CMLS, Cell. Mol. Life Sci. 57 (2000) 186–194
1420-682X/00/020186-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Regulation of eye formation by the *Rx* and *pax6* homeobox genes

P. H. Mathers[a,*] and M. Jamrich[b]

[a]Departments of Otolaryngology, Biochemistry, and Ophthalmology and the Neuroscience Graduate Program, West Virginia University School of Medicine, P.O. Box 9200, Morgantown (West Virginia 26506, USA), Fax + 1 304 293 2902, e-mail: pmathers@wvu.edu
[b]Departments of Cell Biology and Molecular and Human Genetics, Baylor College of Medicine, T707, One Baylor Plaza, Houston (Texas 77030, USA)

Abstract. The *Rx* and *pax6* homeobox genes are among the earliest genes expressed in the eye primordia and play crucial roles in the specification of ocular fate. These genes exhibit strong conservation of sequence and expression patterns among vertebrates. As transcription factors, Rx and Pax6 exert their effects through the activation and repression of downstream targets. Overexpression of each protein induces ectopic retinal tissue formation, as well as induction of the other. *pax6* gene mutations have been correlated with an array of human diseases, and a similar array of mutations potentially exists for the human *Rx* gene. Based on functional studies, the vertebrate *Rx* and *pax6* genes are likely to regulate cell proliferation and are required for the initial commitment to retinal and lens cell fate, respectively, while *pax6* appears to play additional roles in the formation of the retina and cornea.

Key words. Eye; development; embryo; Rx; Pax6; homeobox; transcription factor.

Cloning and structure

With the first papers published in 1991, the history of *pax6*-related research is much better elaborated than that of *Rx*-related work, which began in 1997. Identified initially in mice and humans [1, 2], *pax6* has gone through two 'bursts of cloning,' the first in a series of vertebrate species. After the discovery that the *Drosophila pax6* gene corresponds to the *eyeless* mutation [3], a second, invertebrate 'wave of cloning' has started, leading to the identification of *pax6* genes in nematodes, squid, ascidian, *Amphioxous*, and planaria, as well as a second *pax6* gene in *Drosophila* [4–10]. In comparison, the initial expression patterns and functional studies for *Rx* were limited to three independent reports on both vertebrate and invertebrate *Rx* genes [11–13].

The *pax6* and *Rx* genes belong to a large family of factors that are related to the homeodomain region of the *Drosophila* Paired protein [14]. The homeodomain confers DNA-binding capabilities to these factors through a helix-turn-helix structure at the carboxy-terminal end of the homeodomain. *pax* class genes, including the *paired* gene itself, are characterized by a second DNA-binding motif, referred to as the *paired* domain. When the homeodomain is present in Pax class proteins, it carries a serine at position 9 of the third helix. In contrast, the Rx protein (along with several related homeodomain proteins) contains only a *paired*-like homeodomain, with a glutamine at position 9 of the third helix, but no *paired* domain. In addition, the Rx proteins contain small regions of conserved sequence, including the octapeptide and the *paired* tail (or OAR domain) [12, 13]. In all species studied to date except holometabolous insects and zebrafish [10, 15], there is only one *pax6* gene, and its gene copy number is tightly controlled, as demonstrated by the haploinsufficiency seen with *pax6* mutants (see below) and overexpression phenotypes [16]. *Rx*, on the other hand, demonstrates a range of known gene copy numbers, from one in mammals and flies to three in zebrafish. In the case of

* Corresponding author.

zebrafish, the composite embryonic expression pattern of the three *Rx* genes is strikingly similar to the pattern of single-copy *Rx* species, suggesting a diversification of *Rx* gene functions after gene duplication [13].

Embryonic expression patterns

At the beginning of vertebrate neurulation, *Rx* and *pax6* are among a set of genes expressed in a broad, overlapping domain called the anterior neural plate [17]. This region contains the primordial forebrain and a portion or all of the future midbrain. These genes are strongly expressed in the retinal field, which at early stages extends across the midline [11–13, 18, 19] (fig. 1A,B). Additional areas of gene expression exist outside the retinal field for both genes. *Rx* is also expressed in the pineal gland and the ventral neural tube, which gives rise to the hypothalamus and pituitary gland, but is excluded from lens and corneal precursor cells [11–13, 20–22]. *pax6* shows expression in the surface ectoderm corresponding to the lens primordia [19, 23, 24], as well as the olfactory epithelium, presumptive dorsal forebrain, hindbrain, and spinal cord [1] (fig. 1C). As development proceeds, expression of these genes becomes more spatially restricted to distinct domains within the anterior neural plate, and both are down-regulated along the midline, presumably through the actions of Hedgehog, transforming growth factor-β, and epidermal growth factor family members [25–30]. The result of this regulation at the midline is bilaterally symmetric optic vesicles (fig. 1D). Disruptions in the formation and evagination of the optic primordia can result in defects in both ocular and craniofacial development [13, 31, 32].

From the earliest stages of retinal specification to the final mitotic divisions in the differentiating retina, *Rx* and *pax6* are expressed in proliferating cells. High levels of *Rx* expression are found in retinal progenitor cells in the frog, fish, and mouse [11–13]. During retinal differentiation, both genes are expressed in the *Xenopus* retinal ciliary margin [11, 13, 19], the only region in the differentiated frog retina that possesses mitotic retinal progenitor cells [33–36]. *Rx* and *pax6* transcripts are

