Specification of the vertebrate eye by a network of eye field transcription factors

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Summary

Several eye-field transcription factors (EFTFs) are expressed in the anterior region of the vertebrate neural plate and are essential for eye formation. The Xenopus EFTFs ET, Rx1, Pax6, Six3, Lhx2, tll and Optx2 are expressed in a dynamic, overlapping pattern in the presumptive eye field. Expression of an EFTF cocktail with Otx2 is sufficient to induce ectopic eyes outside the nervous system at high frequency. Using both cocktail subsets and functional (inductive) analysis of individual EFTFs, we have revealed a genetic network regulating vertebrate eye field specification. Our results support a model of progressive tissue specification in which neural induction then Otx2-driven neural patterning primes the anterior neural plate for eye field formation. Next, the EFTFs form a self-regulating feedback network that specifies the vertebrate eye field. We find striking similarities and differences to the network of homologous Drosophila genes that specify the eye imaginal disc, a finding that is consistent with the idea of a partial evolutionary conservation of eye formation.

Key words: Neural patterning, Eye field specification, Ectopic eye formation, Genetic network, Noggin, Otx2, ET, Rx1, Pax6, Six3, Lhx2, Tll, Optx2, Xenopus laevis, Transcription factor cocktails

Introduction

The first morphological evidence of eye formation in vertebrates is a bilateral expansion of tissue from the early forebrain to form the optic vesicles. It has been known for nearly 70 years, however, that presumptive eye tissue (eye field) exists prior to optic vesicle formation. In the salamander, a small piece of anterior neural plate can be isolated 6 hours prior to optic vesicle formation, and remarkably, after another 24 hours in vitro this tissue will transform into a single small but histologically normal eye (Lopashov and Stroeva, 1964). Modern molecular evidence shows that the eye anlage is specified at the neural plate stage when a group of eye field transcription factors, EFTFs, are expressed in the anterior neural plate. The EFTFs include ET, Rx1, Pax6, Six3, Lhx2, tll and Optx2 (also known as Six6). Genetic evidence clearly demonstrates the importance of these EFTFs in vertebrate eye formation. Mutations of Pax6, SIX3 and OPTX2 in human result in malformations affecting the eyes (Wawersik and Maas, 2000). The targeted, or spontaneous mutation of Pax6, Rx (Rax – Mouse Genome Informatics) Lhx2, Tll, SIX3 and SIX6 in mice, results in animals with abnormal or no eyes (Hill et al., 1991; Lagutin et al., 2003; Li et al., 2002; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001; Yu et al., 2000). Similar phenotypes have been observed when homologues ofSix3, Pax6, tll, Rx1 and Optx2 genes have been functionally inactivated in other vertebrate species (Carl et al., 2002; Chow et al., 1999; Hollemann et al., 1998; Isaacs et al., 1999; Loosli et al., 2001; Zuber et al., 1999). Not only are these EFTFs necessary for eye formation, but in some contexts they are also sufficient. Overexpression of Pax6, SIX3, REX and OPTX2 homologues can expand or induce eye tissues in the nervous system of vertebrates (Andreazzoli et al., 1999; Bernier et al., 2000; Chow et al., 1999; Chuang and Raymond, 2001; Loosli et al., 1999; Mathers et al., 1997; Oliver et al., 1996; Zuber et al., 1999).

Many of these EFTFs were originally identified as homologs of genes required for eye formation in Drosophila melanogaster. For example, Pax6 is a homologue of Drosophila eyeless and twin of eyeless (Quiring et al., 1994), and Six3 and Optx2 are homologues of Drosophila sine oculis (Oliver et al., 1995). The Drosophila genes, twin of eyeless (toy), eyeless (ey), eyes absent (eya), sine oculis (so), dachshund (dac), eye gone (eyg) and optix either induce ectopic eyes or are required for normal eye formation (Hanson, 2001; Heberlein and Treisman, 2000; Kumar, 2001; Wawersik and Maas, 2000). The expression patterns of toy, ey, so, eya, dac and eyg overlap in the Drosophila eye field during its specification (Kumar and Moses, 2001a). It has been proposed that the overlapping expression patterns of these genes drives eye specification and is regulated by the Notch and EGFR signaling systems (Kumar and Moses, 2001a). Dominant-negative Notch receptor blocks compound eye formation, while constitutively activate Notch induces ey and toy expression and ectopic fly eyes (Kurata et al., 2000). A role for Notch signaling in vertebrate eye formation is
Table 1. Primer sets used for PCR analysis

