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Original Article

Isolation and growth factor inducibility of the *Xenopus laevis Lmx1b* gene

CAROLINE E. HALDIN, SARJIT NIJJAR[1], KARINE MASSÉ, MARK W. BARNETT[2] and ELIZABETH A. JONES*

Cell and Molecular Development Group, Department of Biological Sciences, Warwick University, Coventry, U.K., [1]School of Biosciences, University of Birmingham, U.K. [2]Department of Anatomy, University of Edinburgh Medical School, University of Edinburgh, U.K.

ABSTRACT This paper reports the cloning of the full length *Xenopus laevis Lmx1b* gene, *Xlmx1b.* Xlmx1b is a LIM homeodomain protein with high conservation to homologues identified in human, mouse, hamster and chick. *In situ* hybridisation and RT-PCR analysis showed that *Xlmx1b* has a specific temporal expression pattern which can be separated into three main spatial domains. An *Xlmx1b* probe hybridized to regions of the nervous system from stage 13 onwards; these regions included the placodes and otic vesicles, the eye and specific sets of neurons. Sectioning of *in situ* hybridised embryos confirmed the location of transcripts as discreet regions of staining in ventro-lateral regions of the neural tube. From stage 27, transcripts could be detected in the capsule of pronephric glomus. Finally, transcripts were detected by Northern blot analysis in the developing fore and hind limbs. *Xlmx1b* transcripts were also detected by Northern blot analysis in eye, brain, muscle and mesonephros tissue in metamorphosing tadpoles. RT-PCR analysis showed that zygotic expression of *Xlmx1b* is initiated at stage 10.5 and the temporal sequence of *Xlmx1b* expression is identical in both neural and presumptive pronephros regions. The effects of the growth factors activin A, retinoic acid (RA) and basic fibroblast growth factor (bFGF) on the regulation of *Xlmx1b* were also studied. *Xlmx1b* was found to be upregulated by activin A and RA inhibited this upregulation in a concentration dependant manner. In contrast, bFGF had no effect on the regulation of *Xlmx1b*.

KEY WORDS: *LIM homeodomain, Lmx1b, pronephros, kidney, Xenopus*

Introduction

Lmx-1 was first identified from hamster in a screen for cDNAs to find proteins that bind to the FLAT element, part of a minienhancer, in the rat insulin gene *Isl-1* (German *et al.*, 1992). Since then, two distinct forms of the gene have been identified, *Lmx1.1* and *2.1* (German *et al.*, 1992, Johnson *et al.*, 1997). Homologues of these LIM homeobox genes are known in several other species including, human, mouse, chick and *C. elegans* and the Lmx proteins are known to have several important developmental roles that seem to be conserved between the species.

LIM-HD transcription factors have been shown to be important in development in a number of different organisms. The acronym comes from the first three genes to be isolated that contained this motif; *C. elegans lin-11*, where disruptions of this gene prevent the worm from laying eggs (Freyd *et al.*, 1990), mouse *Isl-1* (Karlsson *et al.*, 1990), where mutants are deficient in motor neuron and interneuron formation (Pfaff *et al.*, 1996) and *C. elegans mec-3*, where the gene is required for differentiation of touch receptor neurons (Way and Chalfie, 1988).

Other examples of LIM-HD genes include the *Drosophila* gene *Apterous,* that is required for imaginal disc development and dorsoventral patterning in the wing (Cohen *et al.*, 1992; Diaz-Benjumea and Cohen, 1993; Blair *et al.,* 1994), correct axon guidance in the nervous system (Lundgren *et al.*, 1995), and development of embryonic muscles (Bourgouin *et al.*, 1992); the mouse *Lhx3* gene, mutants of which lack the anterior and intermediate lobes of the pituitary gland (Sheng *et al.*, 1996) and the *Xenopus Xlim-1* gene which is thought to be required for growth and elongation in the development of the pronephric tubules (Chan *et al.*, 2000).

The LIM domain is a highly conserved cysteine rich region at the N- or C-terminus of the protein. Each domain is composed of two finger-like structures binding one Zn(II) ion each (Michelson *et al.,* 1993, Kosa *et al.*, 1994) held in configuration by hydrophobic

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; HD, homeodomain; IsO, isthmic organiser; NPS, nail patella syndrome; RA, retinoic acid; RT-PCR, reverse-transcription polymerase chain reaction; Xlmx1b, Xenopus LIM homeobox 1b.

***Address correspondence to:** Dr. Elizabeth A. Jones. Cell and Molecular Development Group, Department of Biological Sciences, Warwick University, Coventry, U.K. Fax: +44-2476-5237-01. e-mail: eoliver-jones@bio.warwick.ac.uk

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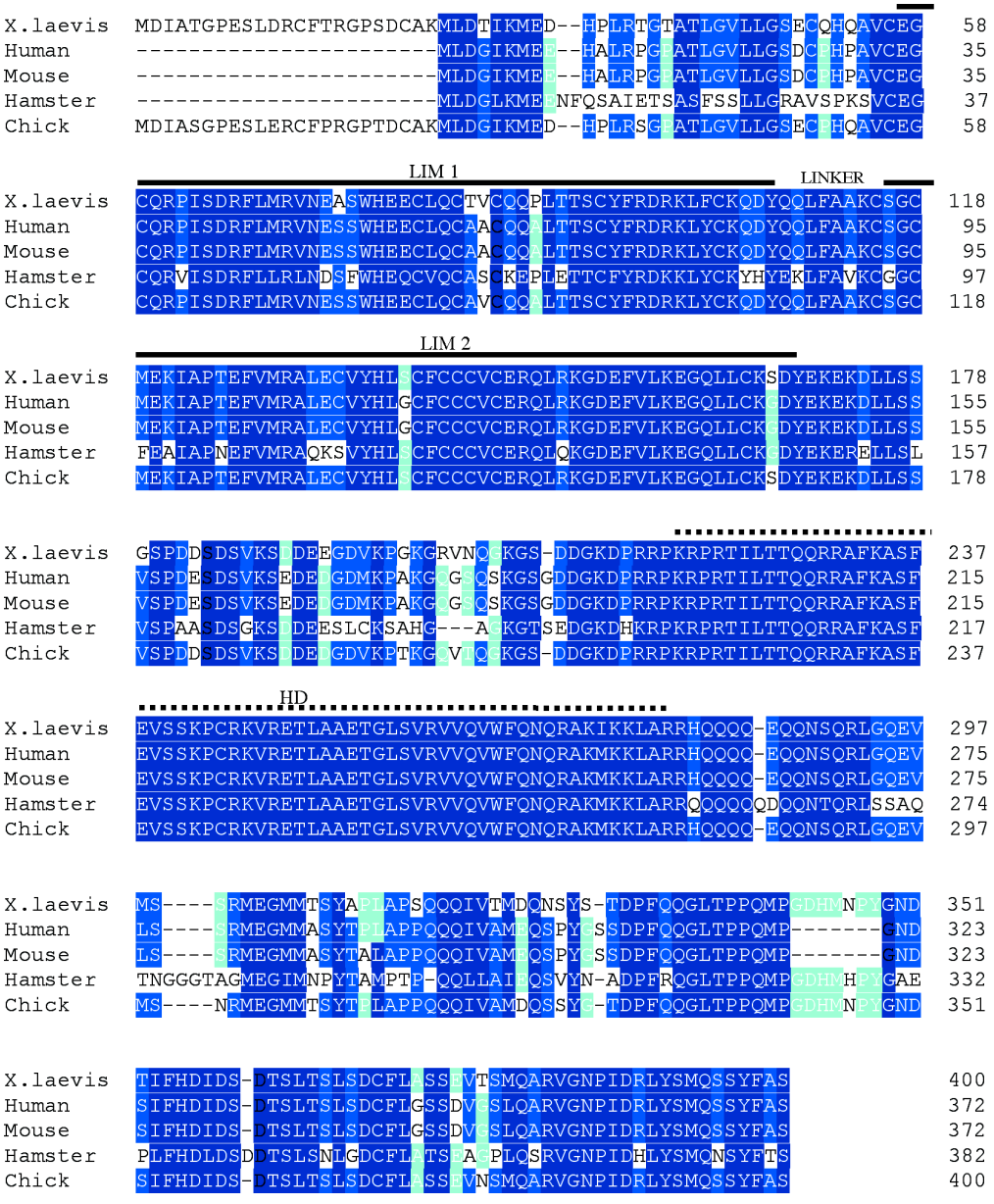