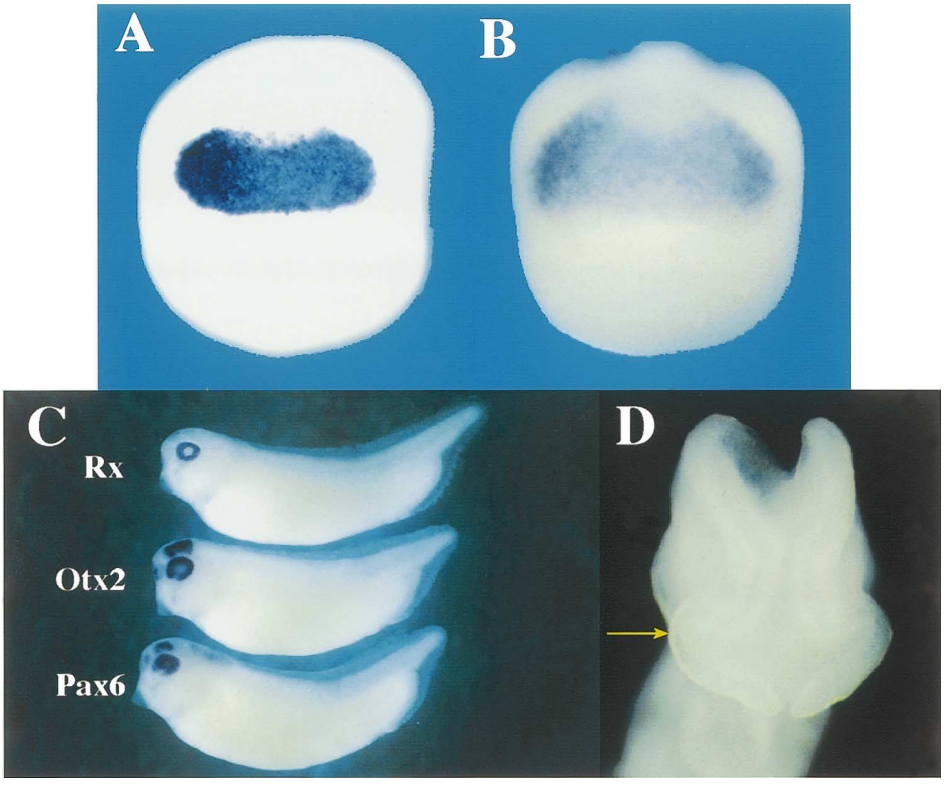

Figure 1. Expression of *Rx* and *pax6* during early development of the eye. (*A*) Pattern of *Rx* expression in early *Xenopus* neurulae as indicated by whole-mount *in situ* hybridization staining. (*B*) Pattern of *pax6* expression in early *Xenopus* embryo. Note the overlapping expression between *Rx* and *pax6* in the anterior neural plate region. (*C*) Comparison of expression patterns for the *Rx*, *otx2*, and *pax6* genes in stage 35 *Xenopus* embryos. (*D*) Frontal view of an unstained E9 mouse embryo showing optic vesicle formation (arrow) prior to neural tube closure.

also found in the proliferating cells at the ventricular zone of the developing mouse retina [1, 12, 13]. A thorough analysis of the frog ciliary margin found that *Rx* and *pax6* (along with the *six3* homeobox gene) are the only transcription factors out of a group of 15 analyzed that are expressed in the most peripheral region [37]. This is the domain where the self-renewing retinal stem cells are found. Similarly in zebrafish, *pax6* [38] and the *Rx1* and *Rx2* genes [20] are expressed in the retinal progenitor cells of the embryonic retinal primordia and in the ciliary margin of the differentiated retina, where self-renewing stem cells are found in fish [39, 40], as in frogs. These expression patterns suggest a role for these genes in regulating retinal stem cell proliferation and fate.

The parallels between *Rx* and *pax6* expression seen in the vertebrate retina do not extend to the *Drosophila* compound eye. Despite the stronger sequence conservation from flies to vertebrates for the *Rx* versus *pax6* homeodomains (97–100% vs 90%), *Rx* is not expressed in the fly eye-antennal imaginal disc [13, 41]. The *Drosophila Rx* gene and both *pax6* genes [*eyeless* and *twin of eyeless* (*toy*)] are all expressed in the anterior region of the early embryo [3, 10, 13, 41], in a region fated to give rise to the eye-antennal disc [42]. Once definitive eye primordia are established, however, *Rx* expression is lost in the eye, but remains in the brain and the clypeolabrum [13, 41]. In comparison, both *pax6* genes continue to be expressed in the eye-antennal disc primordia, as well as the third-instar disc itself [3, 10]. The expression pattern of *eyeless* and *toy* within the third-instar, eye-antennal disc corresponds to the undifferentiated, proliferating cells in front of the morphogenetic furrow. While the role of *Rx* in *Drosophila* eye development has been called into question [41], it is possible that *Rx* acts in the initial specification of the fly eyes, but plays no role in the subsequent proliferation or differentiation of these cells. Development of a *Drosophila Rx* mutant strain would help to clarify this issue.

Adult expression patterns

Like many other homeobox-containing genes, *pax6* and *Rx* appear to have multiple functions during development. In addition to its roles in early ocular cell specification and proliferation, *pax6* shows continued expression in the mature retina, lens, conjunctiva, and corneal epithelium [19, 38, 43, 44]. Within the adult neural retina in mammals, *pax6* transcripts are localized to the ganglion cell layer and amacrine cells within the inner nuclear layer [43, 44]. While the targets of this expression have yet to be elucidated within the retina, we can assume that *pax6* regulates expression of cell-type-specific markers in these layers.

While original reports suggested that *Rx* was not expressed in the differentiated retina, we have determined that *Rx* expression continues throughout postnatal mouse development (V. Voronina and P. H. Mathers, unpublished observations). Levels of *Rx* RNA are high during embryogenesis, but diminish at or near the time of terminal differentiation [12, 13], then recover to abundant levels in the adult human and mouse retina [45] (V. Voronina and P. H. Mathers, unpublished observations). The zebrafish *Rx1* and *Rx2* genes, which are specifically expressed in retinal primordia during embryogenesis [13, 20], are selectively activated in cones within the adult eye [20]. This is the first report of a cone-specific transcription factor. The zebrafish and medaka *Rx3* genes are also expressed in the mature retina; however, their expression is restricted to the inner nuclear layer [20, 21]. As seen with the embryonic expression, the composite pattern of these three genes is similar to the expression of *Rx* in postmitotic frog retinal cells, with low transcript levels found in the photoreceptor and inner nuclear layers [37]. In contrast, the mouse and human *Rx* genes are expressed in all three neural retinal layers [45] (V. Voronina and P. H. Mathers, unpublished observations). Therefore, the pattern of *Rx* expression within the adult retina differs across species and between different genes.

Downstream target genes

Induction of *Rx* and *pax6* during early embryogenesis initiates a cascade of gene activation and repression that leads to the formation of the mature eye. Determining the mechanism of this initiation of ocular development is one of the major goals of numerous research groups. By determining the downstream targets for *Rx* and *pax6*, the primary and secondary components of the cascade that creates the eye may be identified.