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Upstream primer (5' to 3')</th>
<th>Downstream primer (5' to 3')</th>
<th>Cycle number</th>
<th>Number of bp</th>
<th>Accession number/reference</th>
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<tr>
<td>ET</td>
<td>CCT ATC CTT GAC TGG CTA CA</td>
<td>GTT TGG GAG GAG GGT AT</td>
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<td>255</td>
<td>AF173940</td>
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<tr>
<td>Pax6</td>
<td>GCA ACC TGG CAG TGA AGC</td>
<td>CTT GCC GTC TCT GTC TCC GTA GTT</td>
<td>28</td>
<td>450</td>
<td>U76386</td>
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<tr>
<td>Six3</td>
<td>TTT TCT GTC TGC TCC TGG TT</td>
<td>TTT TGT GCT TGG TTT ACT TC</td>
<td>30</td>
<td>369</td>
<td>AF183571</td>
</tr>
<tr>
<td>Rx1</td>
<td>CCC CAA CAG GAG CAT TTA GAA GAC</td>
<td>AGG GCA CTC ATG GCA GCA GGT T</td>
<td>28</td>
<td>416</td>
<td>AF017273</td>
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<tr>
<td>tll</td>
<td>ACT TGC CTC TCG TGC TGC TCT ACT G</td>
<td>ATC CGG TCG TGC TGC TCA TCT T</td>
<td>30</td>
<td>351</td>
<td>U67886</td>
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<tr>
<td>Otx2</td>
<td>ACC CTC CTC CCC CAT TAC TCA C</td>
<td>AGG GCA TAT CTG GCG ATC TCC TA A</td>
<td>30</td>
<td>461</td>
<td>AX141037</td>
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<tr>
<td>Optx2</td>
<td>ACA GAG CAG CGG CAA AGA</td>
<td>GAG GGC TCC CTG GTG TGG TGG A</td>
<td>30</td>
<td>296</td>
<td>AF813352</td>
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<tr>
<td>NCAM</td>
<td>CAC AGT TCC ACC AAA TGC</td>
<td>GGA ATC AAG CGG TAC AGA</td>
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<td>Xenbase</td>
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<tr>
<td>Otx2</td>
<td>GGA TGG ATT TGT TAG ATC GTG C</td>
<td>CAC TCT CCG AGG TCA CTT CCC</td>
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<td>315</td>
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<td>XAG</td>
<td>GAC TGG TGC TGC TCA AAG TGG ACC</td>
<td>CAT TGG GAA ATA ACT GGG ACC</td>
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<td>U76752</td>
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<tr>
<td>H4</td>
<td>CGG GAT AAC ATG CAG GGT ACT ACT</td>
<td>ATC CAT GGC GGT AAC TGC CTT CCT</td>
<td>24</td>
<td>189</td>
<td>Hollemann et al., 1998</td>
</tr>
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</table>

Suggested by similar experiments. Mice homozygous for a hypomorphic Notch2 mutation have bilateral microphthalmia (McCrigh et al., 2001), while activation of Notch signaling induces the expression of Pax6, Six3 and Rx and causes eye duplications and ectopic eye tissue formation (Onuma et al., 2002).

In Drosophila, it has been possible using genetics to show that these genes act as a network with hierarchical components and multiple steps of feedback regulation including functional protein interactions (Chen et al., 1997; Pignoni et al., 1997). More recently, overexpression and inactivation studies have begun to shed light on the transcriptional network of EFTFs, that these genes act as a network with hierarchical components and multiple steps of feedback regulation including functional protein interactions (Chen et al., 1997; Pignoni et al., 1997). More recently, overexpression and inactivation studies have begun to shed light on the transcriptional network of EFTFs.

In this paper, we test the idea proposed by Kumar and Moses that the coordinated expression of EFTFs can also specify the vertebrate eye field. We find that EFTF cocktails not only induce ectopic eye fields in Xenopus, but generate ectopic eyes at high frequency outside the nervous system. In addition we provide an initial characterisation of the functional interactions among the EFTFs involved in vertebrate eye field specification.

Materials and methods

Animals

Fertilised eggs were obtained from pigmented Xenopus injected with 500 U of human chorionic gonadotropin (Sigma-Aldrich Company, UK) to induce egg laying. Embryos were dejellied with 3.3 mM DTT in 200 mM TrisHCL (pH 8.8) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

RNA microinjection

Capped RNA was synthesised in vitro from pCS2.Xnoggin, pCS2.XOptx2, pCS2.RX., RXpax6, pCS2.XSix3, pCS2+XRx1, pCS2.XLhx2 (3), pCS2+mt-X-tll, pCS2.XOptx2, pCS2.uxac/6 or pCS2GFP template DNA using the Message Machine kit (Ambion, Austin, TX). X-Gal staining was performed on embryos injected with 200 ng beta-gal as previously described (Turner and Weintraub, 1994). GFP was sometimes used (500 ng per embryo) in place of beta-gal to label injected embryos, when there was a concern that beta-gal staining would obstruct in situ staining.

RT-PCR analysis

For animal cap assays, embryos were injected at the two-cell stage with the indicated RNA(s). Ectodermal explants (animal caps) were isolated from stage 8.5 embryos using the Gasteromaster (XENOTEK Engineering, Belleville, IL). Total RNA was isolated from embryos or pools of ten stage 21 animal caps by extraction with RNAzol B reagent (Tel-Test, Friendswood, TX, USA). After treatment with RQ-1 DNase (Promega, Poole, UK) to remove contaminating genomic DNA, first-strand cDNA synthesis was performed by reverse transcription with random hexamers in a volume of 20 μl. Histone H4 PCR was performed using 1 μl of template in a final reaction volume of 12.5 μl to determine the relative amount of cDNA in each sample. Subsequent PCR was performed using normalised amounts of template. Cycling conditions were: 92°C, 2 minutes then 92°C, 45 seconds; 56 or 65°C, 45 seconds; 72°C, 45 seconds, for 24-30 cycles and ended with a single extension step of 72°C for 10 minutes. An annealing temperature of 65°C was used for the Optx2 primer set; all other primer sets were annealed at 56°C. The primers used are shown in Table 1. Radiolabelled PCR products were separated on 7% polyacrylamide gels, expression levels were determined using a Storm Phosphorimagerr with ImageQuant ver. 4.1 software (Molecular Dynamics, Sunnyvale, CA) and normalised to H4 as a loading control.

