The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision

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Although the development of the vertebrate eye is well described, the number of transcription factors known to be key to this process is still limited. The localized expression of the orphan nuclear receptor Tlx in the optic cup and discrete parts of the central nervous system suggested the possible role of Tlx in the formation or function of these structures. Analyses of Tlx targeted mice revealed that, in addition to the central nervous system cortical defects, lack of Tlx function results in progressive retinal and optic nerve degeneration with associated blindness. An extensive screen of Tlx-positive and Tlx-negative P19 neural precursors identified Pax2 as a candidate target gene. This identification is significant, because Pax2 is known to be involved in retinal development in both the human and the mouse eye. We find that Pax2 is a direct target and that the Tlx binding site in its promoter is conserved between mouse and human. These studies show that Tlx is a key component of retinal development and vision and an upstream regulator of the Pax2 signaling cascade.

Tlx is a member of the *tailless* class of orphan nuclear receptors, a highly conserved family in both vertebrates and invertebrates, suggesting this family's importance during evolution (1). The lack of identified ligands for most members of this group has hampered progress in understanding their function. One way to circumvent this problem is through analyses of their expression patterns in conjunction with loss- or gain-of-function experiments in mice and flies. We and others have used this approach to investigate the role of Tlx during embryonic development (2–4).

Another approach is the characterization of the pathways affected by these transcription factors. The evolutionary conservation of the pattern of Tlx expression in the embryonic forebrain, midbrain, and optic vesicle in vertebrates suggested that Tlx may participate in the formation of anterior central nervous system-derived structures (2, 3, 5). Our observation that Tlx mutant mice have visual defects that are accompanied by retinal and optic nerve degeneration prompted us to search for target genes involved in the development and maintenance of eye function. The P19 teratocarcinoma system was selected to examine genes that respond to Tlx expression, because P19 cells are multipotential and can differentiate into neuronal cells with central nervous system-like properties on aggregation and treatment with retinoic acid (6). Because Tlx is highly expressed in mitotically active neural precursors in the periventricular zone of the developing brain and retina, gradually becoming more restricted as differentiation progresses (2, 3), we thought that the P19 teratocarcinoma system might simulate, to some extent, conditions that would reveal Tlx target genes.

Materials and Methods

Analyses of Tlx−/− Mice. Mouse Tlx genomic clones were obtained from a 129 library; construction of targeting vectors encoding a *lacZ* knock-in gene and heterozygous mice were obtained as described (7). Homozygous mice were obtained by mating and were genotyped by PCR or Southern blot analysis. Electroretinograms were performed by standard procedures (8).

Identification of Tlx Target Genes. P19 lines were transfected with pCDNA3-chick Tlx (full-length), and 24 neomycin-resistant clones were isolated. Tlx-expressing clones ($n = 11$) were confirmed by Northern blot and reverse transcription–PCR (RT-PCR) analyses (9). Differential expression of marker genes was determined by RT-PCR and Northern blot analyses.

Plasmid and Probe Construction. Expression plasmids used for electroporation were constructed by subcloning of full-length chick Tlx cDNA (pCAGGs-cTlx) or chick Tlx DNA binding domain (amino acids 1–136) fused to the engrailed repressor domain (EnR; amino acids 1–298; ref. 10) for pCAGGS-TlxEnR or the VP16 activation domain (Novagen) for pCAGGs-TlxVP into the pCAGGs vector (11). pCAGGs-GFP (GFP, green fluorescent protein) has been described (12). The chick Pax2 template used for *in situ* probe was obtained by RT-PCR from stage 12 chick embryonic RNA with degenerate primers [5′, ATGCACTGCAA(A/G)GCAGACCC; 3′, T(A/G)GA(G/T)GCGCTGGA(A/G)ACAGG(A/T)G], resulting in amplification of a 526-bp fragment, which was confirmed by sequencing and subcloned into a pMOSBlueT-vector (Amersham Pharmacia). Mouse Pax2-promoter-lacZ was kindly provided by G. Dressler (University of Michigan Medical Center, Ann Arbor). Mouse Pax2-promoter-LUC was made by subcloning of a 4.3-kilobase *Bam*HI–*Not*I fragment from pBS4.3BN (G. Dressler) into a promoterless luciferase vector (PGL, Toyo, Japan).