Fig. 1. Alignment of *Xenopus laevis*, human, mouse, hamster and chick Lmx1b protein sequences. *Xlmx1b is highly conserved (shaded regions), 88%, 88%, 63% and 93% respectively. The two N-terminal LIM domains are indicated with solid lines and the homeodomain with a dotted line.*

interactions. LIM domains are often paired and held together by a linker region (Freyd *et al.*, 1990; Karlsson *et al.*, 1990, also see Dawid *et al.*, 1998 for review). LIM domains are thought to mediate protein-protein interactions, which allows proteins containing these domains to have multiple binding partners and function as adapter molecules. As well as binding each other, LIM domains are known to bind to a variety of other structurally distinct protein motifs e.g. helix-loop-helix transcription factors (reviewed in Dawid *et al.*, 1998 and Bach, 2000). Although zinc finger motifs are regularly used as the DNA binding domain, it is not thought that the LIM domain has DNA-binding activity. Instead it is the homeodomain of this type of LIM protein that is thought to bind the DNA, with the activity of the HD being regulated by inter- and intra-molecular interactions mediated by the LIM-domains. LIM-domains positively regulate LIM-HD activity by promoting protein-protein interactions that allow cooperative binding to regulatory regions of tissue-specific promoters and they negatively regulate it by preventing the HD from associating with the DNA (reviewed in Curtiss and Heilig, 1998).

The chicken homologue *C-Lmx1* plays a major role in development of the dorsoventral axis of the limb bud being thought to specify dorsal cell fate during development (Vogel *et al.*, 1995). *C-Lmx1* transcripts have also been detected in the mesonephros (Riddle *et al.*, 1995), regions of the CNS (Adams *et al.*, 2000) and the otic vesicle (Giraldez, F. 1998).

Similarly, the mouse ortholog *Lmx1b* is thought to be involved in several important areas of development. Mutants show abnormal skeletal patterning, a distinctive kidney phenotype affecting the tubules and glomerular basement membrane (Chen *et al.*, 1998a), a marked reduction of dorsally derived structures from the caudal midbrain and rostral hindbrain (Chen *et al.*, 1998b) and specific ocular defects (Pressman *et al.*, 2000). These murine phenotypes are very similar to those seen in patients displaying Nail Patella Syndrome (NPS) and the *Lmx1b* gene maps to the NPS locus in humans.

C. elegans only has one ortholog of mammalian *Lmx1a* and *b* genes, known as *lim-6*. This gene is expressed in a small number of sensory-, inter- and motor-neurons and in epithelial cells of the uterus and excretory system (Hobert *et al.*, 1999).

We set out to isolate and characterise the *Xenopus Lmx1b* homologue with the expectation that it may play an important role in kidney development. In addition, the simplicity of the *Xenopus laevis* pronephros makes it an ideal model for study of the later kidney forms, as many of the genes and pathways known to play a role in later kidney development are also expressed in the pronephros, where they are presumed to have an equally important role. In amphibians and fish the pronephros is the functional embryonic kidney and this induces the adult mesonephros around the time of metamorphosis (Brändli, 1999). There are three major components to the pronephric nephron; the glomus, a capillary network responsible for blood filtration, the pronephric tubules, which collect waste filtered by the glomus and the pronephric duct, which is the exit channel for wastes via the cloaca (Saxén, 1987).

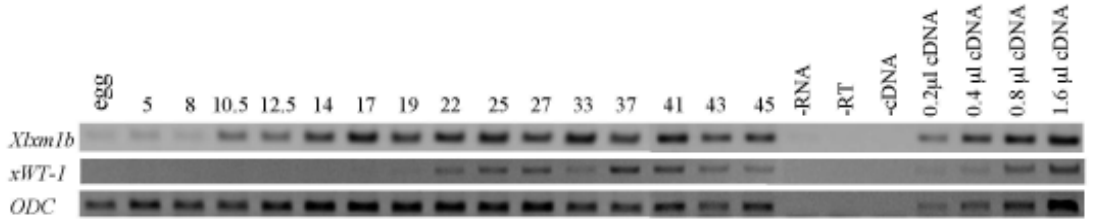

Fig. 2. Temporal expression pattern of *Xlmx1b*. *RT-PCR analysis shows the expression pattern of* Xlmx1b *transcripts in* Xenopus laevis *unfertilised egg and embryo stages. Zygotic expression is upregulated at stage 10.5 with further upregulation after stage 12.5 which is when the glomus is initially specified. Expression is then maintained throughout early development and into tailbud stages. Expression of the glomus marker* xWT-1 *is not initiated until approximately stage 20.*

The amphibian pronephros is derived from the intermediate mesoderm and in the pronephric anlage, tubules and glomus are specified by stage 12.5 and the duct is specified between stages 13 and 14 (Brennan *et al.*, 1998, Brennan *et al.*, 1999). The pronephros starts to function at stage 31 and is fully formed by stage 38.