For multistage analysis, RNA was isolated from three embryos per stage and a total of four sets of RNAs from staged embryos were tested yielding similar results. For animal cap assays, each experiment was performed between three and five times to ensure reproducibility. Control experiments (not shown) with cloned templates demonstrated that the amplification efficiencies did not vary between primer sets.
using these conditions. A no reverse transcription control was included in each reaction to check for the presence of contaminating genomic and plasmid DNA. Subcloning and sequencing confirmed the identities of the amplified products.

cDNA identification and sequence analysis

XSix3 was isolated by screening a stage 42 head cDNA library (a gift from P. A. Krieg, University of Texas, Austin, TX) with an XSix3 PCR-amplified fragment that was obtained as previously described (Andreazzoli et al., 1999). Plating, hybridisation and washing conditions have been described previously (Franco et al., 1991). The XSix3 predicted amino acid sequence is identical to that described by Zhou and colleagues (Zhou et al., 2000). A full-length cDNA was cloned into the EcoRI/XhoI site of pBS(SK-) vector. A complete description of the cloning and sequence of the Xenopus Lhx2 will be given elsewhere (M.E.Z., unpublished). Xenopus Lhx2 sequence has been submitted to GenBank under Accession Number AY141037.

In situ hybridisation

Whole-mount single and double in situ hybridisation on Xenopus embryos was performed as previously described (Andreazzoli et al., 1999; Harland, 1991). Bleaching of pigmented embryos was carried out following color reaction as described by Mayor et al. (Mayor et al., 1995). To determine the change in eye field diameter, the injected side of embryos was first determined by staining for βgal expression

Results

Vertebrate EFTF expression is coordinated and suggests a genetic hierarchy

To determine the relative timing of vertebrate EFTF expression, we used RT-PCR to establish the developmental stage at which each is first and strongly expressed. Only Six3 is expressed at detectable levels in the egg (Fig. 1A). Early Six3 expression is transient and lost by stage 10.5. ET, Pax6, Rx1, tll, Lhx2 and Optx2 were first detected at stages 10, 10.5, 11, 11.5, 12 and 12.5, respectively. In contrast to the first detectable expression, strong expression of Pax6, Six3, Rx1, tll and Lhx2 is nearly simultaneous and starts between stages 12 and 12.5, while strong induction of ET and Optx2 occurs by stages 10.5 and 14/15, respectively. Some variation in the expression of these genes was observed from experiment to experiment (Fig. 1B). However, the relative timing of expression was consistent in each experiment. These results demonstrate a tightly coordinated, strong expression of five EFTFs within a 30 minute time span. In addition, these results suggest that: (1) ET expression does not require the expression of Pax6, Six3, Rx1, tll, Lhx2 or Optx2; and (2) Optx2 is not required for the initial expression of ET, Pax6, Six3, Rx1, tll or Lhx2.

The EFTFs are expressed in overlapping patterns during vertebrate eye field formation

Interactions suggested by the synchronised timing of EFTF expression could only operate if these factors were colocalised. Therefore, we used double whole-mount in situ hybridisation to determine the relative expression patterns of the eye field transcription factors.

We first compared the expression domains of these genes with Otx2, which is required for the establishment of presumptive forebrain and midbrain territories (Kablar et al., 1996; Pannese et al., 1995). Because the eye field originates within the forebrain, mice deficient in Otx2 lack eyes (Acampora et al., 1995; Matsuo et al., 1995). At gastrula stages, Otx2 is expressed in the entire presumptive anterior neuroectoderm (Fig. 2A), but between the end of gastrulation and the beginning of neurulation (stage 12.5/13) it is
downregulated in the medial region of its expression domain (Fig. 2B). This ‘hole’ in the Otx2 expression domain, is the approximate location of the eye field.

ET, Pax6, Six3, Rx1 and Lhx2 are all first detectable in the presumptive eye field before the completion of gastrulation and the beginning of neurulation (stage 12) (not shown). Rx1 is expressed neatly within the inner limits of the ‘hole’ in the Otx2 expression domain (Fig. 2C), and within the Rx1 domain are the even smaller expression domains of Lhx2 and ET (Fig. 2D,E). Both Pax6 and Six3 expression domains are slightly larger that the Rx1 domain and overlap that of Otx2 (Fig. 2F-I). To define the anterior and lateral expression boundaries of these genes more clearly, we used the homeodomain-containing transcription factor Emx1 as a positional marker. Emx1 is expressed in the rostral neural plate in the telencephalic primordium at early neural stages (Pannese et al., 1998). The expression domains of Rx1 and Emx1 do not overlap, although both Pax6 and Six3 overlie Emx1 expression confirming that the lateral expression of Rx1 (and therefore Lhx2 and ET) lies within both the Pax6 and Six3 expression domains (Fig. 2J-L). Although the Six3 expression domain clearly extends beyond the anterior limit of Emx1 (Fig. 2L), the most anterior limit of Pax6 expression is coincident with Emx1 expression confirming that the lateral expression of Rx1 (and therefore Lhx2 and ET) lies within both the Pax6 and Six3 expression domains (Fig. 2J-L). Both Emx1 and ET borders are indicated by a broken line. A schematic summary of the overlapping expression patterns of the eye field transcription factors at stage 12.5/13 (U) and 15 (V) is shown. Scale bars: in A, 300 μm for A-L; in M, 300 μm for M-T.

