Convergence of a head-field selector Otx2 and Notch signaling: a mechanism for lens specification

Hajime Ogino, Marilyn Fisher and Robert M. Grainger*

Xenopus is ideal for systematic decoding of cis-regulatory networks because its evolutionary position among vertebrates allows one to combine comparative genomics with efficient transgenic technology in one system. Here, we have identified and analyzed the major enhancer of FoxE3 (Lens1), a gene essential for lens formation that is activated in the presumptive lens ectoderm (PLE) when commitment to the lens fate occurs. Deletion and mutation analyses of the enhancer based on comparison of Xenopus and mammalian sequences and in vitro and in vivo binding assays identified two essential transcriptional regulators: Otx2, a homeodomain protein expressed broadly in head ectoderm including the PLE, and Su(H), a nuclear signal transducer of Notch signaling. A Notch ligand, Delta2, is expressed in the optic vesicle adjacent to the PLE, and inhibition of its activity led to loss, or severe reduction, of FoxE3 expression followed by failure of placode formation. Ectopic activation of Notch signaling induced FoxE3 expression within head ectoderm expressing Otx2, and additional misexpression of Otx2 in trunk ectoderm extended the Notch-induced FoxE3 expression posteriorly. These data provide the first direct evidence of the involvement of Notch signaling in lens induction. The obligate integration of inputs of a field-selector (Otx2) and localized signaling (Notch) within target cis-regulatory elements might be a general mechanism of organ-field specification in vertebrates (as it is in Drosophila). This concept is also consistent with classical embryological studies of many organ systems involving a ‘multiple-step induction’.

KEY WORDS: Competence, Genomics, Induction, Lens, Notch, Xenopus

INTRODUCTION

Although many signaling factors and transcriptional regulators are essential for organ formation in vertebrates, little is known about how these multiple inputs are integrated to generate the specific locations and identities of particular organs. This is mainly because of the laborious nature of analysis of the cis-regulatory elements on which transcriptional and signaling inputs directly converge; enhancer searching by traditional genome walking is a slow and painstaking process. However, recent remarkable advances in genome analysis tools have revolutionized the process (Wasserman and Sandelin, 2004). Putative enhancers are predicted in silico within entire genomes as clusters of transcription factor-binding motifs and/or intergenic sequences conserved among related species. Especially in vertebrates, enhancer activities are often mapped to conserved non-coding elements associated with developmental regulatory genes (Woolfe et al., 2005).

From this perspective, we have investigated mechanisms of lens induction in Xenopus, where essential tools for genomics-based cis-regulatory analysis, i.e. the whole genome sequence (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) and a highly efficient transgenesis technique, have become recently available (Offield et al., 2000; Smith, 2005). The relatively large evolutionary distance between Xenopus tropicalis and mammalian genomes (350 million years) allows sequence comparison to highlight the most conserved elements involved in vertebrate genomic regulatory networks (Muller et al., 2002).

Embryological studies have shown that lens induction is a stepwise process that begins when a broad domain of the animal cap ectoderm acquires a lens-forming competence at mid-gastrula stages (Grainger, 1992). This lens-competent field is subsequently narrowed down to the non-neural ectoderm surrounding the anterior margin of the newly formed neural plate. Accompanying this field-restriction process, a lens-forming bias is established in this region by planar signals provided by the adjacent anterior neural plate. This biased region corresponds to (or includes) the part of the non-neural ectoderm termed pre-placodal ectoderm, which will give rise to the lens, otic, olfactory and adenohypophysial placodes at later stages. After neural tube formation, only the lateral part of the lens-biased ectoderm makes contact with the developing optic vesicle and at this stage determination occurs, followed several hours later by differentiation into lens tissue (lens placode and, subsequently, the lens vesicle). This stepwise commitment process is likely to be mediated, at least in part, by several transcription factor genes that exhibit distinct but overlapping expression patterns during the course of lens-field specification (Ogino and Yasuda, 2000). Otx2, the earliest of these genes, exhibits expression from the end of gastrulation in the pre-placodal ectoderm and the adjacent anterior neural plate (Blitz and Cho, 1995; Zygar et al., 1998). Pax6 and Six3 show more-restricted expression in the lens-field within the pre-placodal ectoderm (Zhou et al., 2000; Zygar et al., 1998). After neural tube formation, their expression is followed by activation of lens-specific transcription factor genes such as MafB, L-maf and Ptx3, exclusively in the presumptive lens ectoderm (PLE) overlying the optic vesicle (Ishibashi and Yasuda, 2001; Pommerreit et al., 2001).

