

***Pbx* genes are required in *Xenopus* lens development**

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ABSTRACT Development of the vertebrate eye is dependent on multiple tissue interactions and distinct sets of transcription factors and signalling molecules. The latter are thought to be FGF8 and BMP4, proteins secreted from the neural plate to induce lens formation in the overlying ectoderm. Here we show that the *Pbx* transcription factor plays a crucial role in this process, as it is required for FGF8 expression in the neural plate and is thus part of the complex hierarchy of genes involved in lens induction.

KEY WORDS: *Pbx*, eye development, lens induction, FGF8, BMP, HOX

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* (Fujiwara *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-Hopker *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

Abbreviations used in this paper: BMP, bone morphogenetic protein; EDC, 1-ethyl-3-[3-(dimethyl-amino)propyl]carbodiimide; FGF, fibroblast growth factor; Q-PCR, quantitative Polymerase Chain Reaction.

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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

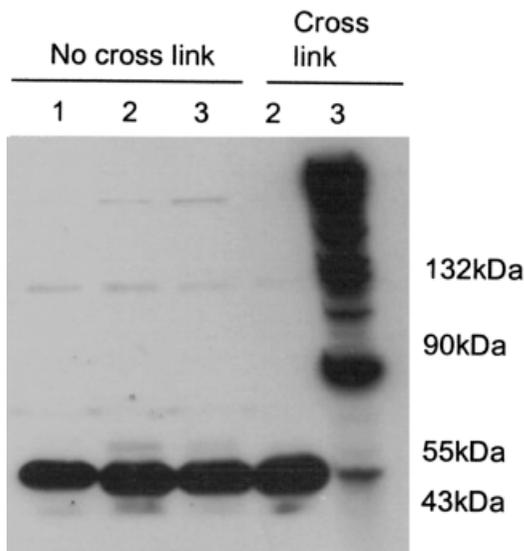


Fig. 1. Disruption of HOX and PBX binding by HXP4. Western blot of protein extracted from the dorsal ectoderm of neurula stage *Xenopus* embryos and probed with an antibody raised against PBX1, 2 and 3 isoforms. 'Cross-linked' - protein was extracted in 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to cross link non-covalently associated proteins. (1) Untreated ectoderm; (2) CXP4 peptide-treated ectoderm; (3) HXP4 peptide-treated ectoderm. The presence of HXP4 disrupted the formation of PBX / HOX dimers from PBX monomers (52 kDa).

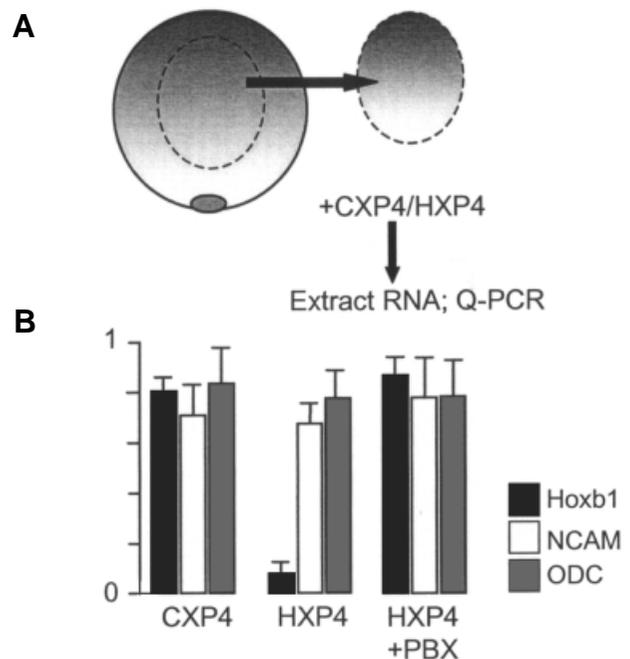


Fig. 2. HXP4 inhibits *Hoxb1* expression in the neural plate. (A) The neural plate was isolated from early neurula (stage 12.5) *Xenopus* embryos and incubated with HXP4 or CXP4, as shown. Subsequently RNA was extracted for Q-PCR analysis. (B) The relative expression levels of *Hoxb1*, *NCAM* and *ODC* as determined by Q-PCR, standardised to that of *ef1alpha*. The amount of each transcript is shown relative to that in untreated, control neural plates. The error bars represent the SEM from three experiments.

