Introduction

The development of the vertebrate eye is one of the most extensively studied processes in embryology due to its accessibility in a number of different model systems. These studies have shown that eye development involves multiple tissue interactions that are regulated by a set of highly conserved developmental genes. The first of these interactions occur during gastrulation, when the involuting mesoderm and endoderm interact with the head ectoderm to give it a lens-forming bias (reviewed by Saha et al., 1989). Subsequently an extension of the diencephalon, the optic vesicle, induces the overlying head ectoderm to form a lens placode which then invaginates to form the lens. The optic vesicle gives rise to the optic cup, the outer layer of which ultimately differentiates into the pigmented retina, and the inner cell layer gives rise to the multitude of cell types that together form the neural retina.

The competence of the head ectoderm to form a lens is dependent upon the expression of the paired box / homeodomain containing transcription factor Pax6 (Fujiiwara et al., 1994; Li et al., 1994), and indeed, the ectopic expression of Pax6 in the ectoderm of Xenopus embryos results in the formation of additional eye structures (Onuma et al., 2002). Pax6 expression is not required in the optic vesicle to induce lens formation in the overlying ectoderm though; this process depends instead on other genes that have not yet been fully characterised, but they include the secreted proteins BMP4 and FGF8. The former induces the expression of the Sox2 and Sox3 transcription factors in the overlying ectoderm, whilst FGF8 induces the expression of the L-Maf gene (Furuta and Hogan, 1998; Ogino and Yasuda, 1998; Vogel-Hopfer et al., 2000), and the combination of Pax6, Sox2, Sox3 and L-Maf ensures that the responding ectodermal cells differentiate into lens (Zygar et al., 1998).

In addition to BMP4 and FGF8, a number of transcription factors that have conserved functions in development are also expressed in the Xenopus forebrain, including Pbx1b (Maeda et al., 2002). This is a homologue of the Drosophila extradenticle gene, a homeodomain-containing transcription factor. It can bind to a diverse set of other transcription factors as part of a heteromeric complex, including engrailed (Peltenburg and Murre, 1997), the myogenic factor TFS (Knoepfler et al., 1999), and the glucocorticoid receptor (Subramaniam et al., 2003). The most extensively characterised Pbx interaction is with the HOX proteins, however. These are a family of homeodomain-containing transcription factors that determine cellular identity during development (reviewed by Carroll 1995; Gehring 1998; Burke 2000), and which also regulate cell proliferation and differentiation in the adult, most notably that of hematopoietic progenitor cells (Antonchuk et al., 2002; Kyba et al., 2002). The interaction between Pbx and HOX / Engrailed proteins is dependent on a short stretch of highly conserved amino acids in the amino terminal of the HOX protein, known as the hexapeptide sequence (Phelan et al., 1995; Passner et al., 1999; Piper et al., 1999; Neuteboom et al., 1995). Pbx binding to HOX proteins significantly increases their affinity and specificity of DNA binding (Chang et al., 1996).

The antisense-mediated depletion of Pbx genes in the primitive chordate Ciona results in severe developmental abnormalities (Yamada et al., 2003). A similar approach in Zebrafish that blocks the function of the Pbx2 and Pbx4 genes leads to a ‘ground state’ being imposed on the hindbrain, whereby segmentation is prevented and the entire structure takes on the identity of the first
rhombomere (Waskiewicz et al., 2002; Cooper et al., 2003). Blocking Pbx1b activity in Xenopus also results in neural defects, whereby the induction of gene expression associated with the posterior neural tube and the neural crest is prevented (Maeda et al., 2002).

We have used a novel strategy for blocking PBX binding to its cofactors to inactivate it in the isolated neural plate from Xenopus embryos. This in turn prevents the expression of FGF8, and blocks lens induction in adjacent head ectoderm explants.