The expression of Otx2, Pax6 and Six3 implies their involvement in the establishment of lens bias, and recent significant progress made by mouse genetic studies supports this view (Lang, 2004). However, little is known about how their activities are sequentially integrated to narrow down the lens-field, and how signaling from the optic vesicle is involved in this process. To address this question, we chose to study the regulation of a forkhead-family gene, Lens1, which is the earliest of the genes that are expressed primarily in the PLE overlying the optic vesicle (Kenyon et al., 1999), and which is
activated at the time when commitment to the lens fate occurs (Grainger, 1992). Synteny analysis using Metazone 1.1 (http://www.metazone.net/) showed that the X. tropicalis Lens1 locus is orthologous to mouse FoxE3 and we therefore refer to it as Xenopus FoxE3 in this study. Analyses of mouse mutants have shown that Foxe3 is essential for lens epithelial proliferation and lens vesicle closure (Blixt et al., 2000; Brownell et al., 2000).

Using in vivo and in silico approaches, we identify and characterize an enhancer of X. tropicalis FoxE3 responsible for its PLE-specific expression, and demonstrate that Xenopus-mammalian genome comparison is a powerful strategy for prediction and for further detailed analysis of vertebrate cis-regulatory elements. A ‘co-transgenesis’ assay, in which separate enhancer and reporter element constructs are co-injected, was also developed to facilitate the rapid survey of possible enhancer activities of the predicted cis-regulatory elements. Our analysis has led to the first recognition that Notch signaling (Lai, 2004) is a lens-inducing signal, and revealed a role for Otx2 acting in concert with Notch signaling to specify the lens field. The data presented here reveal one of the first molecular mechanisms found to underlie stepwise determination of the lens, as well as suggesting a general mechanism for how organ progenitor cells are segregated within a broader ‘zone of responsiveness’ during vertebrate development.

**MATERIALS AND METHODS**

**Plasmid constructs**

pBSSK+EGFP was generated by introducing an EGFP-poly(A) cassette excised from pEGFP-1 (Clontech) into pBlueScript SK+ (Stratagene). –10.6kEGFP was generated by introducing the FoxE3 promoter region (–10,632 to +118) isolated from a X. laevis FoxE3 (Lensi) (Kenyon et al., 1999) in X. tropicalis genomic assembly 4.1 (scaffold 1: 2902082-2903173, http://genome.jgi-psf.org/Xentr4/Xentr4.home.html). We assigned the transcription start site (+1) to the 5′ end of the putative 5′ untranslated region (UTR) (scaffold 1: 2901906-2902081), which was predicted from its homology to the 5′ UTR of X. laevis FoxE3 (94% identity), to number the flanking sequence. BGFP was generated by introducing the chicken β-actin basal promoter (–55 to +53) excised from pCS2+GR-β-actin (Promega) into the pGEM-T Easy vector (Promega). pCS2+GR-Otx2-En was generated by introducing the coding sequence of a GR ligand-binding domain isolated from pCS2+GR-Su(H)Vp16 (Rones et al., 2000) into the 5′ end of the Otx2 coding sequence of pCS2+Otx2-En (Gammill and Sive, 2001).

**Xenopus transgenesis**

Transgenic embryos were generated by a sperm nuclear transplantation method (Kroll and Amaya, 1996), and their GFP expression was detected by in situ hybridization (Sive et al., 2000) for maximum sensitivity. The fraction of embryos that developed normally until scoring stages (stages 22-24) (Nieuwkoop and Faber, 1967) varied between 10-20% of total injected embryos depending on egg quality. However, the frequency of reporter gene expression within the group of normal embryos was fairly constant. In the case of three independent assays using X462-βGF, the average fraction of embryos with PLE-specific GFP expression in a normal group was 21±1.7%. This construct was injected in parallel with a series of its mutant constructs (mt1-m9) as a control to monitor transgenesis efficiency. For co-transgenesis, the 462 bp enhancer fragment of Xenopus FoxE3, amplified by PCR, was mixed with a β-actin promoter-GFP cassette excised from BGFP in a molar ratio of 4:1 and directly used for transgenesis.

**In situ hybridization and RNA injections**

In situ hybridization analyses of Delta1, Delta2, Otx2, γl-crystallin and Rx were performed as described (Blitz and Cho, 1995; Chitnis et al., 1995; Jen et al., 1997; Mathers et al., 1997; Offield et al., 2000). The antisense probes for GFP, FoxE3, Serrate1 and Notch2 were generated using pBSSK+EGFP, pGEM-XFoxE3, pBS-XSerrate1 (a gift from Dr C. Kintner, The Salk Institute for Biological Studies, La Jolla, CA) and an EST clone (NCBI accession BX855335), respectively.

Capped mRNAs for microinjections were transcribed from pCS2+GR-Su(H)VP16, pCS2+GR-Su(H)DBM (Rones et al., 2000), pCS2+NICD (Chitnis et al., 1995), pCS2+XOtx2-GR (Gammill and Sive, 1997), pCS2+GR-Otx2-En, pCS2+XOtx2 (Blitz and Cho, 1995), pCS2+XDelta1, pCS2+XDelta1Su(H) (Chitnis et al., 1995), pCS2+XDelta2, pCS2+XDelta2Tr (Jen et al., 1997), pCS2+EGFP and pCS2+lacZ. For lacZ staining, magenta-gal was used as the substrate (Rones et al., 2000).