By midneurula stages (stage 14/15), tll and Optx2 expression can be detected by WISH. tll is first observed in a narrow stripe of cells in the prechordal region of the neural plate. As described by Holleman et al. (Holleman et al., 1998), the expression domain of tll overlaps the posterior and lateral Pax6 expression domain (Fig. 2M), distinct from the eye field. By contrast, Six3 expression overlaps tll expression medially (Fig. 2N). The expression domains of Rx1, ET and Optx2 closely border, but do not significantly overlap the expression domain of tll (Fig. 2O-Q). These results suggest that tll is unlikely to be required for eye field specification as it is expressed after the eye field forms and only partially overlaps the eye field region. Optx2 transcripts are detected within the Pax6, Six3, Rx1 and Lhx2 expression domains (Fig. 2R-T and not shown).

Clearly, some of the EFTFs are expressed outside the definitive eye field, consistent with the roles of genes like Pax6 and Six3 in the development of other nearby structures, such as the olfactory epithelium and the hypothalamus (Lagutin et al., 2003; Oliver et al., 1995; Van Heyningen and Williamson, 2002). Within the eye field – the expression patterns of the EFTFs are dynamic and follow the morphogenesis of the neural plate, including the lateral migration of the eye field as it begins to separate. This is illustrated by comparing their expression patterns at stage 12.5/13 and stage 15 only 3 hours later (Fig. 2U,V). These results demonstrate that the anterior neural plate is subdivided into molecularly distinct domains that express specific subsets of the EFTFs.

Fig. 2. Comparison of EFTF expression patterns by double whole-mount in situ hybridisation. Otx2 expression at stage 12 (A) and 13 (B). In C-I and K-T, the dark blue stain is the expression pattern of the gene named on the left, while the magenta stain is the expression pattern of the gene named on the right, at the stages shown. For example, in C, Otx2 is dark blue and Rx1 is magenta. (J) Both Emx1 and Rx1 stain dark blue. (J-L) The Rx1 (J), Pax6 (K) and Six3 (L) expression borders are indicated by a broken line. A schematic summary of the overlapping expression patterns of the eye field transcription factors at stage 12.5/13 (U) and 15 (V) is shown. Scale bars: in A, 300 μm for A-L; in M, 300 μm for M-T.
The coordinated overexpression of EFTFs is sufficient to generate secondary eye fields and ectopic eyes outside the nervous system

To determine if the coordinated expression of EFTF genes is sufficient to generate eye fields and eyes in vertebrates, we expressed a cocktail of seven of the EFTFs in developing *Xenopus* embryos. We injected *Otx2*, *ET*, *Pax6*, *Six3*, *Rx1*, *tll*, *Optx2* and β-gal RNAs simultaneously into one blastomere at the two-cell stage with β-gal to identify the injected side of the embryo. *Lhx2* was intentionally left out of the cocktail, as we needed an early marker to identify the presence of ectopic eye field. Preliminary experiments demonstrated that the absence of *Lhx2* from the cocktail had little effect on the observed phenotypes. Coordinated expression of the EFTF cocktail induced ectopic expression of *Lhx2* in 100% of injected embryos. Ectopic *Lhx2* was detected both within and outside the nervous system (Fig. 3B-D), whereas its normal expression domain is limited to the anterior neural plate (Fig. 3A). When the injected embryos were grown to stage 45, we found ~90% of these embryos expressed ectopic retinal pigment epithelium (RPE) on the injected side. Sections taken through this ectopic tissue and immunostained for opsin, revealed that photoreceptors were often associated with the ectopic pigment. Approximately 20% of injected embryos clearly developed quite large ectopic eyes, the most striking aspect of which was their location. Ectopic eyes were detected near the CNS, but were also often found at locations far from the CNS, e.g. in the belly region and even at the anus (Fig. 3E-H). These tissues expressed markers for differentiated retinal ganglion, rod and cone photoreceptor cells, RPE and lens (Fig. 3I-J and not shown), indicating that they were indeed eyes as defined by the cell types detected as well as their morphology.

Cocktail subsets reveal crucial circuit components

The high efficiency with which cocktails of EFTFs generate ectopic eye tissue enabled us to determine those most crucial for eye formation. To do this, we systematically injected cocktail subsets lacking one of the EFTFs and determined their efficiency at inducing ectopic eye tissues. The most dramatic reductions in ectopic eye tissue were observed when *Pax6* was
removed (Fig. 3K), followed by Otx2, Six3 and ET. Removal of Rx1, Optx2 or tll from the EFTF cocktails affected ectopic eye tissue induction to a lesser extent (Fig. 3K). The strong effect of removing Pax6, Otx2 and Six3 from the cocktails may have been predicted, as numerous studies have demonstrated these genes are required for eye formation. However, a crucial role for ET in early eye formation has not been reported. Conversely, the relatively small effects of removing Rx1, Optx2, tll and Lhx2 from the cocktails is intriguing because each of these genes has been shown to be required for normal eye formation. Remembering that this is non-mutant tissue, a possible explanation is that Rx1, Optx2, tll and Lhx2 can be induced to sufficient levels by the remaining EFTFs – compensating for their removal. The strong ectopic expression of Lhx2 seen in embryos injected with EFTF cocktails (Fig. 3B-D) certainly supports this hypothesis. The genetic hierarchy

**Fig. 4.** Noggin but not Otx2 regulates eye field transcription factor expression while Otx2 blocks the repression of ET by noggin. (A) RT-PCR was used to detect changes in the expression of the EFTFs in response to noggin (10 pg) and Otx2 (200 pg). The effect of Otx2 on its own expression was not determined (ND). The presence of ET and Six3 in uninjected animal caps (U) was not a result of DNA contamination as neither transcript was detected when duplicate samples were amplified in the absence of reverse transcriptase (–RT). Uninjected sibling embryos ‘E’ were used as a positive control for PCR. Histone H4 was used as a loading control. (B) RT-PCR was used to determine the relative expression of ET in ectodermal explants from embryos injected with Otx2, noggin or both. The percent of ET expression relative to un.injected controls is shown above each bar of the graph. (C) Interpretation of the combined results from A and B.