In this work we describe the identification and characterisation of the *Xlmx1b* gene. We have identified three different embryonic domains of expression; the nervous system, the pronephric glomus and developing limb buds. We show that the temporal control of *Xlmx1b* is the same in both the presumptive pronephros and in the nervous system, although *in situ* detection in the glomus is not obvious until stage 27. Furthermore, we have analysed the growth factor inducibility of the gene in an animal cap assay.

Results

Isolation and Sequencing of Xlmx1b *using Degenerate Primers*

Xlmx1b was cloned from a *Xenopus laevis* stage 17 embryonic cDNA library using degenerate primers designed from the conserved LIM domains in the chick, hamster and mouse Lmx1 homologues. Conceptual translation and alignment in Fig. 1 shows that Xlmx1b is a LIM homeodomain protein with two N-terminal LIM domains and a homeodomain nearer the C-terminus. The LIM domains both agree with the consensus sequence (Freyd *et al.*, 1990) whereas the homeodomain fits less well to its consensus, but these variations are expected and distinguish it as a LIM-type homeodomain (Gehring *et al.*, 1994). The protein is highly conserved showing similarities of 88%, 88%, 63% and 93% with human, mouse, hamster and chick Lmx1b homologues respectively. A database search revealed a 101 amino acid long partial coding sequence for the *C. elegans* homolog *Lim-6*. This aligned with a 47% similarity to the central region of the Lmx proteins. A general classification scheme has been established for LIM proteins and Xlmx1b falls into group 1, proteins with an 'A' and a 'B' class LIM domain in tandem (Taira *et al.*, 1995). The Genbank accession number for *Xlmx1b* is AF414086.

Expression of Xlmx1b *is initiated at Stage 10.5 and persists through Metamorphosis*

RT-PCR analysis was carried out to analyse the temporal expression pattern of *Xlmx1b*. Figure 2 shows that *Xlmx1b* transcripts are not expressed maternally but are present at very low levels during the early cleavage stages of development. Zygotic expression is initiated at stage 10.5 and significantly upregulated between stages 12.5 and 14, concomitant with the time when the glomus is initially specified (Brennan *et al.*, 1999). *Xlmx1b* is expressed continually throughout neurulae, tailbud and swimming tadpole stages.

In addition to an established role in kidney and neural development, *Lmx1b* is known to be involved in limb development. During the course of this work, Matsuda *et al.* (2001) have shown *Xenopus laevis Lmx-1* transcripts are present in dorsal mesenchyme of limb buds between stages 52-55 by *in situ* hybridisation. Using RT-PCR, we confirm *Xlmx1b* is still present in later fore and hind limbs (stage 60 and 56 respectively, data not shown). Northern blot analysis of mRNAs isolated from dissected metamorphosing tadpole was also carried out, Fig. 3. *Xlmx1b* transcripts are approximately 2kb and expression was clearly detected in eye, brain, spinal cord, mesonephros, fore and hind limb tissues.

Xlmx1b *is expressed in the Nervous System and Pronephric Glomus in Premetamorphosing Tadpoles*

In situ hybridisation was carried out to establish the expression pattern of *Xlmx1b* and to allow a comparison with *Lmx* genes of other species, Fig. 4. Expression was first observed at stage 13 as diffuse patches in the placodal regions, (A). At stage 15 the staining becomes more defined and can also be seen down the ridges of the forming neural folds and on the neural plate, (B). By stage 19 the staining is still obvious in the placodal regions but within this area are two more intense regions of expression where the otic vesicles

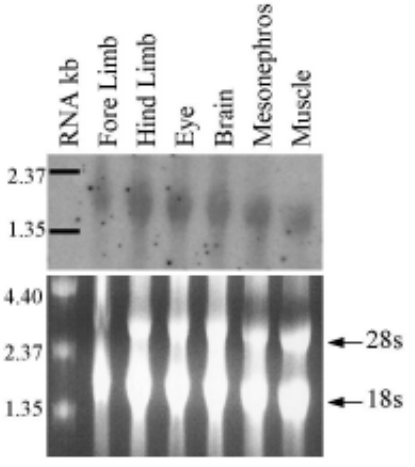

Fig. 3. *Xlmx1b* is expressed at metamorphosis and in limb buds. Xlmx1b *transcripts are identified in metamorphosing tadpole* Xenopus laevis *RNA by Northern blot analysis. The transcript is approximately 2 kb in length. Confirming RT-PCR results,* Xlmx1b *transcripts are found in metamorphosing tadpole limbs and the mesonephros.* Xlmx1b *was also detected in eye, brain and muscle RNA.*