***In Vitro* DNA Binding and Cell Transfection Assays.** *In vitro* translation of Tlx protein from pCMX-TLX and DNA-binding assays were carried out as described (13). Transient cell transfection assays were performed by the calcium phosphate method as described (14). All points were performed in triplicate with variations of less than 10%.

***In Ovo* Electroporation of Chick Embryos.** Fertilized chicken eggs were obtained from Yamagishi Farms (Mie Prefecture, Japan) incubated at 38°C and staged according to the method of

Abbreviations: RT-PCR, reverse transcription–PCR; EnR, engrailed repressor domain; GFP, green fluorescent protein; β-gal, β-galactosidase.

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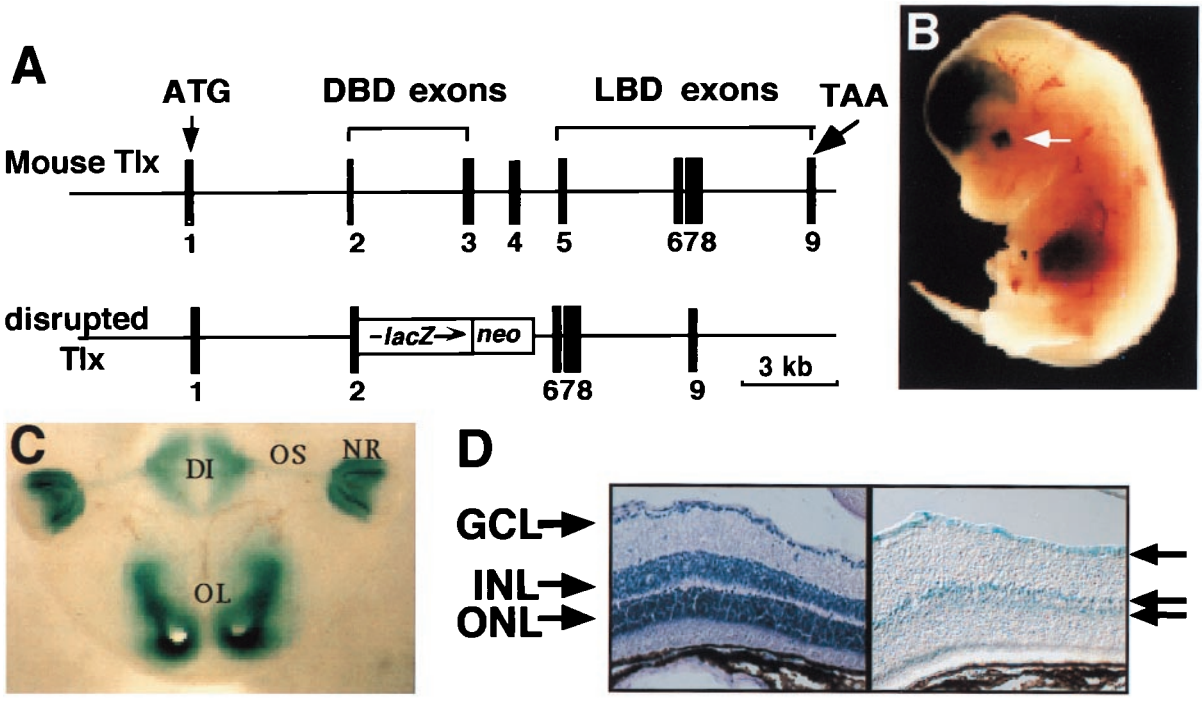

Fig. 1. Tlx expression in the mouse retina and optic stalk. (*A*) Schematic of the Tlx genetic locus (*Upper*) with predicted structure of the recombinant mutant allele (*Lower*). Exons are numbered. DBD, DNA binding domain; LBD, ligand binding domain. (*B*) Whole-mount LacZ staining of an embryonic day 13 heterozygous embryo showing Tlx expression in the neural retina, optic stalk, and forebrain. The arrow indicates the level of section shown in *C*. (*C*) DI, diencephalon; OS, optic stalk; NR, neural retina; OL, olfactory. (*D*) Retina sections of 3-week-old Tlx heterozygous mice have LacZ-positive cells (indicated by arrows) in the ganglion cell layer (GCL) as well as in the inner and outer nuclear layers (INL and ONL, respectively).

Hamburger and Hamilton (15). General conditions for *in ovo* electroporation have been described (12). For assays with the mouse Pax2–lacZ constructs, the ratio of reporter:effector:GFP plasmids was 15:7:1 at a total DNA concentration of 4.0 μg/μl. Whole-mount detection of lacZ signals and *in situ* hybridization were performed as described (2, 12).