Results and Discussion

Pbx genes are expressed in distinct tissues of the developing embryo, and the phenotype associated with a general ablation of Pbx activity reflects this. A different approach is therefore required to study the role of Pbx in a specific tissue, especially at a relatively late stage of development. For this reason, we developed a strategy for blocking the binding of PBX protein to its cofactors, taking the highly conserved HOX hexapeptide sequence WYPWMKKHH - which is known to mediate this process (reviewed by Morgan et al., 2000) - and making a synthetic peptide containing both it and a 16 amino acid sequence ('penetratin') based on the Drosophila Antennapedia protein, previously shown to mediate efficient movement of proteins across cell membranes (Derossi et al., 1998). This peptide is referred to as HXP4. In addition, a control peptide was made consisting of a non-hexapeptide sequence (WCPWLDRHGR) linked to penetratin (CXP4). The ability of HXP4 to prevent HOX / PBX interactions was tested by adding it to anterior neural plate isolated from neurula stage Xenopus embryos. Total cell protein was then analysed for PBX by western blotting (Monica et al., 1991), with or without cross linking (Fig. 1). HXP4, but not the control peptide CXP4, prevented binding of PBX to other proteins under these conditions. Hence HXP4 can act as a competitive inhibitor of PBX function by blocking its interaction with other co-factors. In order to test that HXP4 does indeed have this property, its affect on the expression of Hoxb1 in the neural plate was assessed. Hoxb1 expression is maintained in this tissue by an autoregulatory loop that is dependent on an interaction between HOXB1 and PBX proteins (Di Rocco et al., 1997). Isolated neural plates excised from early neural (stage 12.5) embryos were incubated with HXP4 or CXP4 for two hours, whereupon the total RNA was extracted and assayed for the expression of Hoxb1 and NCAM (which is expressed in both Hoxb1-expressing and non-expressing neural cells). Treatment with HXP4, but not CXP4, resulted in a significant decrease in the expression of Hoxb1, whilst neither peptide affected the expression of NCAM (Fig. 2). Furthermore, neural plates taken from Pbx over-expressing embryos did not show any reduction in Hoxb1 expression (Fig. 2). Hence HXP4 is a specific inhibitor of PBX interactions.

In order to further characterise the activity of HXP4, we also examined the expression of a reporter gene driven by a previously characterised HOXB4 / PBX responsive element (Morsi El-Kadi et al., 2002; White et al., 2000). This was microinjected into fertilised eggs together with Hoxb4 RNA, and naive ectoderm was subsequently removed from these embryos at the late gastrula stage (12.5). In the presence of HOXB4 the reporter construct shows a high level of activity, which is abolished if an altered HOXB4 / PBX consensus site is used. CXP4 has no affect on reporter activity, whilst HXP4 causes a significant reduction (Fig. 3). Furthermore, the co-injection...
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of Pbx into embryos rescues reporter activity, indicating that the effects of HXP4 are specific for protein interactions involving the hexapeptide sequence.

The optic vesicle is derived from the anterior neural plate, and in Xenopus the anterior neural plate alone is sufficient to induce the expression of lens specific genes in naive ectoderm (Zygar et al., 1998). One of the genes expressed in the anterior neural plate is Pbx1b (Maeda et al., 2002), and we decided to use HXP4 to test whether it is required for lens induction. Anterior neural plate was removed from mid neurula (stage 14) embryos and cultured for three hours in either high salt buffer (MMR) alone, MMR with CXP4, or MMR with HXP4 (Fig. 4A). After this time the total RNA was extracted from the explants and analysed for the expression of a number of different genes using quantitative – PCR (Q-PCR). HXP4 treatment caused a significant decrease in the number of FGF8 transcripts, whilst the expression of both BMP4 and the housekeeping gene ornithine decarboxylase (ODC) was unaffected (Fig. 4B). Hence of the two previously identified lens-inducing factors, only FGF8 is dependent on Pbx for its expression.

We made tissue recombinants of ectoderm removed from the animal pole of mid-gastrula (stage 11.5) embryos, and the anterior neural plate taken from neurula (stage 14) embryos which had been treated with HXP4 or CXP4 (Fig. 5A). These recombinants were cultured until their untreated sibling embryo had reached the late neurula (stage 21). Recombinants incubated without peptide or with the control peptide (CXP4) both expressed L-Maf and the lens-specific gene gamma-crystallin, whilst the HXP4 treated recombinants did not (Fig. 5B).

In order to further characterise gene expression in the lens-competent ectoderm, RNA was also extracted from ectoderm removed when sibling embryos had reached the mid-neurala stage, and the relative amounts of ODC, Pax6, Sox2, Sox3 and L-Maf were assayed by QPCR (Fig. 5C). This revealed a significant reduction only in L-Maf expression (Fig. 5C), a finding that concurs with previous studies indicating that FGF8 is required for the induction of L-Maf expression in competent lens ectoderm (Ogino and Yasuda, 1998; Vogel-Hopker et al., 2000), whilst BMP4 is required to induce Sox2 and Sox3 expression (Furuta and Hogan, 1998).
Fig. 5. HXP4 treated neural plate cannot induce lens formation in ectodermal explants. (A) Conjugates were made between ectoderm taken from mid gastrula (stage 11.5) embryos (left) and the anterior neural plate from neurula (stage 14) embryos. These were cultured until the sibling embryos had reached stage 21 (late neurula). (B) The explants were analysed by in situ hybridisation for gamma-crystalline and L-Maf expression (purple staining). (C) The explants were also analysed for Pax6, L-Maf, ODC, Sox2 and Sox3 expression by Q-PCR. The amount of each transcript is shown relative to that in untreated, control neural plates. The error bars represent the SEM from three experiments.

