eye defects, ectopic formation of retinal pigment epithelium, and ectopic formation of eye and lens structures at high frequencies at stage 48. Following the same conditions, we injected *Notch* ΔE, *eyeless*, and *twin of eyeless* RNA into one animal blastomere at the 16-cell stage and allowed the embryos to develop until stage 48 (Fig. 3 and Table 2). As

shown in Fig. 3*D*, the activation of *Notch* signaling induced the proximal eye defects, which appeared as an extension of the endogenous RPE toward the midline. The phenotype was similar to that observed with overexpression of *Pax 6* and Rx in *Xenopus* embryos (15, 24). These results suggest that *Notch* signaling participates in the eye development via induction of *Pax 6* and Rx expression.

Induction of Eye-Related Gene Expression Including *Pax 6* in the Isolated Animal Caps of *Xenopus* by the Activation of *Notch* Signaling. In *Drosophila*, the expression of *eyeless* is restricted to the region anterior to the morphogenetic furrow in the eye part of wild-type eye-antennal discs (12). The ectopic expression of *eyeless* is induced in eye-antennal discs by the activation of *Notch* signaling resulting from the expression of a constitutively activated form of *Drosophila Notch* receptor (*N^act^*) in eye-antennal discs (29). To examine the potential role of *Notch* signaling on the induction of the *Pax 6* gene in vertebrates, we injected RNA for *Xenopus* Notch ΔE into both blastomeres of 2-cell *Xenopus* embryos and analyzed the expression of *Pax 6* in the animal cap ectoderm isolated at late blastula (stage 9). In this well established animal cap assay, the animal caps are not exposed to the inductive signals from the mesodermal tissues, and they develop as epithelia that fail to express eye-related genes including *Pax 6*. As shown in Fig. 4*B*, RT-PCR analysis revealed that the activation of *Notch* signaling induces the expression of two isomers of *Pax 6* in the isolated animal caps from 12 h after the isolation of animal caps. Almost no expression of *Pax 6* was observed in the animal caps isolated from uninjected control embryos. Next, we investigated whether the activation of *Notch* signaling induces other eye-related genes like N-CAM, *Six 3*, Rx, and β-crystallin under these conditions. In vertebrates, the homeobox genes Rx and *Six 3* are expressed in the neural plate and in the developing eyes (24, 39). Injection of Rx RNA into *Xenopus* embryos induces ectopic RPE (24). *Six 3* is a member of the *sine oculis* family and is sufficient to induce ectopic lens formation in medaka and zebrafish (40, 41). As shown in Fig. 4*C*, the activation of *Notch* signaling induced the expression of N-CAM, *Six 3*, and Rx but not the expression of β-crystallin in the isolated animal caps. No expression of N-CAM, *Six 3*, and Rx was observed in the animal caps isolated from uninjected embryos. A very weak or no expression of these genes was observed in the animal caps isolated from GFP encoding RNA-injected control embryos. These results indicate that the activation of *Notch* signaling is capable of inducing eye-related gene expression including *Pax 6* in the isolated animal caps of *Xenopus*.