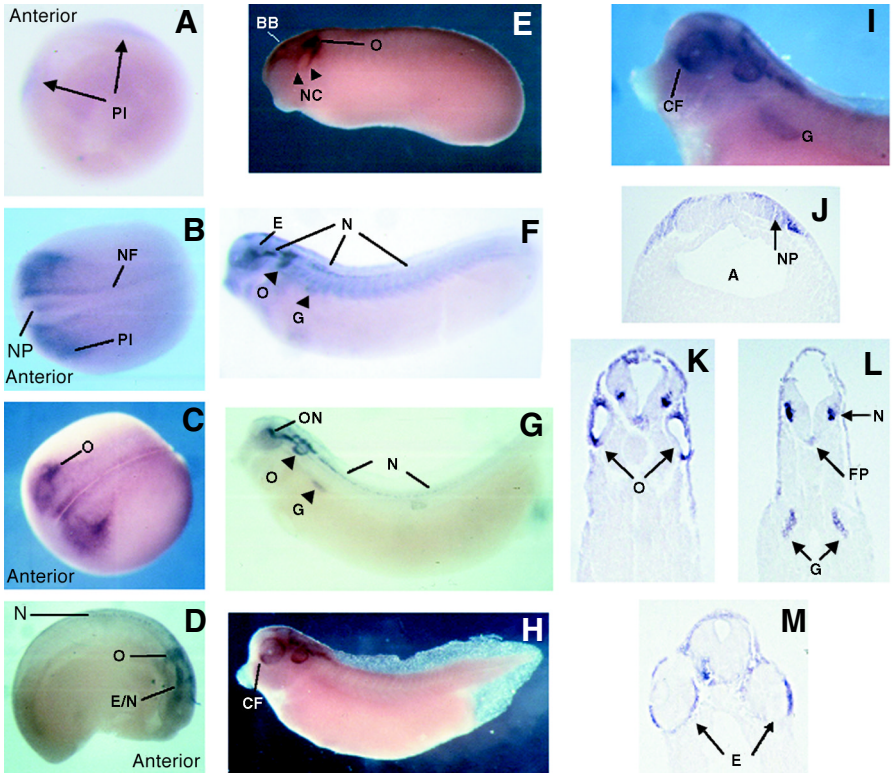

Fig. 4. Wholemount *in situ* hybridisation of *Xlmx1b*. **(A)** *Dorsal view of a stage 13 embryo showing placodal region expression.* **(B)** *A stage 15 embryo showing expression on the neural plate, along the neural folds and in the placodal regions.* **(C)** *A stage 19 embryo showing two intense patches of expression in the placodal regions, the otic anlagen. These will eventually develop into the otic vesicle and the embryos hearing system.* **(D)** *A cleared stage 21 embryo with neurons visible all down the dorsal side of the embryo. Some staining is noticeable in the anterior region, which may develop into the eye expression or the midbrain neurons.* **(E)** *A stage 24 embryo, the first stage at which expression is visible in the dorsal region of the eye. Staining is also seen in a population of migrating neural crest cells near the otic vesicle.* **(F)** *A stage 27 embryo showing neuron expression down the back. Staining is also apparent in the otic vesicle, eye and in a population of neurons in the midbrain region.* **(G)** *A stage 32 embryo showing more defined glomus and otic vesicle expression, staining in the optic nerve and neurons all down the back.* **(H)** *A stage 36 embryo showing precise expression in the eye and otic vesicle.* **(I)** *A stage 39 embryo close-up showing neuron and otic vesicle expression and a well developed glomus.* **(J)** *Cross-section through a stage 15 embryo showing exact areas of expression of* Xlmx1b *in the placodes and neural plate.* **(K,L)** *Cross-sections through a stage 33 embryo showing exact areas of expression of* Xlmx1b *in the otic vesicles, neurons, floor plate and glomus.* **(M)** *Cross-section through a stage 36 embryo showing expression of* Xlmx1b *in the eye. Abbreviations: A, archenteron; BB, mid-hindbrain boundary; CF, choroid fissure; E, eye; FP, floor plate; G, glomus; N, neurons; NC, neural crest cells; NF, neural folds; NP, neural plate; O, otic vesicles; ON, optic nerve; Pl , placodal regions.*

are forming. There is also still expression along the fusing neural folds, (C). By stage 21 the placodes have condensed considerably and the developing otic vesicle is apparent as a discreet small patch. Upon clearing, neurons are visible along the dorsal side of the embryo and some staining is visible in the head region, which could be a developing patch of neurons or the optic nerve seen in the later stages, (D). By stage 24-25, there is a patch of expression on the dorsal surface of the head that looks v-shaped from the lateral view and diamond-shaped from the dorsal view that may correspond to the mid- hindbrain boundary. Expression is first visible in the dorsal edge of the eye and there are also finger-like projections of stain extending from the otic anlage into the pharyngeal arch region that may be migrating neural crest cells, (E). At stage 26, the otic vesicle is visible as a semi-circular structure and the v-shape is still obvious in the head. The clusters of neurons in the midbrain region, between the eye and otic vesicle, are strongly stained and there is expression in the fin fold epidermis that could have come from another population of migrating neural crest cells. At stage 27 the pattern can be divided into two major domains of localised expression, one in the capsule of the glomus and the other in regions of the nervous system including specific mid- and hindbrain neuronal populations, the optic nerve and the otic vesicle, (F). At this stage and until stage 32, the neurons are visible throughout the majority of the length of the tadpole giving the appearance of darkly stained spots at regular intervals down the anterior/posterior axis. By stage 32, the otic vesicle is a fully formed ring and staining in the dorsal eye is stronger. Upon clearing, staining can be seen in the optic nerve that leads from the eye, directly into the brain region, (G). By stage 33/34 both domains are more clearly defined, although expression of the neurons does not seem to extend as far down the embryo. At stages 35-36 the expression is stronger in the ventral part of the otic vesicle, there is staining both dorsally and ventrally on the edges of the eye and also in lines that lead to the pupil that may be the choroid fissure, (H). At stage 39 all the organs are clearly defined, especially the glomus, (I). Embryos were also hybridized with a control sense probe. No staining was observed in any of the stages tested (data not shown).

In order to study the expression pattern more precisely, representative embryos from the stages were sectioned. At stage 15 the staining is visible on the neural plate and as a slightly thickened area where the neural folds are forming, (J). By stage 21, as in the whole mounts, the neurons are first visible as discreet patches laterally in the neural tube. The otic anlage is clearly identifiable in the stage 24 sections and the neurons can also be seen in more ventral regions of the neural tube. Sections through a stage 26 embryo show *Xlmx1b* expression in the floor plate and epidermis, particularly in the developing fin folds. There are also more neurons visible in the sections, in a range of ventro-lateral positions and the otic vesicle is now visible as a fully formed cavity. At stage 33, as expected, the otic vesicle can be clearly distinguished and discrete groups of neurons show as obvious, darkly stained patches in lateral and ventro-lateral regions of the neural tube, (K). Expression is still noticeable in the floor plate and the glomus is intensely stained, (L). Sections through the eye region at stage 33 and 36 (M), show staining around the edges and the optic nerve is visible on some sections (data not shown).

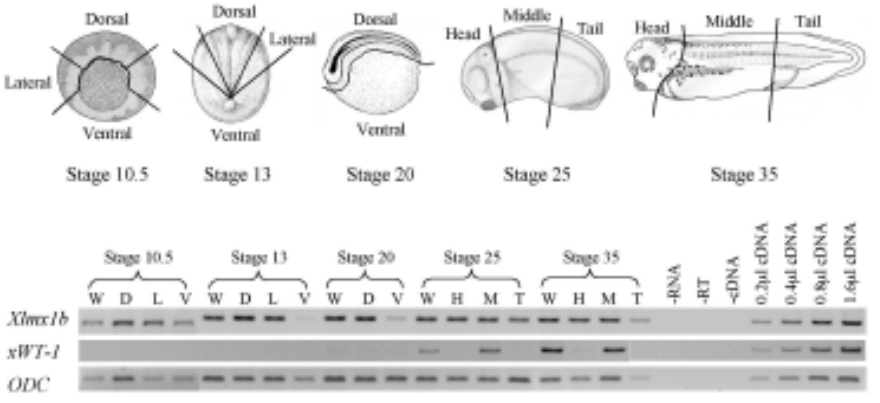

Fig. 5. *Xlmx1b* is expressed in the nervous system and glomus domains at the same time. *RT-PCR analysis shows* Xlmx1b *expression is initiated ubiquitously at stage 10.5. Transcripts are then found in dorsal and lateral regions of stage 13 and 20 embryos, as these two regions will eventually form the nervous system and glomus tissues respectively. At stage 25 and 35, expression is seen in the head from the nervous system expression domain, in the mid region from both nervous system and glomus domains and in the tail region from the neurons.* xWT-1 *was used to identify the glomus domain and show that* Xlmx1b *expression in these regions is initiated prior to* xWT-1. *This experiment cannot rule out expression in those regions being due to some contamination with the neural domain. Dissection was as follows; W, whole embryos; D, dorsal; L, lateral; V, ventral; H, head; M, middle and T, tail regions. (cDNA was not equalised in this experiment in order to establish a genuine relative expression level between components).*