Results and Discussion

Tlx Mutant Mice Have Visual Defects. To investigate the function of Tlx, we obtained and analyzed mice carrying a null mutation. Fig. 1 shows the knock-out vector and the associated knock-in lacZ indicator allele. Initial analyses of these mice identified defects in the rhinencephalic and limbic structures of the brain (ref. 4 and data not shown). However, it was perplexing that Tlx did not seem to be required for eye development, because the optic vesicle and retina expression are highly conserved among vertebrates (2, 3, 5). The lacZ knock-in marker allowed confirmation of Tlx expression at embryonic day 13 in the neural retina, optic stalk, and adjacent diencephalon (Fig. 1 *B* and *C*) and, at postnatal week 3, in the ganglion as well as in the inner and outer nuclear cell layers of the retina (Fig. 1*D*).

These expression patterns prompted us to examine the visual function of the Tlx$-/-$ mice in more detail. In general, the mice had a nearly complete lack of response to light stimuli (15 of 15 mice), accompanied by abnormal electroretinograms. The waves corresponding to the responses for photoreceptor, Muller, bipolar, and amacrine cells were all absent in 3-month-old homozygous mice (Fig. 2*A*). Examination of younger mice starting at 3 weeks of age identified similar patterns (data not shown). Histological examination of the retinas identified a patchy pattern of retinal degeneration that is detectable by 3 weeks after birth and is progressive thereafter. Most prevalent are hypocellularity and disorganization of the ganglion, inner, and outer nuclear cell layers, correlating with the pattern of Tlx expression. The outer plexiform and photoreceptor segment layers are also often missing (Fig. 2*B*). Furthermore, the optic nerve is greatly reduced in diameter at all stages examined, with an approximate 6-fold reduction in cross-sectional area (in 12 of 12 mice; Fig. 2*C*).

Identification of Potential Tlx Target Genes. Given a role for Tlx in eye function, we sought to identify target genes that respond to Tlx expression in the P19 teratocarcinoma cell line. Stable Tlx-expressing P19 lines were established and used to examine an extensive selection of brain and eye marker genes for alteration in their levels of expression relative to parental Tlx-negative cells. RNAs from monolayer cultures and retinoic acid-treated aggregates were screened by RT-PCR, and potential positives were confirmed by Northern blot analyses. Three independent Tlx-positive lines were examined to confirm that the alteration in target gene expression is consistent. Among over 100 genes examined, several transcription factors seemed to be affected (Fig. 3*A*), including Otx1, Chx10, NURR1, and Pax2. Interestingly, like its fly homologue, Tailless (16), Tlx seemed capable of both activation and repression of target genes.

The repression of Pax2, a paired box homeodomain transcription factor, was particularly interesting, because its mutation has been shown to result in a combined syndrome of optic nerve colobomas, various retinal anomalies, and multiple kidney defects in both human and mice (17–19). The expression patterns of Tlx and Pax2 in early stage chick embryos are for the most part complementary, although not completely exclusive. Tlx is expressed mainly in the forebrain and dorsal retina, with low levels of expression in the ventral retina and dorsal midbrain, whereas Pax2 is highly expressed in the ventral retina and midbrain–hindbrain junction (isthmus; refs. 2 and 20). During stages 10–13 (15), the patterns of their expression are very dynamic and seem to overlap transiently, particularly in the presumptive optic stalk