**RESULTS**

**Identification of the FoxE3 cis-regulatory element by both classic promoter deletion assays and in silico analysis**

PLE-specific expression of FoxE3 in early tailbud embryos of X. tropicalis (Fig. 1A) was indistinguishable from the previously reported FoxE3 expression in X. laevis (Kenyon et al., 1999). This expression was closely recapitulated by GFP expression in transgenic X. laevis embryos generated with a reporter construct carrying a 10.6 kb upstream sequence of X. tropicalis FoxE3 (Fig. 1B, –10.6kGFP; see Fig. S1 in the supplementary material). Sequences responsible for PLE-specific expression were narrowed down by a series of promoter deletion assays (Fig. 1C-G), which identified a 901 bp element (–6313 to –5413) as the one that essentially recapitulates the activity of the –10.6 kb promoter when linked to the basal promoter region (–640 to +118) (Fig. 1G).

We also approached the same question from a bioinformatic perspective. To examine a possible relationship between conserved non-coding elements distributed around vertebrate FoxE3 loci and
the functionally identified 901 bp enhancer, we aligned a ~60 kb sequence of the human FOXE3 locus with orthologous mouse, chicken and \textit{X. tropicalis} sequences using a genome alignment program, MultiPipmaker (http://pipmaker.bx.psu.edu/pipmaker/) (Fig. 2A). In this alignment, the percent identity plot (pip) shows both the position in the human sequence and the degree of similarity for each aligning segment between the human and other sequences. The pip in the human-mouse alignment (Fig. 2A, top row) indicates extensive sequence conservation between these two species. However, the pip in the human-chicken and the human-\textit{Xenopus} alignments (Fig. 2A, second and third rows) indicates that only one region is conserved in all four species besides the coding region (Fig. 2A, red box). This conserved region corresponds to the 462 bp sequence between –6159 and –5698 in the \textit{X. tropicalis} FoxE3 locus, and is included in the 901 bp element (~6313 to –5413) described above.

Sequence conservation in the 462 bp element was further analyzed by phylogenetic footprinting (Fig. 2B). The evolutionary distance from \textit{Xenopus} to human resolves the 462 bp element into the 462 bp element necessary for PLE-specific expression is boxed with a dotted red line. *The expression in D was positive in the PLE but very spotty and broad, as shown in the left-hand panel.

Fig. 1. In vivo deletion analysis identifies a 901 bp enhancer that directs PLE-specific expression of FoxE3. (A) FoxE3 expression in \textit{X. tropicalis} embryos (stage 23) detected by in situ hybridization. (B-G) GFP expression detected by in situ hybridization in representative transgenic embryos (stages 22-24) generated with the reporter constructs shown to the left. White and black arrows in A-G indicate the PLE. The arrowhead in B indicates ectopic GFP expression in the presumptive oral ectoderm. Numbers of embryos with GFP expression in the PLE and the total number of normally (or near normally) developing embryos injected with each construct are indicated to the right, along with the percentage of GFP-positive cases. The 901 bp element necessary for PLE-specific expression is boxed with a dotted red line. *The expression in D was positive in the PLE but very spotty and broad, as shown in the left-hand panel.

Fig. 2. In silico analysis of FoxE3 cis-regulatory elements. (A) Genomic sequence of the human FOXE3 locus with orthologous mouse, chicken and \textit{X. tropicalis} sequences using a genome alignment program, MultiPipmaker (http://pipmaker.bx.psu.edu/pipmaker/) (Fig. 2A). In this alignment, the percent identity plot (pip) shows both the position in the human sequence and the degree of similarity for each aligning segment between the human and other sequences. The pip in the human-mouse alignment (Fig. 2A, top row) indicates extensive sequence conservation between these two species. However, the pip in the human-chicken and the human-\textit{Xenopus} alignments (Fig. 2A, second and third rows) indicates that only one region is conserved in all four species besides the coding region (Fig. 2A, red box). This conserved region corresponds to the 462 bp sequence between –6159 and –5698 in the \textit{X. tropicalis} FoxE3 locus, and is included in the 901 bp element (~6313 to –5413) described above.

Sequence conservation in the 462 bp element was further analyzed by phylogenetic footprinting (Fig. 2B). The evolutionary distance from \textit{Xenopus} to human resolves the 462 bp element into...
discontinuous stretches of conserved sequences of 6-11 bp, each of which may predict transcription factor-binding motifs. Eight of these stretches are identical or similar to known transcription factor-binding motifs and include target sequences of three signaling pathways: a Smad1-binding motif for BMP signaling (Kusanagi et al., 2000); a Su(H) (also known as CBF1/RBP-Jc/Lag-1) motif for Notch signaling (Tun et al., 1994); and a Tcf3/Lef1-binding motif for canonical Wnt signaling (Eastman and Grosschedl, 1999). Other predicted motifs are two Otx-binding motifs (Gan et al., 1995), two Fox motifs (Kaufmann et al., 1995) and a GATA motif (Ko and Engel, 1993).