Since *Rx* was originally cloned through its ability to bind to a defined oligonucleotide sequence [46], it would not be unreasonable to expect that binding sites and downstream targets would be identified for the Rx protein. To date, this has not been the case, primarily due to the nature of the Rx-binding site. Rx is able to bind to the core homeodomain binding consensus sequence, TAAT, as are almost all other classes of homeodomain-containing proteins. Therefore, the specificity and significance of any binding interaction must be interpreted cautiously. Only recently, a report has identified vertebrate Rx as binding to the Ret1/PCE1 site found in a wide variety of promoters for photoreceptor-specific genes [45]. The Ret1 binding site consists of CTAATTG, which includes the homeodomain core consensus [47, 48]. Through its binding to the Ret1/PCE1 site, Rx is able to directly activate the transcrip-

tion of the mammalian *β-arrestin* and *IRBP* retinal genes [45]. However, recent reports suggest that two other homeodomain proteins, Erx and Crx, are also able to bind to the Ret1/PCE1 site and function as transcriptional activators [49–51]. Therefore, a complex interaction or competition between multiple regulatory factors is likely to govern transcriptional activation and quantitative output from these target promoters.

The ability of Rx to activate the *β-arrestin* and *IRBP* genes suggests a function for the *Rx* gene in the adult, but what are the targets of the Rx protein in activating retinogenesis early in development? This aspect of Rx function has been studied recently by Barssacchi and colleagues. By injecting *Xenopus Rx1* RNA into embryos and analyzing the molecular responses, Rx was found to induce ectopic expression of *pax6* and *six3*, albeit at late embryonic stages, while repressing the activity of the p*ax2* and *En* genes in the midbrain-hindbrain region [52]. In a complex pattern of repression and activation, *Rx* injection resulted in a reduction of *otx2* and *XAG-1* RNA levels in early embryos, but later induced ectopic expression of these genes. These and other [13] studies suggest that Rx is able to induce retinogenesis, either by specifying retinal fate and/or by activating retinal stem cell proliferation. While it is not known whether these responses are direct or indirect effects, their significance is clear based on the nature of the responsive genes. We should not interpret these results to mean, however, that *Rx* is necessarily upstream of *pax6* and *six3*, only that it has inductive activity.

While Rx targets are now beginning to be identified, the role of *pax6* in activating transcription has been established for several years. For *pax6*, regulatory targets include both lens-specific and retinal-specific promoters. The binding site for Pax6 is complex, due to the presence of both the *paired* domain and homeodomain in the protein. Consensus binding sites for the *paired* domain and the homeodomain of Pax6 have been determined [53, 54]. Pax6 is able to bind to these sites within the various *crystallin* promoters and activate their transcription [55]. However, Pax6 binding in the case of the *β-crystallin* promoter acts to inhibit rather than activate transcription [56]. In the cornea, Pax6 has a direct stimulatory effect on the transcription of the corneal-specific *K12* keratin gene [57, 58].

The role for Pax6 in controlling vertebrate retinal transcription is less well established than for other tissues, but there are numerous Pax6 (*eyeless*)-binding sites in photoreceptor-specific promoters in fruit flies [59]. Given the strong conservation of activity between vertebrate and invertebrate Pax6 proteins, it is formally possible that targets in adult photoreceptor genes will also be identified for vertebrate Pax6. However, *pax6* shows a weak or absent expression pattern in mature photoreceptor cells [19, 38, 43, 44] and there is a shift away from photoreceptor development when *pax6* is overexpressed [16]. These findings suggest that *pax6* will not play an important role in controlling vertebrate photoreceptor-specific gene expression and, instead, downstream targets in the vertebrate retina are likely to be restricted to ganglion cells and amacrine cells.

Embryonic targets of Pax6 have been determined in both flies and vertebrates. The second *Drosophila pax6* gene, *toy*, has been proposed to act at the top of the gene cascade that leads to eye formation [10]. *toy* can activate *eyeless*, which in turn acts to induce *sine oculis*, *eyes absent*, and *dachshund* [10]. These three factors then work in a feedback regulatory loop to control *eyeless* expression, but not that of *toy*. A similar feedback mechanism has been proposed in vertebrates based upon evidence from medaka *six3* and *Xenopus pax6* RNA injection studies. In both cases, overexpression of the appropriate RNA induces ectopic *Rx* gene expression, as well as *pax6* expression when *six3* is injected [60], or *six3* expression when *pax6* is injected [61]. When the results of *Rx* injection are included [52], it appears that all three genes, *Rx*, *pax6*, and *six3*, can activate the transcription of each other. Presumably, other targets will be identified that lead to structural changes in the formation of the optic vesicle and lens placode, and that commit these tissues towards ocular differentiation.

Functional studies and disease correlation

A combination of genetic and molecular techniques has allowed significant progress in establishing a function for the *pax6* and *Rx* genes in vertebrate eye development. Determination of *pax6* function began from the study of two autosomal semidominant mutations, aniridia in humans and *Small eye* in mouse. Both mutant phenotypes result from haploinsufficiency at the *pax6* locus. The human *pax6* gene is located within the region responsible for aniridia [2]. This mutation is characterized by a lack of iris formation and is frequently associated with corneal opacification and cataracts. In addition, the mouse *pax6* gene is the target for mutations leading to the similar *Small eye* phenotype [62]. Specific mutations in the human *pax6* gene have been shown to lead to aniridia and cataract formation [63–65], and Peter's anomaly [66].