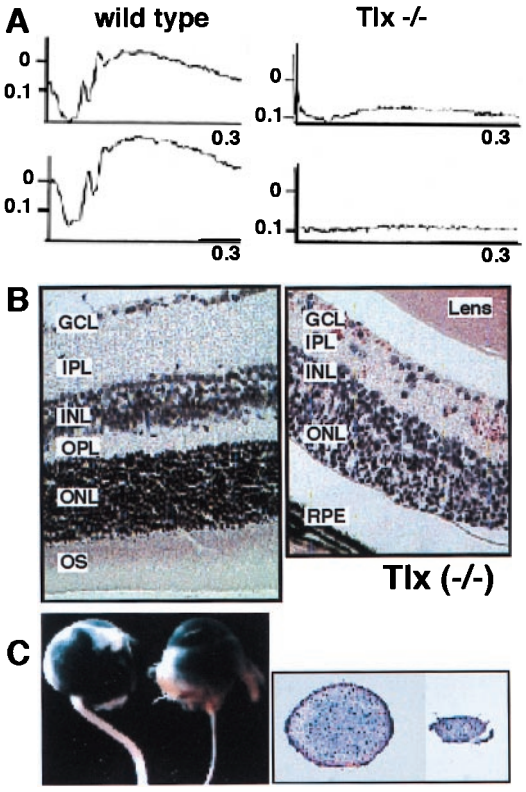

Fig. 2. Retina and optic stalk defects in Tlx mutant mice. (*A*) Electroretinograms of 6-month-old homozygous Tlx mutant mice (*Right*) compared with those of wild-type siblings (*Left*); no a-, op-, nor b-waves are found in Tlx−/− mice. Abnormal electroretinograms were obtained for homozygous mice at all ages examined (3 weeks to 6 months). (*B*) Histological sections through the eye of a 2.5-month-old Tlx−/− mouse (*Right*) showing hypocellularity and disorganization of the ganglion cell layer (GCL) and the inner and outer nuclear layers (INL and ONL, respectively) as well as absence of outer plexiform and outer segment layers. Hematoxylin-eosin staining of section from wild-type sibling is also shown (*Left*). IPL, inner plexiform layer; OPL, outer plexiform layer; OS, outer segments; RPE, retinal pigmented epithelium. (*C*) Optic nerve defects are observed in homozygous mice (stages examined are postnatal day 10 through 6 months). The difference in diameter of nerve as examined by transverse section is over 6-fold. Whole eyes with attached optic nerves from 3-week-old siblings (*Left*: wild type on left and Tlx−/− on right). Section through optic nerves (*Right*: wild type on left and Tlx−/− on right).

and ventral diencephalon. As discussed below, the ability of Tlx to repress Pax2 is likely to be dose-dependent, and thus, the accumulation of a certain level of Tlx protein may be required for effective repression.

A Conserved Tlx Binding Site Is Present in the Pax2 Promoter and Mediates Tlx Repression. Examination of the reported sequences for the Pax2 regulatory regions identified the presence of a strictly conserved Tlx consensus binding site of AAGTCA (1) in both human and mouse promoters upstream of the TATA box (refs. 18 and 21; Fig. 3*B*). Using a probe encoding this region for gel shift analyses, we showed that Tlx can specifically recognize and bind to this sequence *in vitro* (Fig. 3*C*), indicating that the Pax2 repression may be direct.

Using a mouse Pax2 promoter fused to luciferase, we demonstrated that cotransfection with Tlx in CV1 cells results in repression of the basal activity of the reporter gene (Fig. 3*D*). To confirm that the interaction is direct, we conducted a ''one-hybrid'' experiment in which Tlx was fused to the herpes virus VP16 activation domain (Tlx-VP) or to the EnR (Tlx-EnR). Cotransfection of the Tlx-VP construct indeed resulted in strong