**Fig. 5.** ET, Rx1 and Pax6 regulate Otx2 expression. Embryos were injected into one blastomere at the two-cell stage with RNA of the indicated gene. Whole-mount in situ hybridisation was used to detect Otx2 expression in embryos injected with 100 pg ET (B), 400 pg Rx1 (C), 200 pg Pax6 (D), 200 pg Six3 (E) or 500 pg Lhx2 (F) RNA. Embryos in A,D-F were co-injected with βgal RNA to identify the injected side. In B and C, the embryos were not stained for βgal expression so that the repression of Otx2 could be more easily visualised. Scale bar: 300 μm. (G) Quantitation of the effect of EFTFs on Otx2 expression. Percent of embryos with an increase (↑), decrease (↓) or no change (NC) in Otx2 expression. ET induces Rx1 expression. (H,I) Rx1 injection did not effect ET expression, while ET induced Rx1 expression in Xenopus animal caps in a dose-dependent manner. Histone H4 was used as a loading control; U, uninjected; E, parallel, uninjected embryo. (J-M) Whole-mount in situ hybridisation was used to detect ET (J-K) and Rx1 (L-M) expression in stage 13 Xenopus embryos injected with 200 pg Rx1 (K) or ET (M) RNA. In (J,K), embryos were injected with βgal RNA. In L,M, GFP RNA was used to detect the injected side of the embryo. The right side is the injected side in J-M. Scale bar: 300 μm. (N) Interpretation of the results of Figs 4, 5.
suggested by the timing of EFTF expression (Fig. 1) is also consistent with this idea as the four EFTFs that are deemed most crucial by the cocktail subset method are expressed earlier than, and may therefore induce the expression of, Rx1, Otx2, tll or Lhx2.

**EFTFs are induced by the combined action of noggin and Otx2**

The above results suggest that eye field formation might result from a series of progressive inductions. Extending this hypothesis prior to eye field specification, the ectoderm is converted into the neural plate in response to neural inducers. Next, presumptive forebrain is specified by the regulated expression of Otx2. Finally, the eye field forms within the presumptive forebrain. If this model were correct, one would expect that both noggin and Otx2 are upstream of the EFTF genes, and may activate them either directly or indirectly. We therefore used the animal cap assay to test the effect of noggin and Otx2 on the expression of the EFTFs.

In untreated animal caps, only ET and Six3 were detected (U, Fig. 4A), consistent with their early expression in the embryo (Fig. 1). The neural inducer, noggin, dramatically increased the expression of many of the eye field transcription factors including Pax6, Six3, Rx1, Lhx2, tll and Optx2. Interestingly, noggin strongly repressed ET expression. Similar results were found with another neural inducer, chordin (data not shown). Both noggin and Otx2 induced the neural marker NCAM and the cement gland marker XAG. Unlike noggin, however, Otx2 did not alter the expression of ET, Pax6, Six3, Rx1, Lhx2, tll or Optx2. The inability of Otx2 to induce any EFTFs suggests that their regulation is Otx2 independent.

The strong repression of ET by noggin and chordin, however, raised the question of how the initial expression of ET is turned on in the eye field. We therefore considered the possibility that Otx2 inhibits the repression of ET by noggin. To test this idea, noggin mRNA was injected with and without Otx2 mRNA, and the effect on ET expression was determined in animal caps. Otx2 alone had no effect on ET, while noggin repressed ET expression to 15% of control levels (Fig. 4B). However, co-expression of Otx2 with noggin not only rescued ET expression, but increased ET levels beyond that of controls in a dose-dependent manner. These results indicate a potentially crucial role for Otx2 in eye field formation. Neural induction alone results in a neural plate resistant to ET expression; however, subsequent expression of Otx2 in the anterior neural plate permits ET expression and perhaps subsequent eye field formation (Fig. 4C).

**ET, Rx1 and Pax6 regulate Otx2 expression in the anterior neural plate and presumptive eye field**

The loss of Otx2 expression between stages 12 and 13 is synchronised with the induction of several EFTFs in the eye field (Fig. 2), suggesting that they may repress Otx2 expression in the anterior neural plate during normal embryonic development. It was, in fact, previously shown that overexpression of Rx1 represses Otx2 expression in the neural plate (Andreazzoli et al., 1999). To test if other EFTFs are also capable of regulating Otx2 expression, we injected the EFTFs into one cell of two-cell stage embryos and determined their effect on Otx2 expression using whole-mount in situ hybridisation.

Otx2 expression normally extends both rostral to the neural plate, in a region corresponding to the cement gland anlagen, and posterior to the eye field, in the region fated to be the primordium of the mesencephalon (Eagleson et al., 1995), Fig. 2B, Fig. 5A). We found that both ET and Rx1 repressed Otx2 expression throughout the entire anterior neural plate (Fig. 5B,C). Otx2 expression was repressed in 100% of embryos injected with ET RNA and 94% of embryos injected with Rx1 RNA (Fig. 5G). In 74% of embryos injected with Pax6, there was an expansion of the Otx2 expression domain (Fig. 5D,G). Interestingly, Otx2 expression was expanded laterally and caudally but not into the eye field by Pax6 (Fig. 5D). Neither Six3 nor Lhx2 altered Otx2 expression (Fig. 5E,F). These results demonstrate that both ET and Rx1 are able to repress the expression of Otx2 in the eye field region.