Discussion

Evolutionary Conservation of the Function of *Pax 6* in Eye Development. In this paper, we demonstrate that the *Drosophila* homologs of *Pax 6*, *eyeless* and *twin of eyeless*, are sufficient to induce eye structures in *Xenopus* embryos, indicating that *Drosophila Pax 6* homologs are functional equivalents of vertebrate *Pax 6* in vertebrate eye development. Because *eyeless* and *twin of eyeless* induce the expression of endogenous *Pax 6* in the isolated animal caps, they presumably activate the positive feedback loop for *Pax 6*, which has been shown to be implicated in eye development. In *Drosophila*, the ectopic expression of mouse *Pax 6* in imaginal discs activates a similar positive feedback loop for *eyeless* that comprises *eyeless*, *sine oculis*, *eyes absent*, and *dachshund* (16, 20). Therefore, the function of *Pax 6* genes seems to be conserved in eye development of vertebrates and invertebrates. We found that both *Pax 6* homologs, *eyeless* and *twin of eyeless*, induce ectopic eye structures in *Xenopus* embryo. However, judging from the frequency of the ectopic induction of eye-related structures, *eyeless* has a stronger activity for the induction than *twin of eyeless*. *Eyeless* is capable of inducing ectopic lenses, whereas *twin of eyeless* failed to do so, even at higher concentrations. Corre-
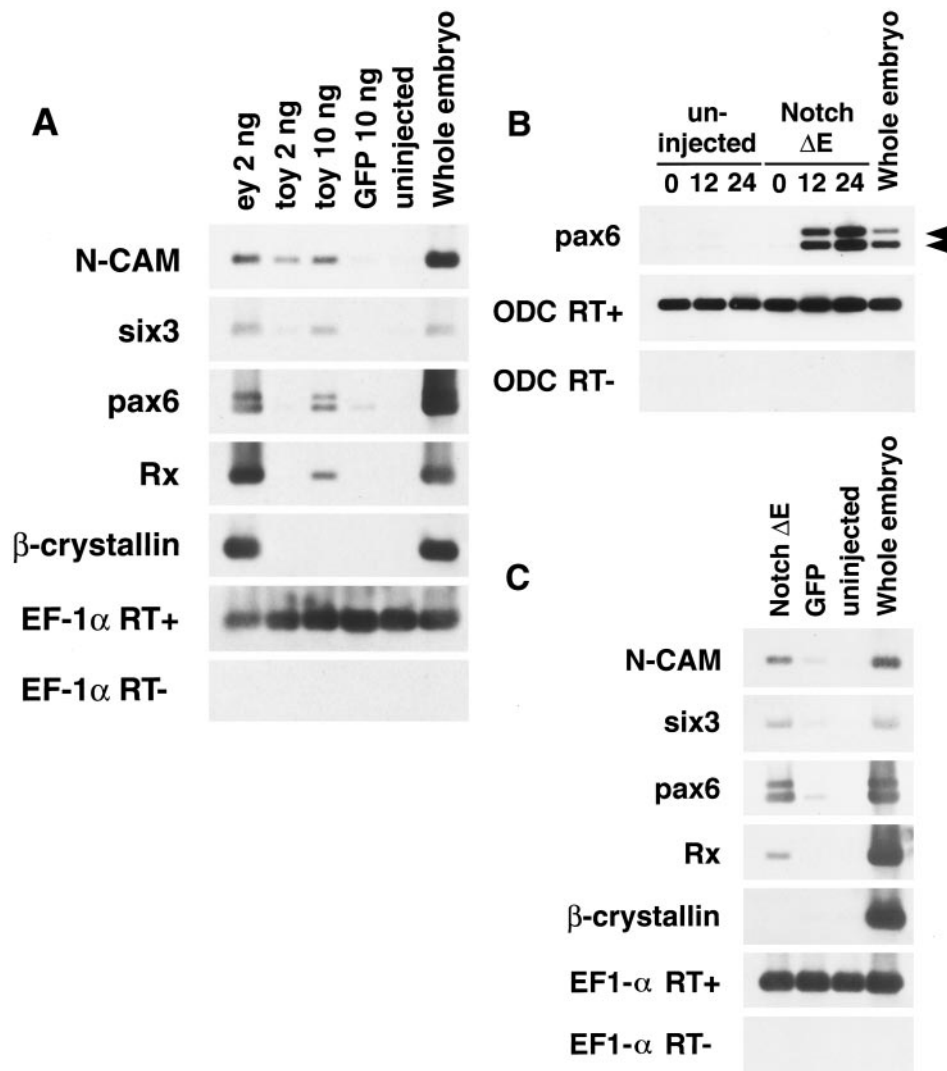
Fig. 4. Induction of eye-related gene expression including *Pax 6* in *Xenopus* animal caps by overexpression of *Drosophila Pax 6* genes, *eyeless* and *twin of eyeless*, and by activation of *Notch* signaling. (*A*) Induction of eye-related gene expression in the animal caps by the injection of *eyeless* and *twin of eyeless* RNA. Embryos were injected with 1 ng of *ey* and 1 and 5 ng of *toy* RNA into each of the two blastomeres at the 2-cell stage. Animal cap ectoderm was isolated at late blastula (stage 9) and processed for RT-PCR at the time when control embryos have reached stage 28. The misexpression of *ey* induces the expression of neural cell adhesion molecule (N-CAM), *Six 3*, endogenous *Pax 6*, Rx, and β-crystallin in the animal caps. *Toy* requires five times more RNA than *ey* to induce the expression of N-CAM, *Six3*, *Pax6*, and Rx, which is in agreement with our morphological analyses (Tables 1 and 2). The expression of β-crystallin was not induced in the animal cap by the injection of *toy* RNA. GFP 10 ng, 5 ng of green fluorescent protein RNA (total 10 ng) was injected into each of the two blastomeres at the 2-cell stage as a negative control. Uninjected, uninjected animal cap control. Whole embryo, whole embryo positive control. An elongation factor 1α (EF-1α) cDNA product was amplified as an internal loading control for animal caps. RT−, reverse transcriptase minus negative control. (*B*) Induction of *Pax 6* expression in the animal caps by the activation of *Notch* signaling. RNA encoding an activated form of *Xenopus* Notch receptor (*Notch* ΔE) was injected into both blastomeres of 2-cell stage embryos. Animal cap ectoderm was isolated at late blastula (stage 9) and processed for RT-PCR after 0, 12, and 24 h. The induction of *Pax 6* expression was observed after 12 h in the animal caps injected with *Notch* ΔE RNA (2 ng). Uninjected, uninjected animal cap control. Whole embryo, whole embryo positive control at the same stage of 24-h culture of animal caps. An ornitin dehydrocarboxylase cDNA product was amplified as an internal loading control for animal caps. RNA for noggin was injected as a positive control for the induction of *Pax 6* in animal caps. RT−, reverse transcriptase minus negative control. Arrow heads indicate two isomers of *Xenopus* Pax 6. (*C*) Induction of eye-related gene expression in the animal caps by the activation of *Notch* signaling. RNA (5 ng) for *Xenopus* Notch ΔE RNA was injected into both blastomeres of 2-cell stage embryos. Animal cap ectoderm was isolated at late blastula (stage 9) and processed for RT-PCR at the time in which control embryos show stage 28. The activation of *Notch* signaling induces the expression of N-CAM, *Six 3*, *Pax 6*, and Rx, but not the expression of β-crystallin in the animal caps. The pattern of the induction is similar to that of animal caps injected with 10 ng of *toy* RNA (*A*), in agreement with our phenotypical analyses (Table 1). GFP, 5 ng of green fluorescent protein RNA was injected into both blastomeres of 2-cell embryos as the negative control. Uninjected, uninjected animal cap control. Whole embryo, whole embryo positive control. EF-1α cDNA product was amplified as an internal loading control for animal caps. RT−, reverse transcriptase minus negative control.