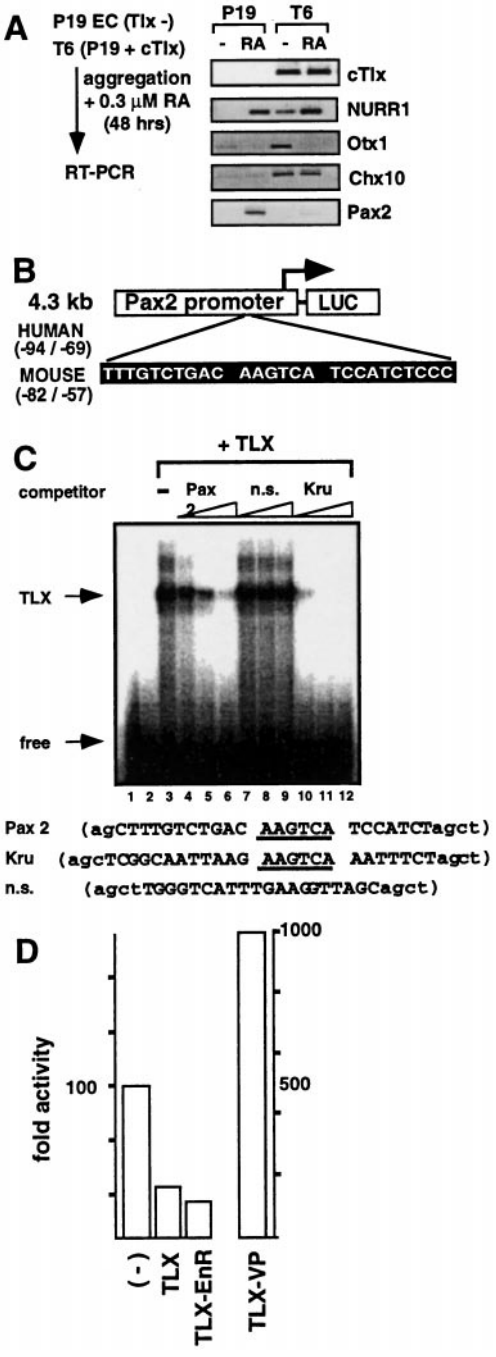

Fig. 3. Tlx regulates Pax2 expression. (*A*) RT-PCR analyses detected up-regulation of several transcription factors such as Otx1, Chx10, and NURR1 in Tlx-positive lines. Repression was observed with Pax2 in retinoic acid (RA)-treated Tlx-positive lines (compared with parental P19 Tlx-negative lines). EC, embryonal carcinoma. (*B*) A Tlx consensus binding site is conserved in human and mouse Pax2 regulatory regions just upstream of the TATA box (≈80 bp). kb, kilobase. (*C*) *In vitro* DNA-binding specificity of Tlx to conserved site in Pax2 promoter. Control lanes (1, probe alone; 2, probe plus control extract) show no binding. Specific protein–DNA complex is formed in the presence of *in vitro* translated Tlx protein and labeled Pax2 promoter probe (lane 3). This complex can be competed by addition of cold Pax2 promoter probe (lanes 3–6) and a probe encoding the Tll binding site identified in the Kruppel (Kru) gene (lanes 10–12) but not by probes that do not encode an AAGTCA site (lanes 7–9; n.s., nonspecific). Cold competitor probes were added at 6-, 30-, and 60-fold excesses. (*D*) Transient transfection assays in CV1 cells show that Tlx can repress the activity of the mouse Pax2 promoter driving a luciferase reporter gene. Tlx-VP results in activation, whereas Tlx-EnR mimics the repression seen with wild-type Tlx.

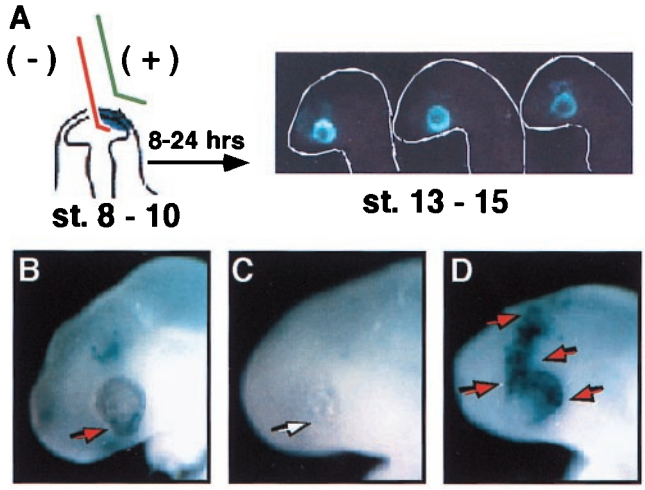

Fig. 4. Tlx can regulate the activity of mouse Pax2 promoter *in ovo*. A mouse Pax2 promoter fused to β-galactosidase (β-gal) construct was introduced alone or in conjunction with pCAGGS-Tlx and pCAGGS-Tlx-VP into the optic vesicles of stage (st.) 9 chick embryos by electroporation. Embryos were stained for β-gal after 24 hrs. (*A*) Schematic of placement of DNA solution (blue dye) and electrodes for introduction of DNA (*Left*). Codelivery of GFP allows easy assessment of the targeting efficiency (*Right*). (*B*) Pax2-lacZ results in positive β-gal staining in ventral retina (arrow). (*C*) The addition of Tlx (or Tlx-EnR, not shown) results in suppression of Pax2-lacZ activity. (*D*) Tlx-VP promotes strong ectopic Pax2-lacZ activity in dorsal retina and diencephalon (arrows).