In a striking conservation of function, a complete loss of *pax6* function in humans, mice, and fruit flies leads to the failure to form an eye [3, 64, 67]. In addition to ocular defects, the mammalian *pax6* mutants show ablation of the olfactory epithelium and a failure to fuse the anterior neural tube, resulting in severe craniofacial anomalies [31, 32]. Analysis of homozygous *Small eye* mouse embryos has clearly demonstrated that the pri-

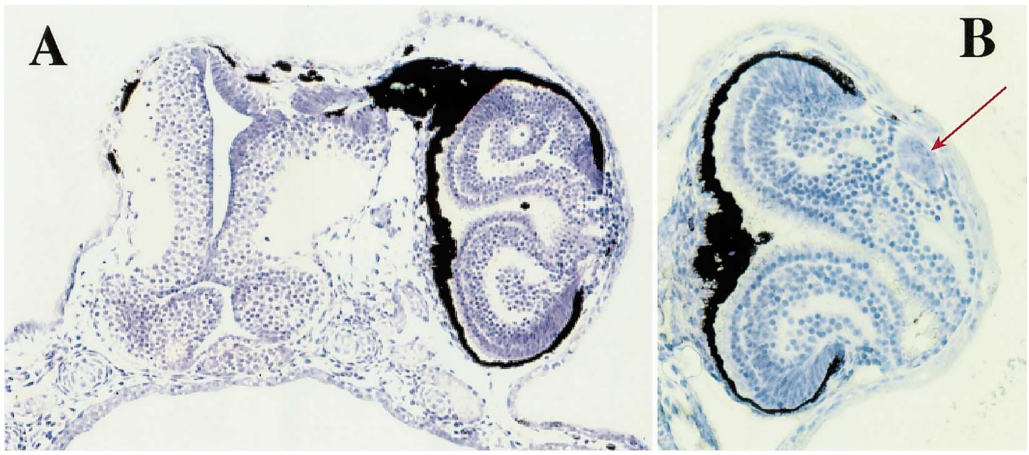

Figure 2. *Rx* overexpression defects. (*A*) Injection of *Rx* RNA causes hyperplasia of the neural retina, pigmented retina, and neural tube. One dorsal blastomere of a four-cell-stage *Xenopus* embryo was injected with 200 pg of synthetic *Rx* RNA. Embryos were grown for 4 days, sectioned, and stained. (*B*) Eye resulting from *Rx* RNA injection showing retinal hyperproliferation, at the expense of lens development. Arrow points to what remains of lens tissue. Note the folding of the photoreceptor layer out to the anterior margin of the eye.

mary defect in ocular development is the failure of the surface ectoderm to form a lens placode [24, 31, 67–69]. Subsequently, the optic vesicle fails to progress to form the optic cup, and degeneration occurs, resulting in anophthalmia. *pax6* certainly plays a role in specifying lens cell fate and in regulating later retinal and corneal cell fate decisions. However, the ability of homozygous *Small eye* embryos to form an optic vesicle shows that the vertebrate *pax6* gene is not required for the initial formation of the optic vesicle or for the movement of the vesicle toward the presumptive lens ectoderm.

pax6 is able to induce defects in eye formation not only when mutated, but also when overexpressed. The most notable of the overexpression studies are those performed in flies, with *Drosophila*, mouse, and squid *pax6* genes capable of inducing ectopic eye formation in multiple adult tissues [6, 10, 70]. The phenotype of transgenic mouse *pax6* expression does not lead to ectopic eyes, but does rescue the *Small eye* phenotype [16]. These transgenic studies reveal an interesting aspect of *Pax6* gene regulation—overexpression can also cause a complex set of ocular abnormalities, including in one line, the ablation of the photoreceptor layer of the neural retina [16]. Surprisingly, these defects are restricted to the eye. Overexpression of *pax6* by RNA injection into early *Xenopus* embryos is able to induce ectopic lens formation [71]. Although similar results were not observed initially for the retina [13, 19, 71], there is now a report describing the induction of ectopic eyes upon *pax6* injection [61]. Adjacent retina and lens are induced ectopically by *pax6*, as are *Rx* and *six3* gene expression [61]. It remains to be fully explained why this phenotype was not observed previously, but the implications are that *pax6* may possess a similar ability in both invertebrates and vertebrates to activate the entire repertoire of genes required for eye formation.

The embryonic expression pattern of the *Rx* gene, from its initial activation to its localization in the stem cells of the differentiated retina, suggests a regulatory role during retinal proliferation. Overexpression results are consistent with a model where *Rx* is regulating ocular and neural cell proliferation. When RNA is microinjected into developing frog embryos, *Rx* is able to induce hyperproliferation of both the neural and pigmented retina [13, 52] (fig. 2A,B). A hyperproliferative phenotype is also observed for a restricted portion of the neural tube (fig. 2A), suggesting that *Rx* may induce cell proliferation in multiple cell types within the anterior neural plate. In rare cases, pigmented retinal epithelial cells are observed in ectopic locations within the head, suggesting that *Rx* is able to induce localized centers of retinal specification [13]. Due to excessive production of retinal cells the lens fails to differentiate properly [13], but it is uncertain whether this results from aberrant recruitment of prospective lens ectoderm toward a retinal fate or the loss of differentiating signals for lens development from specific cells within the retina. The injection phenotypes require the presence of either a competence to form retinal and neural tissue or additional cofactors, as expression of *Rx* RNA in cells that are not fated to give rise to the anterior neural plate gives no phenotype [13, 52].

If *Rx* functions to induce or maintain retinal and neural cell proliferation, then a loss of *Rx* gene activity would likely result in the failure of proper retinal and forebrain development. This is the case, as observed in a strain carrying a targeted deletion of exons 1 and 2 of the mouse *Rx* gene [13]. Embryos with both copies of the *Rx* gene deleted show a complete loss of retinal morphology, including a loss of the early optic pit and vesicle. The *Rx*-knockout phenotype is the earliest known defect in mouse retinal development, preceding those of other anophthalmic or microphthalmic mutations, including *Small eye* and *ocular retardation*, and targeted deletions of *Lhx2*, *BMP-7*, and *HES-1* [67, 72–76]. Unlike embryos carrying the targeted *Rx* deletion, embryos from each of these mutations exhibit optic vesicle formation, though not all progress to form the optic cup.

Embryos and newborn pups from the *Rx* deletion strain also exhibit defects in formation of the ventral neural tube. Based on the expression of *Rx* in the early forebrain and subsequently in proliferating cells of the hypothalamus and posterior pituitary, we might expect to see these regions affected most in *Rx* mutants. Indeed, sections through the cranial region of *Rx*-null pups at birth reveal a range of neural and facial defects, including defects in the formation of the eye, hypothalamus, pituitary, and palate [13]. Given their localized expression in proliferative tissues of the head, the *Rx* genes are likely to play roles in regulating cell fate decisions within the hypothalamus and the pituitary gland, in addition to their roles in retinal stem cell proliferation and in early embryonic patterning of the anterior neural tube.