**Identification of transcription factor-binding motifs essential for PLE-specific expression**

The 901 bp enhancer identified by the deletion analysis contains not only the conserved 462 bp, but also surrounding non-conserved sequences. Transgenic embryos generated with a construct in which the conserved 462 bp sequence alone was linked to a heterologous basal promoter (chicken β-actin) exhibited GFP expression in the PLE similar to the embryos carrying the 901 bp enhancer construct (Xt462-βGFP in Fig. 3A, compare with Fig. 1G), whereas the construct with the β-actin promoter alone (βGFP) did not drive any detectable expression in any embryos (Fig. 3B). Interestingly, PLE-specific expression was also detected when the 462 bp element amplified by PCR was co-injected with the βGFP cassette (Fig. 3C), an approach taken because this transgenic method is known to produce a concatemer of transgenic inserts. This novel, cloning-free ‘co-transgenesis’ strategy is a powerful tool for the rapid survey of enhancer activities of conserved non-coding elements that are widely distributed in vertebrate genomes (see Discussion).

Transgenic embryos generated with a construct in which the 462 bp *Xenopus* element was replaced with the orthologous 423 bp element of mouse *Foxe3* (−3529 to −3107) used for the phylogenetic footprinting (Fig. 2B) exhibited GFP expression that was indistinguishable from that driven by the *Xenopus* element (compare Fig. S2A with B in the supplementary material), suggesting that the sum of the discontinuous stretches of short conserved sequences identified by the phylogenetic footprinting is sufficient to account for the expression. To evaluate the role of each short conserved sequence, we introduced base-substitution mutations individually into all of the eight putative transcription factor-binding motifs (indicated as Factor X motif in Fig. 2B). The mutant constructs were generated from *Xt462*-βGFP, which drove PLE-specific expression in 21% of the generated embryos in transgenic assays as described (Fig. 3A and ‘wt’ in Fig. 3D). None of the mutations led to additional ectopic expression, but the percentage of embryos with PLE-specific expression was decreased to different extents (Fig. 3D). The most striking result was obtained with the construct carrying a mutation in the Su(H) motif (Fig. 3D, mt7), which completely abolished the expression in all cases except one (n=70). Even in this one positive case, the expression was very faint (not shown). The mutation of the 3′-most Otx motif and the mutation of the unknown Factor X motif decreased the positive cases to 6% and 8%, respectively (Fig. 3D, mt5 and mt4). Mutation of the 5′-most Fox motif, Smad1 motif, or GATA motif, somewhat decreased the positive cases, to ~12% (Fig. 3D, mt2, mt3 and mt6). Mutation of the 5′ Otx motif, Tcf3/Lef1 motif, or 3′ Fox motif did not significantly reduce the percentage of positive cases (Fig. 3D, mt1, mt8 and mt9). By χ² test (http://www.graphpad.com/quickcalcs/chisquared1.cfm), the percentage of positive cases in the wild type and the Su(H), 3′-most Otx, or unknown Factor X mutant constructs are significantly different (P<0.0001, P=0.0006 and P=0.0018, respectively), whereas the differences observed in other cases are not significant (P>0.05). These results show that the Su(H), 3′-most Otx, and unknown Factor X motifs are essential for the enhancer activity, whereas other motifs might serve to boost its level and/or are involved in the regulation at different developmental stages.
Regulation of FoxE3 expression and lens placode formation by Notch signaling

Gel retardation assays showed direct binding of Xenopus Su(H) protein to the putative Su(H) motif identified in the FoxE3 enhancer in vitro, and chromatin immunoprecipitation experiments confirmed in vivo binding of the Su(H) protein to the enhancer (see Fig. S3A and Fig. S4A in the supplementary material). Su(H) is ubiquitously expressed and activates transcription only when it forms a nuclear complex with the intracellular domain of Notch receptor (NICD) that is translocated from the cytoplasm upon activation by a ligand (Fig. 7A) (Lai, 2004). We found that a Xenopus homolog of mammalian Notch2, newly identified in this study, is expressed during the course of lens formation in the pre-placodal ectoderm, PLE and developing lens vesicle (Fig. 4A-B and see Fig. S5 in the supplementary material).