As ET is expressed before Rx1, it is possible that the repression of Otx2 by ET is indirect – mediated through Rx1. To test this possibility, we first used the animal cap assay to determine the effect of ET and Rx1 on each other’s expression. Rx1 neither induced nor repressed ET expression in animal caps at concentrations as high as 1000 pg (Fig. 5H). However, ET strongly induced the expression of Rx1 in a dose-dependent manner (Fig. 5I).

To test this pathway in vivo, we injected ET or Rx1 RNA and assayed for changes in Rx1 or ET expression in stage 13 embryos. To target the eye field, we injected ET or Rx1 RNA into dorsal blastomeres at the four-cell stage. Consistent with the animal cap assays, Rx1 had no effect on ET expression at stage 13 (compare Fig. 5J-K). As predicted, ET strongly induced the expression of Rx1 in 93% of injected embryos (n=29). Interestingly, Rx1 induction was only observed in the anterior neural plate in the presumptive eye field region (Fig. 5M). When ET was injected ventrally, Rx1 induction was not observed (not shown). When ET was injected at the two-cell stage (resulting in the expression of ET throughout an entire half of the embryo), Rx1 induction was again only detected in the anterior neural plate, including the presumptive eye field region (not shown).

The downregulation of Otx2 in the eye field can thus be explained by the fact that ET induces the expression of Rx1, which then represses Otx2 (Fig. 5N). However, these results, do not rule out the possibility that ET also represses Otx2 independently of Rx1. In fact, there is some indication that this pathway may also be operative as induction of Rx1 by ET was detected in most but not all embryos while ET repressed Otx2 expression in all embryos tested. In addition, we found that in the presumptive cement gland region, ET represses Otx2 (Fig. 5B) without inducing Rx1 (Fig. 5M), implicating an Rx1-independent mechanism in this tissue.

**Both Otx2 and noggin potentiate functional interactions between the EFTFs.**

The inducing effect of ET on Rx1 is limited to the anterior forebrain, suggesting that Otx2, although not an inducer of EFTFs itself, may provide an environment that primes the anterior neuroectoderm for eye field formation (Fig. 4). If so, co-injection of Otx2 might potentiate the effects of ET on the activation of downstream EFTFs. ET is an obvious candidate for co-injection experiments as it is expressed earlier than all other EFTFs and has the most restricted expression domain.

As predicted by the above model, Otx2 strongly potentiates the induction of Rx1 by ET in the animal cap assay (Fig. 6A).
This is in spite of the fact that Otx2 alone does not induce Rx1 expression in vitro. The effect is dose dependent as co-injection of 50 and 100 pg of Otx2 with ET induced a three- and eleven-fold increase in Rx1 expression. Noggin also potentiates the Rx1 induction by ET. Weak Rx1 expression was detected in animal caps from embryos injected with suboptimal amounts of ET or noggin. However, co-injection of noggin and ET RNAs at these same concentrations induced a greater than five-fold increase in Rx1 expression (Fig. 6B).

We next examined the effect of Otx2:ET co-injection on Rx1 expression in vivo. Otx2 (25 pg) only slightly increased Rx1 expression in stage 13 embryos (Fig. 6D,G), while ET (10 pg) expanded the average domain of Rx1 expression by 15% (Fig. 6E,G). However, co-injection of Otx2 with ET increased the average eye field diameter by nearly 35% (Fig. 6F,G), approximately equivalent to the effect of 25 pg of ET alone (Fig. 6G). These results are consistent with the model of progressive tissue specification described above.

The circuitry of the EFTF network revealed by systematic overexpression studies in animal caps

The relative timing, spatial expression patterns, cocktail subset experiments and directed overexpression studies suggest that early genes such as ET may be required for the expression of later expressed genes in the eye field and rule out the possibility that later genes such as Optx2 are required for the initial induction of earlier EFTFs. Nevertheless, dominant-negative Optx2 and tll constructs and Optx2 knockouts retard eye field growth and thus lead to reduced levels of other EFTFs, including ET and Pax6 (Hollemann et al., 1998; Li et al., 2002; Zhu et al., 2002; Zuber et al., 1999). Because of the possibility of such indirect feedback effects on EFTF expression, it is difficult to unravel the EFTF network using a loss-of-function approach alone, especially when the initial expression of these genes in the eye field is so closely synchronised. To overcome this problem, we used a systematic approach, injecting embryos with one EFTF at a time, and screening the injected caps using RT-PCR to detect changes in the expression of the remaining EFTFs.

A representative experiment and a summary of our results are shown in Fig. 7A,B. This data was then assembled into a circuit using Occam’s razor (Fig. 7C) that shows the most parsimonious set of necessary interactions needed to explain the results. These results confirm many of the predictions made.
from the expression studies (Figs 1, 2) and incomplete cocktail experiments (Fig. 3). ET, positioned at the front of the circuit induces the expression of Rx1, Lhx2 and tll, which in turn induce the expression of Pax6: Lhx2:tll:Optx2, Pax6 and Pax6:Six3:Lhx2, respectively. However, ET is unique in that none of the EFTFs studied here can induce its expression in the animal cap assay. Conversely, Otx2, the last of these genes to be expressed during eye formation, is induced by both Pax6 and Rx1, yet is unable to induce any of the earlier expressed EFTFs. The four EFTFs expressed earliest and deemed most crucial by the incomplete cocktail method are not only situated towards the front end of the circuit, but are also factors like Pax6, Six3 and Otx2 that previous studies have demonstrated are central to eye formation. Pax6 and Six3 induce each other’s expression as well as that of Lhx2 and tll. Pax6 also induces Otx2, Lhx2 and tll were induced by five and four of the six EFTFs, respectively, confirming the hypothesis that a sufficient amount of these genes could be induced by the remaining EFTFs to compensate for their removal from the eye inducing cocktails. In summary, ET at the front of the circuit induces Rx1, which activates a crossregulatory network, including Pax6, Six3, Lhx2 and tll, followed by Otx2 induced by Pax6.