A similar phenotype as that seen with the *Rx*-knockout has been observed when *Xenopus* embryos are injected with repressive or defective versions of *Rx* RNA [52]. When the repressor region of the *engrailed* gene is fused to *Rx* to create a dominant-negative protein with the binding affinity of *Rx*, the resulting embryos show strong defects in eye and neural development. When the normal *Rx* gene is truncated at its end to remove a conserved element called the *paired*-tail or OAR domain [12, 13], a less severe ablation of the eyes and neural tube is observed [52]. From these overexpression and deletion studies, we can conclude that *Rx* must play a crucial role in establishing and regulating early cell fate decisions within the anterior neural plate.

Unlike the situation for *pax6*, *Rx* heterozygous mice have no discernible phenotype, and it remains to be seen if *Rx* mutants in humans (heterozygous or homozygous) will have a phenotype. Three lines of evidence suggest that *Rx* will play a role in human disease formation. First, the mouse knockout gives a strong anophthalmic phenotype [13], suggesting that *Rx* may have a similar effect in humans. Second, Glaser and colleagues have preliminary evidence that the *eyeless* mutation in mice is the result of a missense mutation in *Rx* [77]. The viability of this strain as homozygous mutants, as compared to the *Rx* deletion, indicates that mutations at the human *Rx* locus may lead to viable anophthalmic or microphthalmic patients. Finally, the human *Rx* locus maps to the same interval identified for cone-rod dystrophy-1 (*CORD1*) [78] on chromosome 18 (E. Kozhemyakina and P. H. Mathers, unpublished observations). Based on the correlation between the *crx* homeobox gene and the *CORD2* locus on chromosome 19 [79, 80] and Leber's congenital amaurosis [81], there is a good chance that *Rx* is the *CORD1* gene. Since *Rx* appears to function in the adult retina by activating *β-arrestin* and *IRBP* [45], it is reasonable to assume that a retinal dystrophy phenotype will result when the adult function of the *Rx* gene is altered. A positive correlation between *Rx* and *CORD1* would help to determine the function for the mammalian *Rx* gene in postembryonic retinal development. Analysis of potential *Rx* mutations in anophthalmic, microphthalmic, and CORD patients is currently underway.

Specification of the vertebrate eye

The ever-increasing number of transcription factors involved in early eye development suggests that accurate models for how the eye is constructed will be extremely complex and are likely to be years away. In addition, the intricate autoregulatory and cross-regulatory relationships among these factors compound the complexity associated with early embryonic events. Therefore, we venture into model building with some trepidation. Starting as early as gastrulation in vertebrate embryogenesis, a collection of transcription factors is activated in the presumptive anterior neural ectoderm, including *otx2*, *six3*, and *anf* family members [82–87]. By the beginning of neurulation, these genes are joined in their anterior neural plate expression by *Rx* and *pax6*, among others. The expression patterns of these factors are overlapping in the region fated to become the retina, suggesting that each may play a role in either activating or repressing retinal specification. The question becomes whether any of these factors are sufficient to act independently to specify ocular fate.

Based on the ectopic eye formation in flies caused by *eyeless* and mouse *pax6* [70] and the closely related *toy* [10], *pax6* has been proposed as the master regulator of eye development [88]. The evidence for this conclusion is compelling in *Drosophila*, with *toy* being capable of inducing *eyeless* expression, but insensitive to feedback regulation by the factors *eyeless*, *sine oculis*, *eyes absent*, or *dachshund* [10]. The case in vertebrates for *pax6* regulating all eye specification is less convincing than

that found in flies, although the ability of *pax6* to induce ectopic eyes when overexpressed [61] is certainly intriguing. Of the factors expressed in the anterior neural plate, *pax6* is the last to be activated in the presumptive retinal field, placing it in a temporal position that would be unlikely for the initial induction of *Rx* and *six3*. In addition, the phenotype of the *Small eye* mouse mutant suggests that *pax6* is responsible initially for the formation of lens tissue, and that other factors regulate optic vesicle formation [24, 31, 67, 69]. Finally, the cross-regulation exhibited by overexpression of *Rx*, *pax6*, and *six3* [52, 60, 61] suggests that these genes may be of equal stature with regards to their importance in eye formation. With all of the functional information gathered for these early genes, however, a clear genetic hierarchy has yet to be established that will serve as a blueprint for the construction of the eye.

While complex relationships, such as the *Rx*/*pax6*/*six3* cross-regulation, are best analyzed in genetic organisms such as *Drosophila*, the role of the Rx protein in fruit fly eye development is debated [13, 41]. Therefore, we must rely on gene expression studies in mutant mouse strains until other vertebrate systems, such as zebrafish, provide us with the tools to analyze these genetic relationships. While such studies are being completed, our unpublished observations on the kinetics of gene induction in response to a neural stimulus may provide some clues as to the nature of eye induction. In those studies, uncommitted *Xenopus* ectoderm was treated with NH_4Cl, which specifically induces early anterior markers [13, 17, 85, 89]. *Rx* and *pax6*, along with *otx2* and *xanf2* (*six3* was not analyzed), were induced with similar, early activation profiles by NH_4Cl treatment. This implies that the earliest expression of these factors in the anterior neural tube is a direct response to anterior neural inducers, such as chordin, follistatin, and/or cerberus [90–92], and may not follow a simple hierarchical relationship. Continued expression of these factors after this initial induction may require a complex network of self- and cross-stimulation or repression, helping to maintain distinct regions of gene expression and to establish and refine specific organ domains.

This model for vertebrate eye development would predict the absence of a single master regulator. While many of the players appear to be common in vertebrate and invertebrate eye formation, the developmental script may be edited sufficiently as to require slightly different models for the different modes of eye formation. This divergence from a unifying theory of eye formation across species may be unsettling to those who view the model systems as often interchangeable. However, the variability in *Rx* expression patterns between vertebrates and invertebrates and the presence of a second, independently functioning, *pax6* gene in insects are evidence that the program is not flawlessly conserved. Much remains to be determined in early eye development, including the downstream targets that are necessary for activation and determination of ocular cell fates, the mechanism for commitment of cells to particular differentiated fates, and the role these embryonic transcription factors play in regulating gene expression within the adult eye.