Expression of Xlmx1b *is upregulated in Both Major Domains at Stage 12.5*

In an attempt to establish the temporal control of *Xlmx1b* in both distinct expression domains, RT-PCR was performed on a carefully staged embryo dissection series, following the scheme seen in Fig. 5. At stage 10.5, when zygotic expression is initiated, *Xlmx1b* transcripts are ubiquitous. At stage 13 and 20, the dorsal and lateral explants show the highest levels of expression as would be expected because these regions will go on to form the nervous system and glomus respectively. By comparison, the ventral regions do not show high levels of expression at these stages, as this region is fated to form endoderm, which does not contribute to nervous system or kidney. Stage 25 embryos show expression in the head and mid region. The head dissection will contain transcripts derived from the nervous system expression domain and the mid region will contain transcripts from both this and the glomus. There is also expression in the tail region which is likely to be from the neuronal expression that stretches down the neural tube. Stage 35 embryos follow the stage 25 expression pattern, with transcripts being detected in the head, mid and tail regions as expected.

Two fine dissection experiments were also carried out (data not shown). Firstly, of stage 20 embryos, to define further the regions of *Xlmx1b* expression and in particular, to clarify staining seen in the placodal regions of wholemount *in situ* hybridisations of stage 19 embryos (Fig. 4C). Embryos were dissected into nervous system including neural tube and placodes, notochord, somites including presumptive pronephros, ectoderm including ventral and lateral plate mesoderm and endoderm. RT-PCR analysis showed that *Xlmx1b* is present in all components at this stage so placodal region expression is not responsible for the expression in the presumptive pronephros region. Secondly, to confirm that the earlier expression does indeed come from the pronephric anlagen and not just the neural domain, we dissected the pronephric anlage from a range of embryos (including stages 12.5, 15 and 20) and carried out RT-PCR. *Xlmx1b* expression is seen in the earliest dissection, which includes the pronephric anlagen from stage 12.5, the time when the glomus is initially specified (Brennan *et al.*, 1999) and there is no expression of *xWT-1* until past stage 20, as expected. As *Xlmx1b* expression is initiated much earlier than *xWT-1* in the glomus domain and we have additional preliminary evidence to say it has a functional role in development of the glomus (Haldin *et al.*, manuscript in preparation), we speculate that *Xlmx1b* may have a role in specifying the glomus before *xWT-1* functions.

Xlmx1b *is induced in Animal Caps by Growth Factors*

Under normal conditions, with no addition of growth factors, animal cap explants form atypical epidermis and generally will not express *Xlmx1b*. Figure 6 shows the effects of incubation with activin A and RA at a series of different concentrations. Activin A alone is sufficient to cause induction of *Xlmx1b* at all concentrations tested. The effect of RA seems to be repressive in a concentration dependant manner. *xWT-1* is induced only when there is RA and activin in combination (Brennan *et al.*, 1999), indicating glomus tissue formation. As *Xlmx1b* expression is induced under

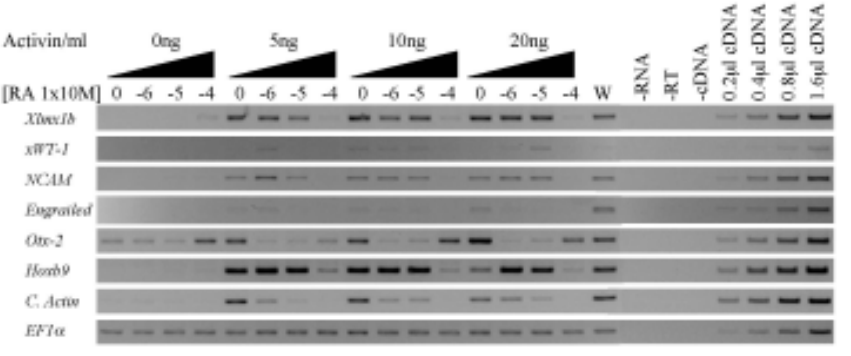

Fig. 6. Activin A and RA affect *Xlmx1b* induction. *Animal cap explants taken at stage 8/9 and treated with activin A show induction of* Xlmx1b *by stage 22 equivalence. This induction is inhibited by the addition of RA in a concentration dependant manner. As* xWT-1 *is not induced in all the samples in which* Xlmx1b *is induced, the expression of* Xlmx1b *may also be from induction of the neural domain.* Otx-2 *and* Hoxb9 *were used to identify formation of anterior and posterior neural tissues that could be contributing to* Xlmx1b *expression. (Expression of* Otx-2 *in untreated caps is an established phenomenon, Lamb and Harland, 1995).* Cardiac actin *expression shows the growth factors were effective in inducing mesoderm.*

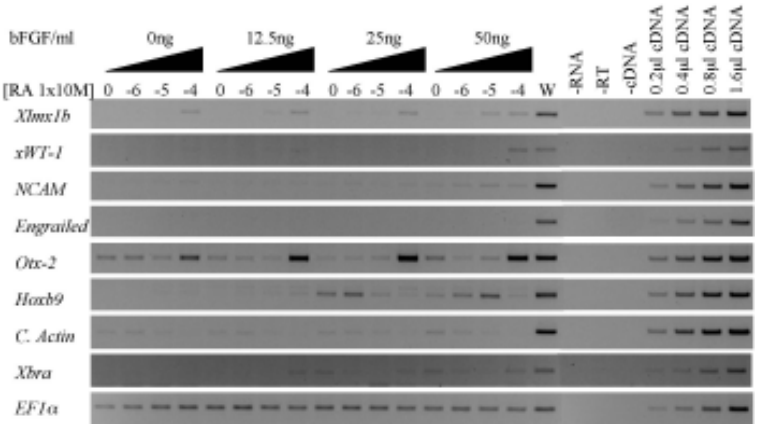

Fig. 7. bFGF and RA affect *Xlmx1b* induction. *Animal cap explants taken at stage 8/9 and treated with bFGF and RA show induction of* Xlmx1b *by stage 22.* xWT-1 *expression at the highest concentrations shows glomus tissue is being induced.* Otx-2 *and* Hoxb9 *were used to identify formation of neural tissues that could be contributing to* Xlmx1b *expression.* Cardiac actin *and* XBra *expression shows that growth factors were effective in inducing mesoderm.*

conditions where *xWT-1* is not, expression may result as a consequence of glomus and/or nervous tissue in the animal caps. In an attempt to answer this, PCR's for several neural markers were carried out. Fore and hindbrain tissues have been induced as shown by the anterior marker *Otx-2* (Lamb *et al.*, 1993) and the marker for posterior neural tissue, e.g. spinal cord, *Hoxb9* (Wright *et al.*, 1990) respectively. However there is very little induction of *NCAM*, an early general marker of the neural plate that later becomes confined to the neural tube (Kintner and Melton, 1987), or the midbrain hindbrain boundary and optic tectum marker *Engrailed* (Hemmati-Brivanlou *et al.*, 1991).