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

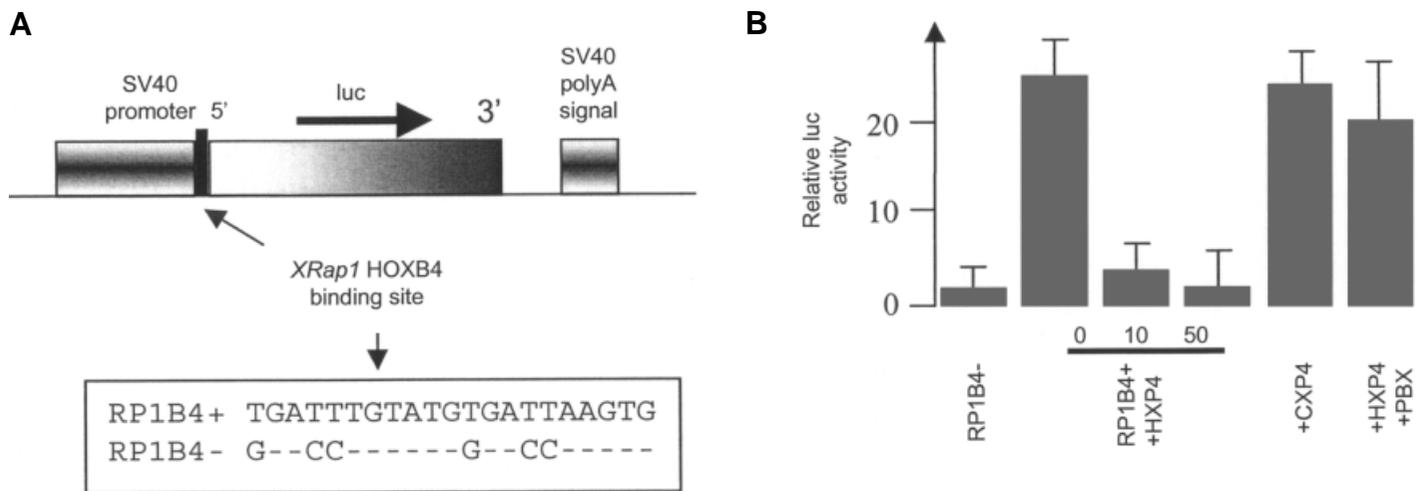


Fig. 3. HXP4 blocks the *Hoxb4*-activated transcription of a reporter construct. (A) The reporter constructs were based on the pGL3 vector which contains the luc gene under the control of the ubiquitously active SV40 promoter. The putative HOXB4 binding site from XRap1 was cloned immediately 5' to the luc gene, as shown. The nucleotide sequence of the putative HOXB4 binding region (RP1B4+) and a non-binding variant used as a control (RP1B4-) are shown. (B) Explants derived from RP1B4+ injected embryos were treated with 0, 10 or 50 microgrammes/ml of HXP4 for 12 hours, after which time luciferase activity was measured. Alternatively, explants were treated with 50 µg/ml CXP4 (+CXP4). As an additional control, embryos were injected with both RP1B4+ and 1 ng Pbx RNA, and excised explants were treated with 50 µg/ml HXP4 (+HXP4+PBX) for 12 hours.

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 naïve 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 neural (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-neurula 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).

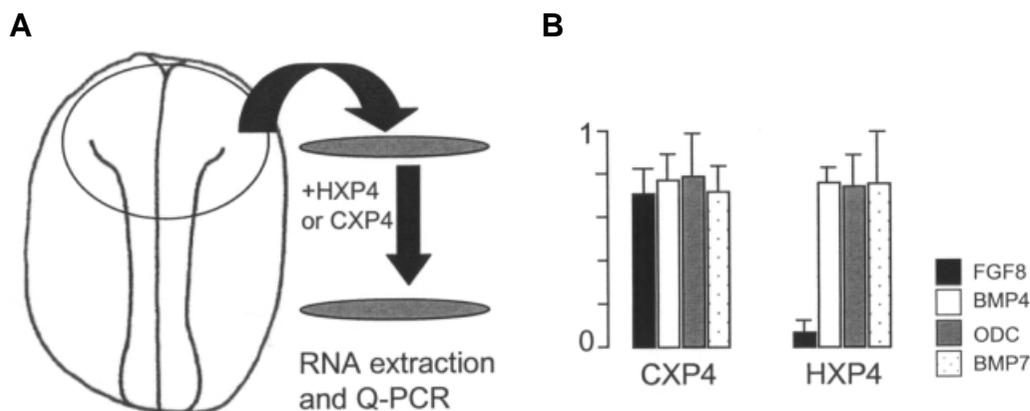


Fig. 4. HXP4 blocks the expression of *FGF8* in the anterior neural plate. (A) The anterior neural plate was excised from neurula (stage 14) embryos and treated with HXP4 or CXP4, as shown. (B) The relative expression levels of *FGF8*, *BMP4* and *ODC* were determined by Q-PCR, and standardised to that of *ef1alpha*. Values are shown relative to the untreated control. The error bars represent the SEM from three experiments.