Acknowledgements. Much of the unpublished work discussed in this review was supported by a grant from the NEI (EY12152) and the Knights-Templar Eye Foundation to P.M.

1 Walther C. and Gruss P. (1991) Pax-6, a murine paired box gene, is expressed in the developing CNS. Development **113:** 1435–1449
2 Ton C. C., Hirvonen H., Miwa H., Weil M. M., Monaghan P., Jordan T. et al. (1991) Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell **67:** 1059–1074
3 Quiring R., Walldorf U., Kloter U. and Gehring W. J. (1994) Homology of the *eyeless* gene of *Drosophila* to the *Small eye* gene in mice and aniridia in humans. Science **265:** 785–789
4 Zhang Y. and Emmons S. W. (1995) Specification of sense-organ identity by a *Caenorhabditis elegans* Pax-6 homologue. Nature **377:** 55–59
5 Chisholm A. D. and Horvitz H. R. (1995) Patterning of the *Caenorhabditis elegans* head region by the Pax-6 family member vab-3. Nature **377:** 52–55
6 Tomarev S. I., Callaerts P., Kos L., Zinovieva R., Halder G., Gehring W. et al. (1997) Squid Pax-6 and eye development. Proc. Natl. Acad. Sci. USA **94:** 2421–2426
7 Glardon S., Callaerts P., Halder G. and Gehring W. J. (1997) Conservation of Pax-6 in a lower chordate, the ascidian *Phallusia mammillata*. Development **124:** 817–825
8 Glardon S., Holland L. Z., Gehring W. J. and Holland N. D. (1998) Isolation and developmental expression of the amphioxus *Pax-6* gene (*AmphiPax-6*): insights into eye and photoreceptor evolution. Development **125:** 2701–2710
9 Callaerts P., Munoz-Marmol A. M., Glardon S., Castillo E., Sun H., Li W. H. et al. (1999) Isolation and expression of a *Pax-6* gene in the regenerating and intact planarian *Dugesia(G)tigrina*. Proc. Natl. Acad. Sci. USA **96:** 558–563
10 Czerny T., Halder G., Kloter U., Souabni A., Gehring W. J. and Busslinger M. (1999) *twin of eyeless*, a second *Pax-6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development. Mol. Cell **3:** 297–307
11 Casarosa S., Andreazzoli M., Simeone A. and Barsacchi G. (1997) *Xrx1*, a novel *Xenopus* homeobox gene expressed during eye and pineal gland development. Mech. Dev. **61:** 187–198
12 Furukawa T., Kozak C. A. and Cepko C. L. (1997) *rax*, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. Proc. Natl. Acad. Sci. USA **94:** 3088–3093
13 Mathers P. H., Grinberg A., Mahon K. A. and Jamrich M. (1997) The *Rx* homeobox gene is essential for vertebrate eye development. Nature **387:** 603–607
14 Bürglin T. R. (1994) A comprehensive classification of homeobox genes. In: Guidebook to Homeobox Genes, pp. 25–73, Duboule D. (ed.), Oxford University Press, New York
15 Nornes S., Clarkson M., Mikkola I., Pedersen M., Bardsley A., Martinez J. P. et al. (1998) Zebrafish contains two *pax6* genes involved in eye development. Mech. Dev. **77:** 185–196
16 Schedl A., Ross A., Lee M., Engelkamp D., Rashbass P., Heyningen V. van et al. (1996) Influence of *PAX6* gene dosage on development: overexpression causes severe eye abnormalities. Cell **86:** 71–82