The Notch-ligand genes, Delta1 (Chitnis et al., 1995), Delta2 (Jen et al., 1997) and Serrate1 (Kiyota et al., 2001) have been identified in Xenopus. To assess the involvement of Notch ligands in FoxE3 regulation, their expression was compared with that of FoxE3 during the course of lens-field formation. In neural plate-stage embryos, neither Delta1 nor Delta2 is expressed in the presumptive retina fields that will give rise to optic vesicles (Fig. 4C,F) (Chitnis et al., 1995). Interestingly, Delta1 expression in the anterior neural ridge is adjacent to FoxE3 expression in the pre-placodal ectoderm at this stage (Bourguignon et al., 1998; Kenyon et al., 1999) (compare Fig. 4C with I), where it may act as an early signal involved in FoxE3 expression. Accompanying neural tube formation, both Delta genes exhibit strong upregulation in the developing optic vesicle (Fig. 4D,E,G-H) (Kenyon et al., 1999). The cells expressing Delta genes are located in the outermost region of the optic vesicle and make contact with the overlying PLE cells expressing FoxE3 (Fig. 4E,H,J-K). Serrate1 is not expressed in these tissues at the neurula or early tailbud stages, but later is expressed in the developing lens placode as reported (not shown) (Kiyota et al., 2001).

To examine possible roles of the Delta genes in FoxE3 regulation, we blocked Delta1 and Delta2 activities using their dominant-negative forms, Delta1SU and Delta2SU, respectively (Chitnis et al., 1995; Jen et al., 1997). mRNA encoding either Delta1SU or Delta2SU or GFP was injected along with a lineage tracer, nlacZ mRNA (nucZ, 50 pg), into one dorsal blastomere of eight-cell stage X. laevis embryos. The injected embryos were fixed at the early tailbud stages (stages 22-24), and stained for lacZ expression to trace distribution of the injected mRNAs. Only the embryos that showed lacZ staining in the optic vesicle were subjected to in situ hybridization with a injected probe. Control injections using GFP mRNA did not have any significant effects on FoxE3 expression (n=55). Embryos injected with Delta1SU mRNA exhibited loss or severe reduction of FoxE3 expression on the injected side (100%, n=22), but this phenotype appeared to be associated with head abnormalities caused by the expression of this construct (not shown). Although the effect seen here is consistent with a role for Delta1 in lens formation, we did not study Delta1 further because of the complexity in interpreting the cause of lens defects in light of the head abnormalities seen in these experiments. By contrast, injection of Delta2SU mRNA led to a very specific phenotype: FoxE3 expression was lost or severely reduced (75%, n=32, Fig. 5A) in the PLE overlying the optic vesicle stained for nucZ, but the optic vesicle itself, which was marked by expression of the retina-specific homeobox gene Rx (Mathers et al., 1997), did not show any detectable abnormalities (100%, n=37, Fig. 5B).

In addition to FoxE3, we examined expression of a lens differentiation marker, yl-crystallin (Offield et al., 2000), by in situ hybridization, to investigate late phenotypes of embryos expressing Delta2SU. At late tailbud stages (stages 29/30), the lens placode of the uninjected sides showed clear yl-crystallin expression (Fig. 5D), but the yl-crystallin-positive cells on the injected sides formed a tiny cell mass or were absent (71%, n=42, Fig. 5C,I). Expression analysis of Rx showed that the optic vesicle of the injected sides still had no significant defects, at least through these stages (100%, n=47, Fig. 5F-H). The downregulation of yl-crystallin by Delta2SU was rescued by co-injection of mRNA encoding wild-type Delta2 (75%, n=67, Fig. 5E,J), but not Delta1 (n=38, Fig. 5I), indicating the specific activity of Delta2 for lens induction. These results show that Delta2 activity in the optic vesicle is necessary for FoxE3 expression in the PLE and for subsequent lens placode formation.

To examine how the responses to Notch signaling might directly impinge on FoxE3 expression in lens cells, we used a construct encoding an inducible dominant-negative form of Su(H), GR-Su(H)DBM (Rones et al., 2000). This construct was generated by fusing the human glucocorticoid receptor ligand-binding domain (GR) to a modified version of Xenopus Su(H), which contains a
point mutation in its DNA-binding domain. This GR-Su(H)DBM protein, which inhibits Notch signaling in response to dexamethasone (Dex) by sequestering NICD from endogenous Su(H), allowed us to circumvent possible head defects that could be caused by constitutive inhibition of Notch signaling. mRNA encoding GR-Su(H)DBM was injected along with nlacZ mRNA into one dorsal blastomere of four-cell stage X. laevis embryos. The injected embryos were cultured in the absence of Dex until stages 15-16, and then maintained with Dex (10 μM) either present (induced) or absent (uninduced) until fixation at early tailbud stages (stages 22-24). This time period was chosen to yield functional GR-Su(H)DBM protein at the time when endogenous FoxE3 expression is upregulated in the PLE following neural tube closure. The fixed embryos were subjected to lacZ staining to select embryos in which expression was targeted to the anterior ectoderm including the PLE. As observed in embryos injected with Delta210 mRNA, FoxE3 expression was lost or severely reduced on injected sides of the Dex-treated embryos (55%, n=33, Fig. 5J). Downregulation of FoxE3 was not observed on uninjected sides of any of these embryos (Fig. 5K) or on injected sides of any sibling embryos untreated with Dex (n=65, Fig. 5L), indicating that Dex itself had no effect on FoxE3 expression and the FoxE3 downregulation in the Dex-treated embryos depended on the activation of GR-Su(H)DBM by hormone treatment.