Figure 7 shows the effects of incubation of animal caps with bFGF and RA. bFGF alone is unable to induce *Xlmx1b* but in combination with RA there is slight induction at the higher concentrations. *xWT-1* is induced at low levels by the highest concentrations of bFGF and RA indicating that glomus tissue is formed. This is also the case for *NCAM*, so expression of *Xlmx1b* is likely to be from both glomus and neural tissue formation. *Hoxb9* is induced by bFGF and inhibited by RA suggesting that some of the neural induction is from posterior tissue types. The forebrain marker *Otx-2* is also induced by the highest concentration of RA, however there is very little induction of the mid-hindbrain marker *Engrailed*.

In each case, RT-PCR's for *Cardiac actin* and/or *Xbra* were carried out to show that mesoderm induction had occurred following growth factor treatment (Mohun *et al.*, 1989; Smith *et al.*, 1991).

Discussion

Isolation and Sequencing of Xlmx1b

As we have seen, *Xlmx1b* shows high homology to the previously identified Lmx1b sequences. Alignments were also carried out to mouse and human Lmx1a protein sequences and although these also showed high homology, they were slightly less conserved confirming that we indeed have *Xlmx1b*. Not surprisingly the LIM and homeo domains show the highest levels of identity, with the homeodomain being 100% conserved. Similarity such as this is an important indicator of conservation of function. So far, most phenotypes associated with mutations in *Lmx1b*, are observed and have been investigated in more than one species. The most documented species are human, mouse and chick. In each of these species Lmx1b has been shown to have a role in dorso-ventral patterning of the limb bud (Vogel *et al.*, 1995, Dreyer *et al.*, 1998, Chen *et al.*, 1998a). We have now shown that *Xenopus* Lmx1b is highly conserved, 88%, 88% and 93% similar to each of these three homologues respectively, and is present in both fore and hind limbs. It has also been shown to be present in the dorsal mesoderm of earlier *Xenopus* limb buds with a possible role in dorso-ventral patterning and regeneration of the limb blastema after amputation (Matsuda *et al.,* 2001*).* Mice showing severe kidney and eye phenotypes have been observed when *mLmx1b* is disrupted (Chen *et al.*, 1998a, Pressman *et al.*, 2000) and similarly, humans with NPS, caused by a mutation in *hLmx1b,* have problems in both these organs (Del Pozo and Lapp, 1970; Hoyer *et al.*, 1972; Pressman *et al.*, 2000). The neural phenotype also seems to be conserved between species. *C-Lmx1* misexpression causes changes in mesencephalic morphology (Adams *et al.*, 2000) and mice with disrupted *Lmx1b* lack dorsally derived structures from the caudal midbrain and rostral hindbrain (Chen *et al.*, 1998b). Whilst reporting *Lmx1b* to be required for dopaminergic neuron development in the mouse, Smidt *et al.* also noted major deficits in the region of the mesencephalon, (Smidt *et al.*, 2000). The *Lmx1a/Dreher* mutation (*dr*), causes dorsal interneurons and spinal cord granule neurons in the cerebellar cortex to be lost (Millonig *et al.*, 2000) and loss of *C. elegans lim-6* affects a number of areas, including differentiation of two classes of GABAergic motorneurons, (Hobert *et al.*, 1999). With such high levels of identity at the amino acid level and so many similarities in the mutant phenotypes of species that have been examined, the fact that we have shown *Xlmx1b* to be expressed in similar organs leads us to believe the functions of this gene will be also be conserved.

Spatial Expression Pattern of Xlmx1b

The expression pattern of *Xlmx1b* can be split into 3 main domains, kidney, neural and limbs and Lmx1b has been documented in all these areas in other species.

We have shown that *Xlmx1b* is highly expressed in the capsule of the pronephric glomus. Lmx1b has been previously linked with the kidneys of chick, mice and humans. *C-Lmx-1* transcripts have been detected from stage 16 chick embryos in the mesonephros but are absent in the earlier pronephros (Riddle *et al.*, 1995). In homozygous mutant mice the glomerular basement membrane is prominently thickened and has occasional regions of discontinuity (Chen *et al.*, 1998a). Similarly, human NPS patients show thickened glomerular basement membrane and renal failure can be a complication (Del Pozo and Lapp, 1970; Hoyer *et al.,* 1972).

Our *in situ* hybridisations also show *Xlmx1b* to be expressed in a region of the mid/hindbrain. *C-Lmx1* is expressed in the rostral CNS with expression persisting in large portions of the dorsal and ventral midline as well as the isthmic organiser (IsO). The IsO is a morphological constriction of the neural tube that eventually defines the mesencephalic/metencephalic boundaries (the embryonic precursors of adult anterior midbrain structures and hindbrain structures respectively) (Adams *et al.*, 2000). At 8.5dpc, *Lmx1b* expression in mice is localised to a thin strip of cells at the intersection of the presumptive midbrain and hindbrain (Johnson *et*

al., 1998). Targeted disruption of *mLmx1b* causes new born brains to have a marked reduction of dorsally derived structures from the caudal midbrain and rostral hindbrain (Chen *et al.*, 1998b) and these deficits in the region of the mesencephalon were also noted by Smidt *et al.*, (2000).

Xlmx1b was also detected in specific populations of neurons that stretch down the neural tube of the embryo. *Lmx1b* has been shown to be required for dopaminergic neuron development in the mouse (Smidt *et al.*, 2000) and the mesencephalic dopaminergic system is for regulation of movement control and behaviour. Also, a mutation in the closely-related *Lmx1a/Dreher* (*dr*) gene, causes dorsal interneurons and spinal cord granule neurons in the cerebellar cortex to be lost (Millonig *et al.*, 2000). The *C. elegans lim-6* gene is expressed in a small number of sensory-, inter-, and motorneurons and in epithelial cells of the uterus and excretory system. Loss of this gene affects a number of areas, including differentiation of two classes of GABAergic motorneurons, as the gene is required to specify the correct axon morphology and regulate expression of the enzyme required for GABA synthesis (glutamic acid decarboxylase) (Hobert *et al.*, 1999). In *Drosophila*, *apterous* (*ap*) is thought to be involved in neuronal pathway selection as it is expressed in a small subset of interneurons. In *ap* mutants these neurons do not follow the correct pathway and cannot fasciculate with one another (Lundgren *et al.*, 1995).

In stage 13 *Xenopus laevis* embryos, *Xlmx1b* staining is seen in the placodal regions and by stage 27 this staining is clear in the otic vesicles. These will eventually develop into the hearing system of the adult frog. Chick *Lmx1* is also detected by *in situ* hybridisation throughout the development of the otic placode and otic vesicle and is thought to be involved in early patterning of this organ (Giraldez, F. 1998).