17 Jamrich M. and Sato S. (1989) Differential gene expression in the anterior neural plate during gastrulation of *Xenopus laevis*. Development **105:** 779–786
18 Li H., Tierney C., Wen L., Wu J. Y. and Rao Y. (1997) A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. Development **124:** 603–615
19 Hirsch N. and Harris W. A. (1997) Xenopus Pax-6 and retinal development. J. Neurobiol. **32:** 45–61
20 Chuang J. C., Mathers P. H. and Raymond P. A. (1999) Expression of three *Rx* homeobox genes in embryonic and adult zebrafish. Mech. Dev. **84:** 195–198
21 Desehet K., Bourrat F., Ristoratore F., Chourrout D. and Joly J. S. (1999) Expression of the medaka (*Oryzias latipes*) *Ol-Rx3* paired-like gene in two diencephalic derivatives, the eye and the hypothalamus. Mech. Dev. **83:** 179–182
22 Ohuchi H., Tomonari S., Itoh H., Mikawa T. and Noji S. (1999) Identification of chick *rax/rx* genes with overlapping patterns of expression during early eye and brain development. Mech. Dev. **85:** 193–195
23 Li H. S., Yang J. M., Jacobson R. D., Pasko D. and Sundin O. (1994) Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. Dev. Biol. **162:** 181–194
24 Grindley J. C., Davidson D. R. and Hill R. E. (1995) The role of Pax-6 in eye and nasal development. Development **121:** 1433–1442
25 Chiang C., Litingtung Y., Lee E., Young K. E., Corden J. L., Westphal H. et al. (1996) Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. Nature **383:** 407–413
26 Macdonald R., Barth K. A., Xu Q., Holder N., Mikkola I. and Wilson S. W. (1995) Midline signalling is required for *Pax* gene regulation and patterning of the eyes. Development **121:** 3267–3278
27 Ekker S. C., Ungar A. R., Greenstein P., Kessler D. P. von, Porter J. A., Moon R. T. et al. (1995) Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. Curr. Biol. **5:** 944–955
28 Rebagliati M. R., Toyama R., Haffter P. and Dawid I. B. (1998) *cyclops* encodes a nodal-related factor involved in midline signaling. Proc. Natl. Acad. Sci. USA **95:** 9932–9937
29 Zhang J., Talbot W. S. and Schier A. F. (1998) Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. Cell **92:** 241–251
30 Gritsman K., Zhang J., Cheng S., Heckscher E., Talbot W. S. and Schier A. F. (1999) The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell **97:** 121–132
31 Hogan B. L., Hirst E. M., Horsburgh G. and Hetherington C. M. (1988) *Small eye* (*Sey*): a mouse model for the genetic analysis of craniofacial abnormalities. Development **103:** 115–119
32 Quinn J. C., West J. D. and Kaufman M. H. (1997) Genetic background effects on dental and other craniofacial abnormalities in homozygous small eye (*Pax6Sey/Pax6Sey*) mice. Anat. Embryol. (Berl.) **196:** 311–321
33 Hollyfield J. G. (1968) Differential addition of cells to the retina in *Rana pipiens* tadpoles. Dev. Biol. **18:** 163–179
34 Wetts R. and Fraser S. E. (1988) Multipotent precursors can give rise to all major cell types of the frog retina. Science **239:** 1142–1145
35 Holt C. E., Bertsch T. W., Ellis H. M. and Harris W. A. (1988) Cellular determination in the *Xenopus retina* is independent of lineage and birth date. Neuron **1:** 15–26
36 Wetts R., Serbedzija G. N. and Fraser S. E. (1989) Cell lineage analysis reveals multipotent precursors in the ciliary margin of the frog retina. Dev. Biol. **136:** 254–263
37 Perron M., Kanekar S., Vetter M. L. and Harris W. A. (1998) The genetic sequence of retinal development in the ciliary margin of the *Xenopus* eye. Dev. Biol. **199:** 185–200
38 Hitchcock P. F., Macdonald R. E., VanDeRyt J. T. and Wilson S. W. (1996) Antibodies against Pax6 immunostain amacrine and ganglion cells and neuronal progenitors, but not rod precursors, in the normal and regenerating retina of the goldfish. J. Neurobiol. **29:** 399–413
39 Johns P. R. (1977) Growth of the adult goldfish eye. III. Source of the new retinal cells. J. Comp. Neurol. **176:** 343–357
40 Fernald R. D. (1990) Teleost vision: seeing while growing. J. Exp. Zool. Suppl. **5:** 167–180
41 Eggert T., Hauck B., Hildebrandt N., Gehring W. J. and Walldorf U. (1998) Isolation of a *Drosophila* homolog of the vertebrate homeobox gene *Rx* and its possible role in brain and eye development. Proc. Natl. Acad. Sci. USA **95:** 2343–2348
42 Younossi-Hartenstein A., Tepass U. and Hartenstein V. (1993) Embryonic origin of the imaginal discs of the head of *Drosophila melanogaster*. Wilhelm Roux Arch. Dev. Biol. **203:** 60–73
43 Koroma B. M., Yang J. M. and Sundin O. H. (1997) The *Pax-6* homeobox gene is expressed throughout the corneal and conjunctival epithelia. Invest. Ophthalmol. Vis. Sci. **38:** 108–120
44 Jones S. E., Jomary C., Grist J., Thomas M. R. and Neal M. J. (1998) Expression of *Pax-6* mRNA in the retinal degeneration (*rd*) mouse. Biochem. Biophys. Res. Commun. **252:** 236–240
45 Kimura A., Singh D., Wawrousek E. F., Kikuchi M., Nakamura M. and Shinohara T. (2000) Both PCE-1/RX and OTX/CRX interactions are necessary for photoreceptor-specific gene expression. J. Biol. Chem. **275:** 1152–1160
46 Kalionis B. and O'Farrell P. H. (1993) A universal target sequence is bound in vitro by diverse homeodomains. Mech. Dev. **43:** 57–70
47 Morabito M. A., Yu X. and Barnstable C. J. (1991) Characterization of developmentally regulated and retina-specific nuclear protein binding to a site in the upstream region of the rat opsin gene. J. Biol. Chem. **266:** 9667–9672
48 Kikuchi T., Raju K., Breitman M. L. and Shinohara T. (1993) The proximal promoter of the mouse *arrestin* gene directs gene expression in photoreceptor cells and contains an evolutionarily conserved retinal factor-binding site. Mol. Cell. Biol. **13:** 4400–4408
49 Martinez J. A. and Barnstable C. J. (1998) Erx, a novel retina-specific homeodomain transcription factor, can interact with Ret 1/PCEI sites. Biochem. Biophys. Res. Commun. **250:** 175–180
50 Chen S., Wang Q. L., Nie Z., Sun H., Lennon G., Copeland N. G. et al. (1997) Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron **19:** 1017–1030
51 Furukawa T., Morrow E. M. and Cepko C. L. (1997) *Crx*, a novel *otx*-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell **91:** 531–541
52 Andreazzoli M., Gestri G., Angeloni D., Menna E. and Barsacchi G. (1999) Role of Xrx1 in Xenopus eye and anterior brain development. Development **126:** 2451–2460
53 Czerny T. and Busslinger M. (1995) DNA-binding and transactivation properties of Pax-6: three amino acids in the paired domain are responsible for the different sequence recognition of Pax-6 and BSAP (Pax-5). Mol. Cell. Biol. **15:** 2858–2871
54 Wilson D., Sheng G., Lecuit T., Dostatni N. and Desplan C. (1993) Cooperative dimerization of paired class homeo domains on DNA. Genes Dev. **7:** 2120–2134
55 Cvekl A. and Piatigorsky J. (1996) Lens development and crystallin gene expression: many roles for Pax-6. Bioessays **18:** 621–630
56 Duncan M. K., Haynes J. I. 2nd, Cvekl A. and Piatigorsky J. (1998) Dual roles for Pax-6: a transcriptional repressor of lens fiber cell-specific beta-crystallin genes. Mol. Cell. Biol. **18:** 5579–5586
57 Shiraishi A., Converse R. L., Liu C. Y., Zhou F., Kao C. W. and Kao W. W. (1998) Identification of the cornea-specific keratin 12 promoter by in vivo particle-mediated gene transfer. Invest. Ophthalmol. Vis. Sci. **39:** 2554–2561