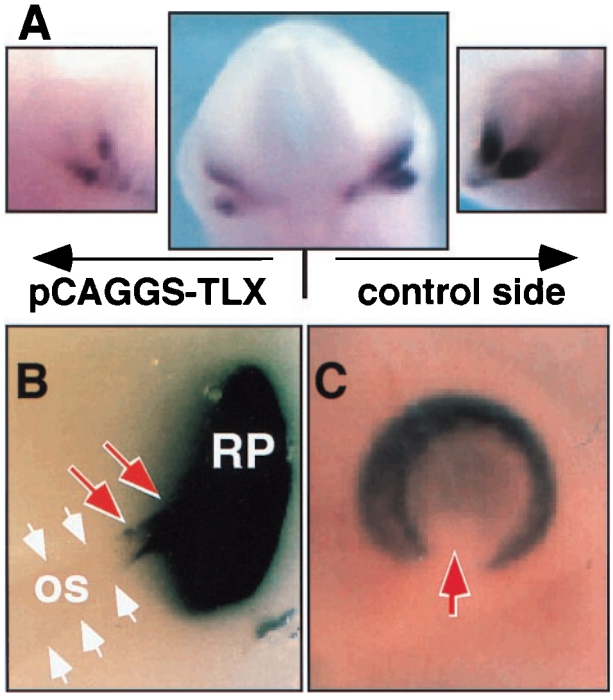

Fig. 5. Ectopic expression of Tlx can repress endogenous Pax2. Optic vesicles of stage 9 chick embryos were unilaterally targeted with pCAGGS-Tlx. (*A*) Embryos were allowed to develop for 12–24 hrs and then fixed and processed for whole-mount *in situ* hybridization with chick Pax2 antisense RNA probes. The level of Pax2 expression is decreased in the ventral retina of the targeted eye (*Left* shows lateral view). Embryos electroporated with Tlx-EnR and Tlx-VP were allowed to develop up to day 4 before fixation and processing for histology. (*B*) Ectopic expansion of pigmented retinal (RP) cells into the optic stalk (OS) region was observed with Tlx-EnR and Tlx (not shown). (*C*) Embryos targeted with Tlx-VP had a widening of the optic fissure (nonpigmented region).

activation of the Pax2 promoter, and reciprocally, the EnR fusion resulted in repression, mimicking the wild-type Tlx activity. The ability of Tlx (full-length, Tlx-VP, and Tlx-EnR) to regulate the mouse Pax2 promoter could also be observed in other cells, including NIH 3T3 and primary cultures of chick fibroblasts and retina cells (data not shown). These observations further support the conclusion that the repression is direct and does not depend on cell type.

Tlx Can Regulate the Mouse Pax2 Promoter in Chick Embryos. To determine whether Tlx could regulate transcription through the Pax2 promoter construct *in vivo*, stage 9 (15) chick embryos were electroporated with a DNA solution containing the mouse Pax2 promoter fused to lacZ, either alone or in conjunction with the expression plasmids mentioned above encoding wild-type Tlx, Tlx-EnR, or Tlx-VP. The efficiency of the targeted DNA incorporation was assessed by codelivery of a GFP expression vector (Fig. 4*A*). After a 24-hr incubation to allow protein expression, electroporated embryos were assayed for β-gal activity. Although some background expression in regions that do not normally express Pax2 was observed with the mouse promoter in chick embryos, overall, the β-gal signals were consistent; 80% (28 of 35) of optic vesicles targeted with Pax2-lacZ showed β-gal activity mainly in the ventral retina (Fig. 4*B*, see arrow). Coexpression of Tlx or Tlx-EnR resulted in dramatic reduction of Pax2 promoter activity (31 of 45 embryos), often accompanied by morphological changes including the loss of ventral retinal characteristics (Fig. 4*C* and data not shown). In contrast, robust ectopic expression of the Pax2-lacZ construct was promoted by Tlx-VP (19 of 25 embryos; Fig. 4*D*). Embryos that received the same concentrations of a control vector plasmid had no anomalies (15 of 15 embryos), indicating that the perturbance of eye morphology is specific to Tlx.