sponding to these phenotypical analyses, in the animal cap assays *eyeless* also shows stronger activity for induction of eye-related gene expression than *twin of eyeless*. Particularly, β-crystallin expression was only induced by misexpression of *eyeless* and not by *twin of eyeless*. Overexpression of vertebrate *Pax 6* induces ectopic eye structures with lens and ectopic expression of

β-crystallin in *Xenopus* embryos (15). These observations indicate that *eyeless* is more closely related to vertebrate *Pax 6* with respect to eye induction than *twin of eyeless*. Because two *Pax 6* genes are only found in holometabolous insects and not in hemimetabolous or apterygote insects (28), the functional divergence of these genes seems to have occurred during insect

evolution. The *twin of eyeless* protein is more similar to the vertebrate *Pax 6* proteins than to the *eyeless* protein, particularly in its overall length and at the C-terminal region (16). However, the eyeless protein is more closely related in the paired domain to vertebrate *Pax 6* proteins than the *twin of eyeless* protein (16). Recently, we found that in *Drosophila* the paired domain of *eyeless* is essential for its ectopic eye induction, and the expression of a truncated *eyeless* protein lacking the homeodomain is sufficient to induce ectopic eyes (42). Therefore, the paired domain of *Pax 6* seems to be critical for its conserved function in eye induction.

Regulatory Mechanisms for Expression of *Pax 6* in Eye Development. We found that activation of *Notch* signaling is sufficient to induce *Pax 6* expression in the isolated animal caps of *Xenopus*. In the embryos, the activation of *Notch* signaling induces eye duplications and proximal eye defects that are also induced by misexpression of *Pax 6* as well as *eyeless* and *twin of eyeless*. These results suggest that *Notch* signaling participates in vertebrate eye development in a *Pax 6*-dependent manner. Overexpression of *Drosophila Pax 6* homologs induces ectopic eye structures in the head and trunk region, whereas activation of *Notch* signaling induces eye defects in a restricted region of the head around the original eyes, suggesting a context-dependent manner for *Pax 6*

induction by *Notch* signaling. In *Drosophila*, *Notch* signaling regulates the expression of several key regulators for eye, antenna, leg, and wing morphogenesis in a context-dependent manner (29). For example, the activation of *Notch* signaling induces *eyeless* and *twin of eyeless* in eye-antennal discs and induces hyperplasia of the original eyes, as well as ectopic eyes on the rostral membrane, which is derived from the antennal discs. In the presence of *Antennapedia* protein, a homeotic gene product specifying the identity of the second thoracic segment (43, 44), the activation of *Notch* signaling induces expression of *vestigial*, a key regulator for wing development (45), and ectopic wing structures on the head (29). These observations suggest the possible conservation of a regulatory mechanism for *Pax 6* gene expression in eye development of vertebrates and invertebrates. Consistent with our findings, Xu *et al.* (46) have reported that cis-regulatory elements of *Pax 6* genes are functionally conserved in vertebrates and invertebrates.

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