58 Liu J. J., Kao W. W. and Wilson S. E. (1999) Corneal epithelium-specific mouse keratin K12 promoter. Exp. Eye Res. **68:** 295–301
59 Sheng G., Thouvenot E., Schmucker D., Wilson D. S. and Desplan C. (1997) Direct regulation of rhodopsin 1 by Pax-6/eyeless in *Drosophila*: evidence for a conserved function in photoreceptors. Genes Dev. **11:** 1122–1131
60 Loosli F., Winkler S. and Wittbrodt J. (1999) Six3 overexpression initiates the formation of ectopic retina. Genes Dev. **13:** 649–654
61 Chow R. L., Altmann C. R., Lang R. A. and Hemmati-Brivanlou A. (1999) Pax6 induces ectopic eyes in a vertebrate. Development **126:** 4213–4222
62 Hill R. E., Favor J., Hogan B. L., Ton C. C., Saunders G. F., Hanson I. M. et al. (1991) Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature **354:** 522–525 [published erratum appears in Nature (1992) **355:** 750]
63 Glaser T., Walton D. S. and Maas R. L. (1992) Genomic structure, evolutionary conservation and aniridia mutations in the human *PAX6* gene. Nat. Genet. **2:** 232–239
64 Glaser T., Jepeal L., Edwards J. G., Young S. R., Favor J. and Maas R. L. (1994) *PAX6* gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. Nat. Genet. **7:** 463–471 [published erratum appears in Nat. Genet. (1994) **8:** 203]
65 Jordan T., Hanson I., Zaletayev D., Hodgson S., Prosser J., Seawright A. et al. (1992) The human *PAX6* gene is mutated in two patients with aniridia. Nat. Genet. **1:** 328–332
66 Hanson I. M., Fletcher J. M., Jordan T., Brown A., Taylor D., Adams R. J. et al. (1994) Mutations at the *PAX6* locus are found in heterogeneous anterior segment malformations including Peters' anomaly. Nat. Genet. **6:** 168–173
67 Hogan B. L., Horsburgh G., Cohen J., Hetherington C. M., Fisher G. and Lyon M. F. (1986) *Small eyes* (*Sey*): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J. Embryol. Exp. Morphol. **97:** 95–110
68 Glaser T., Lane J. and Housman D. (1990) A mouse model of the aniridia-Wilms tumor deletion syndrome. Science **250:** 823–827
69 Quinn J. C., West J. D. and Hill R. E. (1996) Multiple functions for Pax6 in mouse eye and nasal development. Genes Dev. **10:** 435–446
70 Halder G., Callaerts P. and Gehring W. J. (1995) Induction of ectopic eyes by targeted expression of the *eyeless* gene in *Drosophila*. Science **267:** 1788–1792
71 Altmann C. R., Chow R. L., Lang R. A. and Hemmati-Brivanlou A. (1997) Lens induction by Pax-6 in *Xenopus laevis*. Dev. Biol. **185:** 119–123
72 Burmeister M., Novak J., Liang M. Y., Basu S., Ploder L., Hawes N. L. et al. (1996) Ocular retardation mouse caused by *Chx10* homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. Nat. Genet. **12:** 376–384
73 Porter F. D., Drago J., Xu Y., Cheema S. S., Wassif C., Huang S. P. et al. (1997) *Lhx2*, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. Development **124:** 2935–2944
74 Dudley A. T., Lyons K. M. and Robertson E. J. (1995) A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev. **9:** 2795–2807
75 Luo G., Hofmann C., Bronckers A. L., Sohocki M., Bradley A. and Karsenty G. (1995) BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. Genes Dev. **9:** 2808–2820
76 Tomita K., Ishibashi M., Nakahara K., Ang S. L., Nakanishi S., Guillemot F. et al. (1996) Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron **16:** 723–734
77 Glaser T. M., Tucker P., Munson A., Kanekar S., Vetter M. and Laemle L. (1999) Analysis of an eyeless strain of mice: identification of a Rx/rax homeobox gene mutation. Invest. Ophthalmol. Vis. Sci. **40:** S205
78 Warburg M., Sjo O., Tranebjaerg L. and Fledelius H. C. (1991) Deletion mapping of a retinal cone-rod dystrophy: assignment to 18q211. Am. J. Med. Genet. **39:** 288–293
79 Freund C. L., Gregory-Evans C. Y., Furukawa T., Papaioannou M., Looser J., Ploder L. et al. (1997) Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (*CRX*) essential for maintenance of the photoreceptor. Cell **91:** 543–553
80 Swain P. K., Chen S., Wang Q. L., Affatigato L. M., Coats C. L., Brady K. D. et al. (1997) Mutations in the cone-rod homeobox gene are associated with the cone-rod dystrophy photoreceptor degeneration. Neuron **19:** 1329–1336
81 Freund C. L., Wang Q. L., Chen S., Muskat B. L., Wiles C. D., Sheffield V. C. et al. (1998) De novo mutations in the *CRX* homeobox gene associated with Leber congenital amaurosis. Nat. Genet. **18:** 311–312
82 Simeone A., Acampora D., Gulisano M., Stornaiuolo A. and Boncinelli E. (1992) Nested expression domains of four homeobox genes in developing rostral brain. Nature **358:** 687–690
83 Oliver G., Mailhos A., Wehr R., Copeland N. G., Jenkins N. A. and Gruss P. (1995) *Six3*, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. Development **121:** 4045–4055
84 Zaraisky A. G., Lukyanov S. A., Vasiliev O. L., Smirnov Y. V., Belyavsky A. V. and Kazanskaya O. V. (1992) A novel homeobox gene expressed in the anterior neural plate of the *Xenopus* embryo. Dev. Biol. **152:** 373–382
85 Mathers P. H., Miller A., Doniach T., Dirksen M. L. and Jamrich M. (1995) Initiation of anterior head-specific gene expression in uncommitted ectoderm of *Xenopus laevis* by ammonium chloride. Dev. Biol. **171:** 641–654
86 Thomas P. Q., Johnson B. V., Rathjen J. and Rathjen P. D. (1995) Sequence, genomic organization, and expression of the novel homeobox gene *Hesx1*. J. Biol. Chem. **270:** 3869–3875
87 Hermesz E., Mackem S. and Mahon K. A. (1996) *Rpx*: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. Development **122:** 41–52
88 Gehring W. J. (1996) The master control gene for morphogenesis and evolution of the eye. Genes Cells **1:** 11–15
89 Sive H. L., Hattori K. and Weintraub H. (1989) Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. Cell **58:** 171–180
90 Sasai Y., Lu B., Steinbeisser H., Geissert D., Gont L. K. and De Robertis E. M. (1994) *Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell **79:** 779–790
91 Hemmati-Brivanlou A., Kelly O. G. and Melton D. A. (1994) Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. Cell **77:** 283–295
92 Bouwmeester T., Kim S., Sasai Y., Lu B. and De Robertis E. M. (1996) Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature **382:** 595–601