Additional interactions may also exist and are not ruled out by the present data set. The working model illustrated in Fig.
7C forms a framework that further experiments may build on to define more precisely the genetic interactions required for vertebrate eye formation.

Discussion

Eye specification in flies and vertebrates

Kumar and Moses proposed a two-stage mechanism for *Drosophila* eye specification. First the eye-antennal imaginal disk complex is subdivided into two distinct presumptive organ fields. Next, specific organ identities (eye versus antenna) are defined. In the embryo prior to eye specification, *toy, ey, so, eya, dac* and *eya* have only partially overlapping expression patterns. However, at the second larval stage their expression patterns are coordinated and the eye becomes specified (Kumar and Moses, 2001a). In the vertebrate, we have shown that inductive and patterning events prepare the anterior neural plate for formation of the vertebrate eye field. However, it is the coordinated expression of the EFTFs that is required for the specification of the eye field. We find this to be a remarkable example of mechanism conservation, given the large evolutionary distance between these species and the differences in the development and morphology of fly and vertebrate eyes.

In the fly, *toy, ey, so, eya, dac* and *eya* are co-expressed in the second larval stage and the elimination of any of them reduces the probability of eye formation (Kumar and Moses, 2001a). In *Xenopus*, *ET, Rx1, Pax6* and *Six3* are co-expressed in the anterior neural plate and the elimination of any of them from a cocktail of EFTFs injected into the *Xenopus* embryo reduces the frequency of ectopic eye tissue formation.

These remarkable similarities in general developmental design are perhaps logically predicated based on the functional and structural homologies between the *Drosophila* eye genes and the vertebrate EFTFs (Hanson, 2001; Wawersik and Maas, 2000). *orthodenticle* (*otd*) the *Drosophila* homolog of *Otx* genes is required for development of the eye, antenna and anterior brain, and is normally expressed in a wide domain that spans the dorsal midline and encompasses the entire dorsal anterior brain, and is normally expressed in a wide domain that spans the dorsal midline and encompasses the entire dorsal anlagen, later expanding to a larger part of the optic lobes (Finkelstein, 1996). This is strikingly similar to the changes we observe in the *Xenopus Otx2* gene is a member of the *T-box* subfamily. *ET* homolog is not expressed in the larval eye imaginal discs nor the embryonic eye primordium (Eggert et al., 1998; Mathers et al., 1997). However, it is expressed prior to *ey* in the procephalic region from which the eye primordia originates, suggesting a role for *Drosophila Rx* prior to *ey* during eye formation in the fly (Eggert et al., 1998; Mathers et al., 1997). It has therefore been suggested that *Drosophila Rx* may only be required for early brain development (Eggert et al., 1998). Finally, our results showing *Pax6* as the most critical component of the *Xenopus* EFTF cocktail with respect to the induction of ectopic eyes meshes well with the general prominence given to *Pax6* and its *Drosophila* homologues *ey* and *toy* as transcription factors centrally involved in early eye development (Wawersik and Maas, 2000).

The functional interactions among the genes required for *Drosophila* eye formation have been extensively investigated (Heberlein and Treisman, 2000; Kumar and Moses, 2001b). Using the ectodermal explant assay, we identified functional epistatic interactions among the vertebrate EFTFs. There are some striking similarities with the functional interactions among the fly EFTFs. For example, we see induction of *Six3* and *Optx2* by *Pax6* and induction of *Pax6* by *Six3* in ectodermal explants (Fig. 7B). In *Drosophila, ey* can induce ectopic *so* and *optix* expression and ectopic eye formation induced by co-expression of *so* with *eya* results in the activation of the *ey* gene (Halder et al., 1998; Niimi et al., 1999; Pignoni et al., 1997; Seimiya and Gehring, 2000).

Some differences between fly and vertebrate eye formation are also evident. We found that *tll* was able to induce the expression of *Pax6, Six3* and *Lhx2*, and that *Pax6* and *Six3* induce *tll* expression. *Drosophila tll* does not require *ey* or *so* in the embryonic visual system (Daniel et al., 1999; Rudolph et al., 1997). We found *Lhx2* to be induced by all the EFTFs investigated in this report with the exception of *Optx2* (Fig. 7A,B). The gene *apterous* (*ap*) is the most homologous *Drosophila* gene to *Lhx2*; however, *apterous* loss-of-function mutants have no reported defect in eye formation (Bourgouin et al., 1992; Cohen et al., 1992; Lundgren et al., 1995).

Vertebrate EFTFs and their functions

It is interesting to examine the results of this paper in light of studies, particularly knockout studies, on specific EFTFs in other vertebrates. *Otx2* mice lack forebrain and midbrain (Acampora et al., 1995; Matsuo et al., 1995). In *Xenopus*, anterior structures are also lost when *Otx2* fused to the engrailed transcriptional repressor is expressed in embryos (Isaacs et al., 1999). An early requirement for *Otx2* in vertebrate eye formation is implied from studies in which *Pax6, Six3, Optx2* or *Rx* overexpression result in the formation of ectopic eye tissues, because as Chuang and Raymond observed, ectopic eye tissue is only generated in the head region defined by *Otx2* expression (Chuang and Raymond, 2002). Using EFTF cocktails, we were able to generate ectopic eyes outside of the nervous system (Fig. 3). *Otx2* clearly potentiates the functional interaction among the EFTFs and is a crucial component of the mix (Figs 3, 6). However, a more detailed analysis will be required to determine if ectopic eye formation outside the nervous system is a result of including *Otx2* in the cocktail.