Effects of ectopic activation of Notch signaling on FoxE3 expression were examined using an inducible, active form of Su(H), GR-Su(H)VP16 [Xenopus Su(H) fused to GR and to a VP16 activation domain], which mimics Notch pathway activation in response to Dex (Rones et al., 2000). Similar to the experiments with GR-Su(H)DBM, GR-Su(H)VP16 was expressed by injecting its mRNA, and the resulting embryos were cultured with or without Dex until fixation at stages 22-24. Ninety-five percent of the injected embryos treated with Dex exhibited ectopic FoxE3 expression in the anterior region of the injected sides stained with lacZ (n=55, Fig. 5M). In contrast to the extended lacZ staining, the ectopic FoxE3 expression was spotty and localized in a domain of the ectoderm overlying the anterior brain and that surrounding the cement gland. Ectopic FoxE3 expression was not observed on the uninjected sides of any of these embryos (Fig. 5N) or on the injected sides of any sibling embryo untreated with Dex (n=69, Fig. 5O).

The activation and inhibition of Notch signaling using the active and dominant-negative forms of Su(H), respectively, induced upregulation and downregulation of FoxE3. These results demonstrate the essential role of Notch signaling in PLE-specific FoxE3 expression, and suggest that Notch signaling in the PLE is likely to be activated by Delta2 expressed in the adjacent optic vesicle. Interestingly, the ectopic FoxE3 expression induced by the active form of Su(H) was regionally restricted to part of the anterior ectoderm, which suggests pre-localization there of a factor enabling responsiveness to Notch signaling. In addition, this restricted ectopic expression is consistent with a role for Notch signaling as a cue to turn on FoxE3 at the right place within this competent domain.

Fig. 5. Effects of manipulation of Notch signaling on FoxE3 expression and subsequent lens placode formation. (A,B) Frontal view of Xenopus embryos injected with mRNA encoding Delta210 (500 pg), fixed at stage 23, and subjected to lacZ staining (magenta) and in situ hybridization with FoxE3 or Rx probe (purple or deep purple staining). White and black arrowheads in A-H indicate in situ hybridization signals on injected and un.injected sides of embryos, respectively. (C,D,F,G) The injected and un.injected sides of embryos injected with Delta210 mRNA, fixed at stages 29/30, and hybridized with γ1-crystallin or Rx probe. (H) A transverse head section of the embryo shown in F,G. (E) The injected side of an embryo injected with both Delta210 mRNA (500 pg) and wild-type Delta2 mRNA (500 pg), fixed at stage 29, and hybridized with γ1-crystallin probe. (I) Summary of Delta210 mRNA injection experiments. GFP mRNA (1000 pg) was injected as a control. (J,K) The injected and un.injected sides, respectively, of an embryo injected with GR-Su(H)DBM mRNA (1000 pg) and induced with Dex. Arrows in J-O indicate endogenous FoxE3 expression in the PLE. (L) The injected side of an embryo injected with GR-Su(H)DBM but not induced with Dex. (M,N) The injected and un.injected sides, respectively, of an embryo injected with GR-Su(H)VP16 mRNA (1000 pg) and induced with Dex. Black and white arrowheads in M indicate ectopic FoxE3 expression in the ectoderm overlying the anterior brain and that surrounding the cement gland, respectively. (O) The injected side of an embryo injected with GR-Su(H)VP16 but not induced with Dex.
Otx2 confers the ability to activate FoxE3 in response to Notch signaling

We examined Otx2, as a candidate factor responsible for the regional responsiveness to Notch signaling, for two reasons. First, we identified a putative Otx motif as among the most essential of the FoxE3 transcription factor-binding motifs in the enhancer (Fig. 3D, mt5). Second, during the time window chosen for the activation of GR-Su(H)VP16 (from the neural plate to early tailbud stages), Otx2 shows diffuse expression in the head ectoderm, including not only the PLE, but also the surrounding region where the ectopic FoxE3 expression was observed (Fig. 6A-C, compare Fig. 5M and Fig. 6C) (Zygler et al., 1998).

Gel retardation assays showed direct binding in vitro of Xenopus Otx2 protein to the putative Otx motif identified in the FoxE3 enhancer, and chromatin immunoprecipitation experiments confirmed in vivo binding of endogenous Otx2 protein to the enhancer (see Fig. S3B and Fig. S4B in the supplementary material). To test whether Otx2 misexpression confers the ability to respond to Notch signaling in vivo, we used an inducible form of Xenopus Otx2, GR-fused Otx2 (Otx2-GR), whose activity can be controlled by Dex treatment (Gammill and Sive, 1997). This construct allows us to circumvent severe gastrulation defects and spina bifida caused by misexpression of wild-type Otx2 in Xenopus embryos (Blitz and Cho, 1995). mRNAs encoding GR-Su(H)VP16 and Otx2-GR were injected separately or together into a ventral blastomere of four-cell stage embryos to target expression in the trunk region instead of the head ectoderm expressing endogenous Otx2. All injected embryos were treated with Dex at stages 15-16, fixed at stages 22-24, and subjected to lacZ staining and in situ hybridization with the FoxE3 probe.