The requirement for Pbx in FGF8 expression suggests that this transcription factor and at least some of its cofactors are in the hierarchy of genes that regulate lens induction. This hierarchy involves distinct sets of transcription factors and signalling molecules, and two distinct tissues. In the anterior neural plate (including those cells that form the optic vesicle), Pbx activates the expression of FGF8, and this in turn activates expression of the L-Maf transcription factor in the overlying ectoderm (Ogino and Yasuda, 1998; Vogel-Hopker et al., 2000). Other, as yet unidentified transcription factors drive the expression of BMP4 in the neural plate, and this signalling molecule in turn activates the expression of Sox2 and Sox3 in the lens-competent ectoderm (Furuta and Hogan, 1998).

In addition to lens induction, FGF8 also functions within the neural plate to pattern the developing brain, as its release from the midbrain/hindbrain boundary is required for the expression of En2 and Wnt1 in the posterior midbrain (Crossley et al., 1996; Riou et al., 1998). The expression of Pbx is considerably wider than that of FGF8, as it is expressed throughout the neural plate and is not confined to the midbrain/hindbrain boundary. Presumably the greater specificity of FGF8 expression is regulated by specific Pbx co-factors, a likely candidate being engrailed 2, which is co-expressed with FGF8. This is supported by the presence of EN2/PBX binding sites within an intronic enhancer of the FGF8 gene, which are necessary to maintain FGF8 expression in ES cells (Gemel et al., 1999). It is likely that further work will help to identify additional components of these complex regulatory pathways.

Materials and Methods

RNA extraction and RT-QPCR

Total RNA was extracted from whole embryos, isolated neural plate, or ectoderm using a RNeasy mini kit (Qiagen). One µg of RNA was used in subsequent reverse transcription reactions. This was mixed with a poly T15 oligo to 5 µg/ml and heated to 75ºC for 5 minutes. After cooling on ice, the following additional reagents were added; dNTPs to 0.4 mM, RNase OUT (Promega) to 1.6 U/µl, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) RNaseH- point mutant (Promega) to 8 U/µl and the appropriate buffer (supplied by the manufacturer) to x1 concentration. The mixture was incubated for one hour at 37ºC heated to 70ºC for two minutes and cooled on ice.

QPCR reactions were all performed in a total volume of 50 µl. For each we used 1 µl of the M-MLV RT reaction (as described above), 0.2 nmol of each primer and 25 µl of pre-mixed QPCR components (Sigma). All reactions were cycled at 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 60 seconds, for 45 cycles. The primers used for FGF8 amplification were:

FGF8 (5’): 5’ ACC TCC ATC CTG GGC TAT CT 3’ and
FGF8 (3’): 5’ CAC GAT TAA CTT GGC GTG TG 3’ and
GCT (5’): 5’ CAA CAC ACT TTT AAA ATG CTT ACG 3’ and
BMP4 (5’): 5’ GCC ACA TTC CCA TTA GCA GT 3’
BMP4 (3’): 5’ GCT(3’): 5’ GTT GCC ATC AAA CAC ATT GC 3’
L-Maf (5’): 5’ GTG GAG AGT GGC AAC TGG AT 3’
L-Maf (3’): 5’ AGG TAG ATT GTT GCC CAT CG 3’
GCT (5’): 5’ GTG GAG AGT GGC AAC TGG AT 3’
GCT (3’): 5’ AGG TAG ATT GTT GCC CAT CG 3’

The sequences of the other primer pairs can be found on the internet at: http://www.sghms.ac.uk/depts/anatomy/pages/richhmpg.htm/.

QPCR was performed using the SYBR green labelling kit from Sigma., using ROX as the internal standard dye. Thermal cycling and fluorescence detection was by a MX4000 (Stratagene Inc., USA). Semi-quantitative data was obtained by using measurements three cycles after reactions had risen above the base line, and where clearly in exponential increase. Ef1alpha was used as a loading control, and all values are presented as a ratio of target to ef1alpha signal.

Whole mount in situ hybridisation of ectodermal conjugates

The L-Maf reading frame was cloned into vector pGEMT-easy (Promega), and this was linearised using NcoI. A DIG-labelled in situ probe was transcribed from this template using SP6 polymerase. A DIG-labelled gamma-crystalline probe was transcribed as previously described (Godsave et al., 1994). Probe purification and subsequent in situ analysis were performed as described (Sive et al., 2000).

Western blotting of PBX and PBX/HOX dimers

Protein was extracted from HXP4-treated or CXP4-treated (20 µg/ml) neural explants taken from stage 14 embryos. C-20 antibody recognises
the human PBX proteins and cross – reacts with Xenopus PBX1B protein (sc-888, Santa Cruz Biotechnology Inc., USA). Protein cross linking was performed with 2 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Pierce Biotechnology, USA), in cell lysates diluted 1:10 in conjugation buffer (0.1M 2-[N-morpholino] ethane sulfonic acid, pH 5). After 15 minutes at room temperature the reaction was stopped by adding 2 mercaptoethanol to 20 mM and excess reagents were removed using a D-Salt Dextran desalting column (Pierce, USA).

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References

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