Ectopic Expression of Tlx Can Repress Endogenous Chick Pax2. We next examined whether electroporation of the Tlx constructs could alter endogenous Pax2 expression. Tlx DNA was targeted unilaterally into stage 9–10 optic vesicles *in ovo*, and embryos were allowed to develop until stage 14–15 (approximately 12–16 hrs). Keeping the total concentration of the DNA constant (3.5 mg/ml), we delivered varying amounts of Tlx DNA (0, 1, 1.5, and 3 mg/ml) together with control expression vector (pCAGGS) (3.0, 2, 1.5, and 0 mg/ml) and with GFP DNA (0.5 mg/ml) to monitor the electroporated DNA expression. Embryos were allowed to develop for 8 hrs; then those with appropriate expression of GFP in one eye (see Fig. 4*A*) were selected and processed for *in situ* hybridization. At the highest concentration of Tlx DNA (3 mg/ml), the repression of Pax2 was almost always accompanied by malformations of the eye, namely, loss of typical ventral retinal morphology (15 of 20 embryos). Lower concentrations of Tlx DNA (1.5 and 1 mg/ml) resulted in partial repression of Pax2 and fewer morphological defects (Fig. 5*A* and data not shown; 24 of 30 and 21 of 30 embryos, respectively), suggesting that the ability of Tlx to repress Pax2 may be dosage-sensitive.

Although our electroporation assay promotes mainly transient expression of ectopically introduced DNA, with peak expression obtained at about 24 hrs (12), we examined the morphological consequences in vesicles and associated structures allowed to develop until embryonic day 4. In about 65% (13 of 20) of embryos that were targeted with Tlx or Tlx-EnR, ectopic expansion of pigmented cells extending into the optic stalk was observed (Fig. 5*B*). In contrast, embryos injected with Tlx-VP (11 of 20) had a widening of the optic fissure region and a reduction of pigmented epithelium (Fig. 5*C*).

The retinal and optic stalk phenotypes described above are

reminiscent of those reported for the loss and gain of Pax2 function in zebrafish, mice, and humans. Lack of Pax2 function has been shown to result in optic nerve colobomas caused by failure of the optic fissure to close (18, 22–24). In zebrafish, it has been shown that gain of Pax2 function results in an expanded optic stalk and is accompanied by a down-regulation of Pax6 expression (24, 25). This inverse relationship between Pax2 and Pax6 expression has been shown to be critical for partitioning the optic primordia into optic stalk and retinal tissue. We have not been able to observe any significant effect on Pax6 expression by Tlx (data not shown), suggesting that their pathways do not converge except indirectly through Pax2.

In mice, Pax2 has been shown to be required for the establishment and maintenance of proper optic stalk connections as well as appropriate retinal morphology; in the absence of Pax2, the optic nerve is formed but cannot be maintained (23). The degenerate optic nerves observed in the Tlx mutant mice present the possibility that correct interaction or balance between Pax2 and Tlx may be required for proper maintenance of optic nerve function.

In *Xenopus*, injection of a construct encoding the Xtll-DNA binding domain fused to EnR into two-cell stage embryos was found to inhibit eye vesicle formation (5). Assuming that Xtll, like its avian and mammalian counterparts, acts as a repressor, Xtll-EnR should mimic the action of the wild-type receptor; however, no phenotype was obtained with the wild-type Xtll (5). One explanation may be that the timing of the ectopic expression precedes the normal expression of endogenous Tlx by too long a period, and thus, the targeted tissues are not yet competent to respond to signaling by Tlx, i.e., absence of required cofactors or ligands. The ability of Xtll-EnR to inhibit eye vesicle formation supports a crucial role for binding and repression through the Tlx target site within the signaling pathways involved in eye morphogenesis. However, in avians and mammals, it seems that Tlx function is required after optic vesicle formation. The results obtained in *Xenopus* suggest the existence of other Tlx-related receptors, which may be involved in regulating the earlier steps of eye induction.

In *Drosophila*, *tailless* is not only a gap gene involved in segmentation, but, as recent studies also have found, it is essential for embryonic visual development as well (26). Given that *tll*/Tlx and Pax2 related genes are highly conserved and implicated in animal eye development, our results showing that Pax2 is a direct target of Tlx suggest the importance and conservation of this regulatory pathway in evolution. Determining whether the retinal degeneration observed in the Tlx mutant mice may result from abnormal up-regulation of Pax2 during embryonic or postnatal development, thus leading to an imbalance resulting in retinal defects secondary to an optic stalk/optic nerve defect, requires further detailed analysis. Although we are still far from understanding the precise role and the extent and significance of the interaction between Tlx and Pax2, these findings present us with the initial clues and tools to dissect some of the signaling pathways underlying retinal morphogenesis in vertebrates.

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