None of embryos misexpressing either GR-Su(H)VP16 or Otx2-GR exhibited ectopic FoxE3 expression in the trunk region, where the lacZ staining indicated broad distribution of the injected mRNAs (n=66 and n=52, respectively; Fig. 6D,E). This indicates that neither Notch signaling nor Otx2 activity is sufficient for FoxE3 activation. However, when embryos were co-injected with GR-Su(H)VP16 and Otx2-GR mRNAs, 91% (n=54) exhibited striking FoxE3 expression in the trunk region throughout the lacZ-stained region (Fig. 6F, black arrowheads). This trunk expression was restricted to the ectoderm layer, and was absent from the underlying neural and mesodermal tissues (Fig. 6F, inset). Ectopic FoxE3 expression was also detected in embryos that were induced with Dex in the presence of a protein
proposed to bind to the Otx2 and Notch inputs on the unidentified factor(s) (indicated with a question mark) may contribute activator (Co-A). Otx2 synergistically stimulates this transcription. An complex with Su(H) and activates transcription by recruiting a co-
may occur in this system (see Discussion). In the nucleus, NICD forms a
extracellular domain and translocated into the nucleus. Although this signaling, but remain in a quiescent state. In response to stimuli from Delta ligands, the Notch intracellular domain (NICD) is cleaved from its as effectively as the artificial construct GR-Su(H)VP16, if Notch is activated.

Fig. 7. A model of FoxE3 activation in Xenopus. (A) Integration of Otx2 and Notch inputs on the FoxE3 enhancer. Otx2 and Su(H) are proposed to bind to the FoxE3 enhancer prior to receiving Notch signaling, but remain in a quiescent state. In response to stimuli from Delta ligands, the Notch intracellular domain (NICD) is cleaved from its extracellular domain and translocated into the nucleus. Although this model shows a direct interaction between membrane-bound Notch and Delta, we do not know the exact mechanism by which this signaling may occur in this system (see Discussion). In the nucleus, NICD forms a complex with Su(H) and activates transcription by recruiting a co-activator (Co-A). Otx2 synergistically stimulates this transcription. An unidentified factor(s) (indicated with a question mark) may contribute to boosting and refining the PLE-specific expression. (B) Otx2 is broadly expressed in the head ectoderm that includes PLE, while localized Notch signaling is provided to the lens-field from the underlying optic vesicle (upper). FoxE3 is expressed in the PLE, which is the region where there is both Otx2 expression and Notch signaling (beneath).

DISCUSSION
A model of FoxE3 activation
We have presented gain-of-function and loss-of-function strategies demonstrating that Otx2 activity and Notch signaling directly converge on the FoxE3 enhancer to direct PLE-specific expression (Fig. 7A). These are the first data directly showing involvement of Notch signaling in lens induction. Based on these results, we propose a stepwise model of FoxE3 activation (Fig. 7B): (1) Otx2 regionally enables responsiveness to Notch signaling within head ectoderm including PLE; (2) Delta2 signaling from the optic vesicle locally activates Notch signaling in the overlying PLE to turn on FoxE3 expression within this broader competent region. The results of the transgenic assay using the Otx-Su(H) reporter suggest that other transcription factors might contribute to boosting and refining the PLE-specific expression in combination with Otx2 and Su(H). An unknown factor that binds to the Factor X motif (Fig. 2B and Fig. 3D), and FGF, BMP and ZEB2 (SIP1) signaling, the inhibition of which causes a reduction in Foxe3 expression in mouse embryos, might be involved in this process (Faber et al., 2001; Yoshimoto et al., 2005).

Because the dominant-negative form of Delta1 induced head defects in embryos, we could not examine possible roles for Delta1 in Foxe3 regulation and subsequent lens formation. However, neural tube formation is accompanied by a dynamic change in Delta1 expression from the anterior neural ridge to the optic vesicle (Fig. 4C-E’), which might be responsible for the shift of Foxe3 expression from the pre-placodal ectoderm to the PLE (Fig. 4L-K’). Foxe3 expression in the PLE of embryos expressing the dominant-negative form of Delta2 was severely reduced, but remained in some cases, which might be due to some contribution of Delta1 to Foxe3 regulation.
As in Xenopus, Otx2 is expressed in the PLE of mouse embryos, and the lens placode of Otx2 heterozygous mutant mice fail to form a normal lens vesicle on an Otx1 homozygous mutant background (Martinez-Morales et al., 2001). deltaC, a zebrafish Notch-ligand gene that has the highest sequence similarity to Xenopus Delta2, is expressed in the developing optic vesicle in the same manner as Xenopus Delta2 (Smithers et al., 2000), whereas a mammalian homolog of Delta2/deltaC has not been identified yet. Interestingly, Jag1, a mammalian homolog of Xenopus Serrate1, is expressed in the optic vesicle of rat embryos (Bao and Cepko, 1997), and is deleted in the mouse mutant coloboma (Cm), whose lens fails to detach from the ectoderm as in the Foxe3 mutant mouse, dysgenetic lens (dy1) (Blixt et al., 2000; Brownell et al., 2000; Theiler and Varnum, 1981; Xue et al., 1999). Hence the role of Delta2/deltaC in the lens induction of lower vertebrates might be taken over by Jag1 in mammals. Regarding Notch receptors, mammalian Notch2 and Notch3 are expressed in the developing lens (Lindsell et al., 1996), but their expression at earlier stages has not been characterized in detail. In mammalian embryos, cells in the optic vesicle and lens ectoderm are physically separated by a space, but are connected by cytoplasmic extensions (McAvoy, 1980), which may permit direct contact for Notch signaling. It is also possible that Notch ligands might have secreted forms that are involved in Notch signaling (Qi et al., 1999).