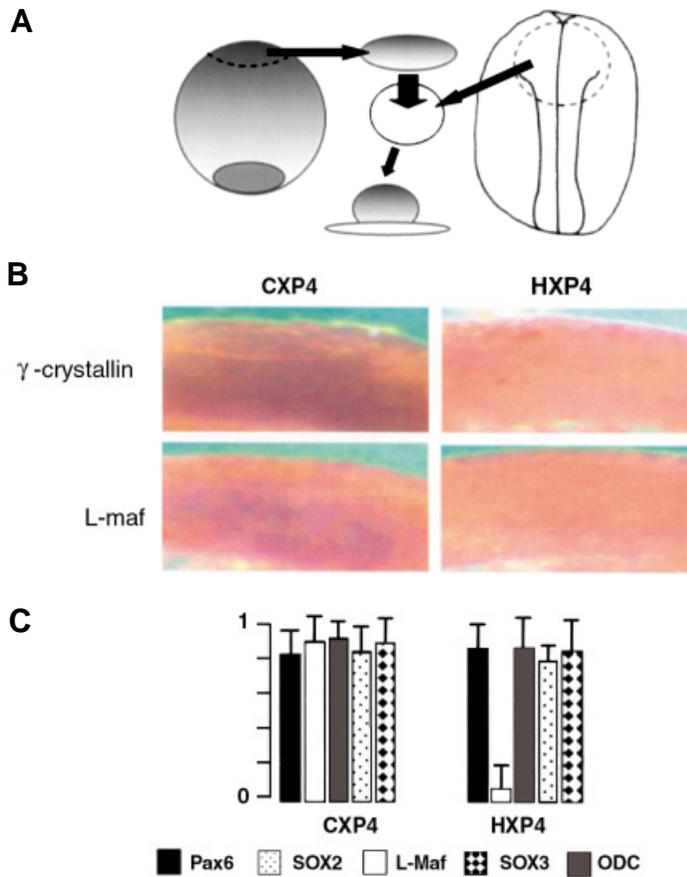


Fig. 5. HXP4 treated neural plate cannot induce lens formation in ectodermal explants. (A) Conjugates were made between ectoderm taken from mid gastrula (stage 11.5) embryos (left) and the anterior neural plate from neurula (stage 14) embryos. These were cultured until the sibling embryos had reached 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 an 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 75°C 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-MLRV) 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 37°C heated to 70°C 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-MLRV reaction (as described above), 0.2 nmol of each primer and 25 μl of pre-mixed QPCR components (Sigma). All reactions were cycled at 94°C for 30 seconds, 55°C for 30 seconds and 72°C 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'; *GCT* (5'): 5' GTG GAG AGT GGC AAC TGG AT 3' and *GCT* (3'): 5' GTT GCC ATC AAA CAC ATT GC 3'; *BMP4* (5'): 5' CAA CAC ACT TTT AAA ATG CTT ACG 3' and *BMP4* (3'): 5' GGC TGG AAT GAA TGG ATT GT 3'; *SOX2* (5'): 5' TCT GCA CAT GAA GGA GCA TC 3' and *SOX2* (3'): 5' CAC TTA TGC CCA CAT GAA CG 3'; *SOX3* (5'): 5' ATG AAC GGC TGG ACT AAT GG 3' and *SOX3* (3'): 5' GGA GCT CTG CTG GTT GTA GG 3'; *L-Maf* (5'): 5' ACT CAG GGC TGT CCC CTA AT 3' and *L-Maf* (3'): 5' TTG GGG ATT TTG AGA CTT GC 3'; *Pax6* (5'): 5' GCC ACA TTC CCA TTA GCA GT 3' and *Pax6* (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-crystallin 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 $\mu\text{g}/\text{ml}$) 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 sulphonic 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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