Expression of *Xlmx1b* is also detectable in the eye and ocular defects have been associated with disruptions in both *mLmx1b* and *hLmx1b*. Homozygous mutant mice show iris and ciliary body hypoplasia and corneal stromal defects and *Lmx1b* is thought to have a role in the development of the anterior segment of the murine eye. (Pressman *et al.*, 2000). Patients with NPS sometimes suffer from glaucoma, which has been found to cosegregate with the NPS locus, but it is not yet known if this is caused by mutations in *hLmx1b* or other closely related genes (Pressman *et al.*, 2000). It is therefore likely that *Xlmx1b* will have a role in the *Xenopus* visual system.

The final area in which we saw *Xlmx1b* expression was limbs. Matsuda *et al.* have shown *Xenopus Lmx-1* to be dorsally restricted in stage 52-55 limbs. We confirm by RT-PCR and northern blot analysis expression in both fore and hind limbs. As well as postulating a role in dorso-ventral patterning, Matsuda *et al.* suggest that *Xenopus* Lmx-1 is involved in regeneration of the limb blastema after amputation. Expression of *Xenopus Lmx-1* is not seen in the forelimb blastema (which is known to only be capable of regenerating a hypomorphic spike-like structure with no differences along the dorso-ventral axis) even by 14 days after amputation, whereas it is detected in the hindlimb blastema (which has higher, or complete, regenerative abilities) as early as 3 days after amputation, (Matsuda *et al.*, 2001). The chicken homologue *C-Lmx1* plays a major role in development of the dorsoventral axis of the limb bud. The transcripts become restricted to the dorsal mesoderm of the developing bud and are thought to specify dorsal cell fate during development (Vogel *et al.*, 1995). This dorsalisation is controlled by the secreted protein Wnt-7a which mediates induction of *C-Lmx1* in mesenchymal cells (Riddle *et al.*, 1995). Similarly, the mouse ortholog *Lmx1b* is thought to be involved in dorsalising limb development and *Lmx1b* mutants show abnormal skeletal patterning including the absence of nails and patella (Chen *et al.*, 1998a). As in the chick, expression of *mLmx1b* is restricted to the dorsal mesenchyme. Ectopic expression of *Wnt-7a* in the ventral ectoderm causes an ectopic expression domain of *Lmx1b* in the ventral mesenchyme and in *Wnt-7a* mutants, regions of dorsal mesenchyme that do not express *Lmx1b* have a ventral fate in the adult limb (Cygan *et al.*, 1997). These abnormalities are very similar to those seen in patients displaying NPS. The *mLmx1b* gene maps to the NPS locus in humans and *hLmx1b* is 99% similar to the mouse homologue. Three mutations have been seen in patients, two of which are thought to disrupt DNA-binding and the third to cause production of a truncated protein which as yet has not been ruled out as a possible dominant negative receptor (Dreyer *et al.*, 1998). *apterous* in *Drosophila* specifies dorsal fate and is involved in distal outgrowth of the wing (Diaz-Benjumea and Cohen, 1993; Blair *et al.*, 1994; Irvine and Wieschaus, 1994; Kim *et al.*, 1995). It has at least two homologues in vertebrates, *Lmx-1* and *Lhx-2*. The two functions of *apterous* are divided between these two homologues, Lmx-1 is necessary and sufficient to specify dorsal cell fate (Riddle *et al.*, 1995; Vogel *et al.*, 1995; Cygan *et al.*, 1997; Chen *et al.*, 1998a; Rodriguez-Esteban *et al.*, 1998) and Lhx-2 is involved in limb outgrowth (Rodriguez-Esteban *et al.*, 1998). In *Xenopus* the function of *Xlmx1b* may overlap with the latter role or it may play a role in bone development as *mLmx1b* is essential for proper patterning and morphogenesis of the calvaria (cranial vault) and *Lmx1b* mutant mice have multiple defects in these bones (Chen *et al.*, 1998c).

TABLE 1

PRIMER SEQUENCES AND PCR CONDITIONS FOR THE REQUIRED MOLECULAR MARKERS

Marker size bp	Primer sequence (5'-3')	Annealing temp. ºC	No. cycles	Reference
Xlmx1b 291	U-GGAGAGTGGCATGGATATTG D-AGTAGCAGCTGGTGGTGAGG	60	28	This work
XWT-1 436	A-CACACGCACGGGGTCT B-TGCATGTTGTGATGACG	55	27	Carroll and Vize, 1996
Hoxb9 259	U-TACTTACGGGCTTGGCTGGA D-AGCGTGTAACCAGTTGGCTG	58	28	Hemmati-Brivalou and Melton, 1994
Otx-2 280	U-CATCGGACATAAAGCAGCTCATC D-CTTTCCCTCCTCTGTTTCCTGG	55	30	Lai *et al.*, 1995
NCAM 342	U-CACAGTTCCACCAAATGC D-GGAATCAAGCGGTACAGA	55	28	Hemmati-Brivalou and Melton, 1994
En-2 302	U-AGACCTTCATCAGGTCCGAGATC D-TGCCGTCCTTTGAAGTGGTCGCG	55	28	Hemmati-Brivanlou and Melton, 1994
Xbra 187	L-GGATCGTTATCACCTCTG R-GTGTAGTCTGTAGCAGCA	55	28	Wilson and Melton, 1994
Cardiac Actin 252	U-TCCCTGTACGCTTCTGGTCGTA D-TCTCAAAGTCCAAAGCCACATA	62	25	Mohun *et al.*, 1989
ODC 131	U-GGAGCTGCAAGTTGGAGA D-TCAGTTGCCAGTGTGTGGTC	55	19	Bassez *et al.*, 1990
EF1α α 270	U-CAGATTGGTGCTGGATATGC D-CACTGCCTTGATGACTCCTA	55	19	Mohun *et al.*, 1989

Growth Factor Induction of Xlmx1b

We decided to investigate the effects of RA, activin A and bFGF on the regulation of *Xlmx1b,* as growth factors such as these are known to have powerful inducing abilities in *Xenopus*. Also,

combinations of these have previously been used in studies of the regulation of other aspects of the pronephros and in particular, Brennan *et al.* (1999) used these to study induction of the glomus. It was therefore of interest to see if the same conditions could induce *Xlmx1b*.