Our results suggest a role for *ET* as an initiator of eye field specification. Originally identified as a T-box family member expressed very early in the eye field, (Li et al., 1997), subsequent overexpression studies showed that *ET* is involved in the dorsoventral patterning of the eye (Wong et al., 2002). The more than 50 T-box family members identified have been classed into five subfamilies (Papaioannou and Silver, 1998; Wilson and Conlon, 2002). *ET* is a member of the *Tbx2* subfamily that includes the *Tbx2, Tbx3, Tbx4* and *Tbx5* genes,
and is most similar to Tbx3. The mouse and chicken orthologues of Tbx2, Tbx3 and Tbx5 are all expressed in overlapping domains within the dorsal neural retina of the embryonic optic cup (Chapman et al., 1996; Gibson-Brown et al., 1998; Sowden et al., 2001) – very similar to the expression pattern of Xenopus ET (Li et al., 1997; Takabatake et al., 2000). Evidence of a role for Tbx3 in mammalian eye formation is limited. Mouse Tbx3 has been detected in preimplantation embryos as early as E9.0 (Zhang et al., 2000). These results are neither Pax6 eye formation and is upstream of Tbx3 locus (Loosli et al., 2001). Rx3 is required for evagination and proliferation of the optic vesicle. In medaka Rx3 mutants, both Tbx2 and Tbx3 expression in the retina is lost, suggesting that Rx3 is genetically upstream of these genes or that Tbx2/3 are expressed in tissues lost or re-patterned in Rx3 mutants (Loosli et al., 2001). This is in contrast to our results, which show Rx1 is downstream of ET.

Two Rx homologues have been reported in Xenopus (Rx1 and Rx2) and medakafish (Rx2 and Rx3), while three have been identified in zebrafish (rx1, rx2 and rx3) (Casarosa et al., 1997; Chuang et al., 1999; Loosli et al., 2001; Mathers et al., 1997; Winkler et al., 2000). Medaka fish Rx3 shares greater sequence homology with Xenopus XRx2 than XRx1 (not shown). In medaka Rx3 mutants, Rx2 expression is unaffected and morphogenetic movements are normal until optic vesicle evagination, Rx2-positive retinal tissue forms and the separation of the single retinal field into the two eye primordial is unaffected (Winkler et al., 2000). Medaka fish Rx2 is exclusively expressed in presumptive and differentiated retinal tissue during and after gastrulation (Loosli et al., 2001; Mathers et al., 1997). These results suggest that medaka fish Rx2, or an as yet unidentified medaka Rx homolog is acting as the Rx1 functional homologue in Xenopus.

Rx–/–, Pax6+/+, Lhx2+/+ and Six3+– mice all lack eyes (Grindley et al., 1995; Mathers et al., 1997; Porter et al., 1997; Tucker et al., 2001), but the morphological defects seen in these embryos also give clues to the order in which they are required during eye formation. Rx–/– embryos do not develop optic sulci, vesicles or cups, which normally form between stages E8.5 and E9.5 (Zhang et al., 2000). Pax6+– (Sey) and Lhx2+/– mice, however, do develop optic vesicles, which form optic stalks and rudimentary optic cups (Grindley et al., 1995; Porter et al., 1997). In Pax6–/– animals, Rx1, Six3 and Lhx2 expression is unaffected as late as E10.5 (Bernier et al., 2001; Zhang et al., 2000). Recently, Lagutin and colleagues demonstrated a requirement for Six3 during forebrain development (Lagutin et al., 2003). Six3+– mice die at birth, and lack head structures anterior to the midbrain, including the eyes. Mouse Six3 expression is first detected at E7.0 to E7.5 in the anterior neuroectoderm and the first morphological abnormalities in Six3–/– mice are seen at E8.0. Rx1 expression, although significantly reduced, is still detected at E8.5 in the anterior neural plate of Six3-null animals, demonstrating that early Rx1 expression does not require Six3. By contrast, neither Rx1 nor Pax6 is detectable at optic vesicle stages, as these structures do not develop. Interestingly, we also detect Six3 expression prior to eye field formation in the frog. Xenopus Six3 is detected weakly until stage 9, is lost, and then increases dramatically during eye field specification (Fig. 1). Perhaps there results point to a twofold role for Six3 in eye formation – an early neural patterning function then as a component of the self-regulating network responsible for eye field specification. Our animal cap analysis indicates that Optx2 does not regulate the expression of any of the EFTFs, consistent with the observation that Pax6, Six3 and Rx expression are normal in the small eyed Six6–/– mouse (Li et al., 2002). Dominant-negative tll constructs inhibit the growth of the optic vesicle in Xenopus (Holleman et al., 1998), and tll–/– mice show signs of retinal degeneration 3 weeks after birth that eventually result in visual defects (Yu et al., 2000). Our results similarly argue that tll and Optx2 are not involved in the earliest steps of eye field formation, but that ET, Rx1, Pax6, Six3 and Lhx2 are part of a self-regulating network of nuclear factors in vertebrates that helps specify the eye field.

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