Otx2-Notch interactions in lens determination programs, and their analogy with selector-signaling system in Drosophila

The data presented here have significant implications for molecular mechanisms underlying the stepwise determination of the lens. Otx2 expression in head ectoderm might constitute a part of the lens-forming bias suggested by embryological studies, and Notch signaling is likely to be one of the inducing signals provided from the optic vesicle to turn on the lens-specification programs in this competent/biased ectoderm. Unlike FoxE3, expression of a lens differentiation marker, γl-crystallin, was not induced in the trunk ectoderm by misexpression of Otx2-GR and GR-Su(H)VP16 (not shown), suggesting that the Otx2-Notch combination is not sufficient to activate the whole lens-differentiation program. However, severe reduction or loss of γl-crystallin-positive lens placode cells in embryos expressing the dominant-negative Delta2 suggests a crucial role for Notch signaling in lens specification. The lens differentiation programs are presumably turned on when a set of all terminal regulators, such as FoxE3 and L-maf, is activated in the PLE by different, but possibly overlapping, mechanisms.

Genetic studies in mouse have shown that Pax6 lies upstream of Mab21l1, and that Mab21l1 lies upstream of Foxe3 (Yamada et al., 2003). We found that the combination of Otx2 and Notch signaling induced ectopic FoxE3 expression in the trunk ectoderm without activating Pax6 (not shown). These findings imply that the Pax6-Mab21l1 pathway controls FoxE3 expression indirectly through regulation of Otx2 and/or Notch signaling. Notch2 might be a downstream target of Pax6, as the broad expression of Notch2 in the head ectoderm is, as development proceeds, gradually localized to the lens and olfactory fields expressing Pax6 (Fig. 4A-B’ and see Fig. S5 in the supplementary material).

Interestingly, the combinatorial mechanism of FoxE3 regulation is similar to the selector-signaling system in Drosophila, in which selective gene activation by signals for cell fate specification is achieved by obligate integration of both inputs of field-specific transcription factors (selectors) and signal-activated transcription factors at the level of their target cis-regulatory elements (Guss et al., 2001). Although this system has not been previously examined in vertebrate development, our study suggests that the same mechanism underlies the ‘multiple-step induction’ of the lens. As classic embryological studies demonstrate similar stages in many vertebrate organ systems (Gurdon, 1992), a selector-signaling system might be broadly used in vertebrates for specifying a variety of organ and tissue identities by reiteratively using a limited number of signaling pathways. While the events we are studying in this paper occur during the bias and specification phases of lens induction, the same principle could apply earlier, during the competence period, when a broadly expressed selector gene may contribute to the period of competence and converge with an early lens-inducing signal.

Xenomics (Xenopus genomics) for analysis of genomic regulatory networks for development

The results of our classic-style deletion analysis are in close agreement with those from the comparative analysis of human and Xenopus genomes, which demonstrates the effectiveness of using the Xenopus genome for in silico prediction of conserved regulatory elements in vertebrates. The conserved enhancer of mouse Foxe3 identified in our study (~3529 to ~3107) is included in the lens element that was independently identified by Kondo’s group by deletion analysis in transgenic mice (~4.40 to ~2.63 kb) (Yoshimoto et al., 2005), showing that Xenopus and mouse assays give consistent results.

An important challenge in the post-genomic era is to untangle the complex wiring of gene regulatory networks controlling development, growth and differentiation. As shown in the pioneering study of the gene regulatory network for sea urchin endomesoderm specification (Davidson et al., 2002), this type of study requires a high-throughput assay system for comprehensive analysis of cis-regulatory elements. The mammalian-Xenopus genome comparison and an approach developed in the course of this study – co-transgenesis – which rapidly tests enhancer activities by co-injection of PCR products along with the basal promoter-GFP cassette, will allow Xenopus to serve as a vertebrate model system that fulfills this requirement.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/2/249/DC1

References