Application of RA to explants causes induction of *Xlim-1* (a marker of pronephric tubules) (Taira *et al.*, 1992) and caudally expands its expression domain in whole embryos (Taira *et al.*, 1994). Induction of *Xlim-1* is also seen when explants are treated with activin A and there is a synergistic effect when both growth factors are applied (Taira *et al.*, 1992). Moriya *et al.* have shown by histological studies that pronephric tubules will form in explants treated with RA and activin but they are not induced when the explants were treated with activin alone (Moriya *et al.*, 1993). This result was confirmed by analysis of the timing of *Xlim-1* and the RA + activin induction is thought to parallel normal development at the molecular level (Uochi and Asashima, 1996). Brennan *et al.,* failed to find pronephric duct to be induced at high frequency by a wide range of RA and activin concentrations in combination (unpublished result, Brennan *et al.*, 1999), although recently, Osafune *et al.*, have shown by immunohistochemistry and *in situ* hybridisation that duct can be formed in some conditions (Osafune *et al.*, 2002).

Brennan *et al.*, used RT-PCR analysis of the marker *xWT-1*, to show the induction of glomus tissue (*xWT-1* is specific for glomus up until stage 38). They showed that neither activin A nor RA alone were able to induce glomus formation, but when activin A was in combination with high concentrations of RA (10^{-4}), *xWT-1* was present and therefore glomus had been induced. In contrast, we show that *Xlmx1b* is induced by activin alone and RA does not augment this, but instead seems to inhibit it in a concentration dependant manner. As it has been shown that *xWT-1* expressing glomus tissue is not induced by activin A alone (Brennan *et al.*, 1999), induction of *Xlmx1b* must either be from one of the other areas of expression, or *Xlmx1b* is one of the earliest genes involved in glomus specification. Induction of the neural markers *Otx-2* and *Hoxb9*, under similar conditions to *Xlmx1b*, show that neural tissues are being induced so it is therefore possible that some of *Xlmx1b* expression does derive from this domain. However, expression from the glomus domain cannot be ruled out at this stage as this assay does not provide a way of separating the two expression domains and we know that both are expressed with the same temporal profile.

Brennan *et al.*, also showed that unlike the pronephric tubules, bFGF in combination with RA (10^{-4} or 10^{-5}) is able to induce *xWT-1* and glomus tissue (Brennan *et al.*, 1999). In concordance, we show that RA and bFGF at high concentrations can induce *xWT-1* and *Xlmx1b* is also up-regulated by this combination. Again, induction of *Xlmx1b* is likely to be from both glomus and neural domains as *Xlmx1b* is present when *xWT-1* is not and neural tissues are formed as shown by the presence of *Hoxb9* and *Otx-2*, although there is very little *Engrailed* or *NCAM* detected.

Xlmx1b *may be involved in Early Specification of the Glomus*

The gene *xWT-1* is thought to have a role in the specification of the glomus by suppressing tubule genes in tissues that are fated to become glomus (Wallingford *et al.*, 1998). We have shown *Xlmx1b* is upregulated around the time and in the region that the glomus is initially specified and this is much earlier than *xWT-1* expression is initiated. Furthermore, we have preliminary evidence to suggest that *Xlmx1b* has a functional role in the development of the glomus (Haldin *et al.*, manuscript in preparation) so we speculate that *Xlmx1b* may have a role in specifying the glomus before *xWT-1* functions. It is possible that *Xlmx1b* expression seen in the animal caps treated with growth factors is not purely neural tissues, but also derived from a form of glomus tissue that is not yet expressing *xWT-1*. The partial induction of differentiated tissue types has already been demonstrated in the induction of neural tissues by Noggin. In this case, neural tissues are induced but these fail to fully differentiate into mature neurons. This shows that development does not necessarily proceed through its full course, presumably because other factors are required (Lamb *et al.*, 1993). On going work in the laboratory includes investigations into the function of *Xlmx1b*.

Materials and Methods

Cloning and Sequencing of Xlmx1b

Degenerate primers, 5'-CACGAGGAGTGTTTGCAGTG-3' (forward) and 5'-ARMAGRTCCTTCTCYTTCTC-3' (reverse), were designed from the conserved LIM domains in the chick, hamster and mouse Lmx1 homologues. PCR using adult kidney cDNA was carried out and the resulting ≈ 300bp fragment cloned into pGEMT-Easy™. This was then used to screen a stage 17 *Xenopus laevis* embryonic library from which *Xlmx1b* was obtained. Sequencing was carried out using the ABI-PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase. The PCR reaction was performed in a Perkin Elmer Gene Amp® PCR system.

Production and Dissection of Xenopus laevis *Embryos*

Embryos were produced by *in vitro* fertilisation by standard procedures, dejellied in 2% cysteine-HCL pH 8.0, washed and cultured to the required stage in 10% Barth X and 10 μg/ml gentamycin. Staging was according to Nieuwkoop and Faber (1994). Where required, embryos were fixed in MEMFA or anaesthetised in MS222 and dissected using forceps and an eyebrow hair knife.

In order to separate the domains of expression by dissection, appropriately staged embryos were dissected into dorsal, lateral, ventral, or head, middle and tail regions as appropriate. To obtain fine dissection samples, stage 20 embryos were dissected in collagenase, into nervous system including neural tube and placodes, notochord, somites including presumptive pronephros, ectoderm including ventral and lateral plate mesoderm, and endoderm. In the pronephric anlagen dissection, the intermediate mesoderm and presumptive pronephros dissections were carried out as described in Brennan *et al.*, (1998).

Growth Factor Explant Assays

Animal caps were taken from embryos at stage 8/9 and incubated in 50% Barth X + 0.001% BSA and 10 μg/ml gentamycin with the required concentrations of growth factors until they were past stage 13 (according to control whole embryos). They were harvested at equivalent to stage 20 - 22 and RT-PCR was performed. Growth factors used were activin A, RA and bFGF in the following concentrations; 0, 5, 10 or 20 ng of activin A with each of 0, 10^{-6}, 10^{-5} or 10^{-4}M RA. 0, 12.5, 25 or 50 ng of bFGF with each of 0, 10^{-6}, 10^{-5} or 10^{-4} M RA.

RT-PCR

Samples were homogenised and total RNA was extracted followed by cDNA synthesis as detailed in Barnett *et al.*, 1998. PCR was carried out according to details in Table 1. Each experiment contained -RNA, -RT and -cDNA negative controls and a linearity series to show the PCR was in the linear range. *ODC* or *EF1α* were used as loading controls and equalised where appropriate. (cDNA was not equalised in the expression domain or fine dissection experiments in order to reflect the proportion of the embryo used in the RNA preparation).

Whole Mount* In Situ *Hybridisation

Hybridisation was carried out on albino embryos. Probes were prepared using a Boehringer-Mannheim digoxygenin (DIG) labelling kit and whole mount *in situ* hybridisation was carried out using a standard protocol (adapted from Hemmati-Brivanlou, 1990 and Harland, R. M., 1991). The antisense probe was *Xlmx1b* :pGEMT-Easy™ cut with *Sal*I and transcribed with T_7 and the sense probe with *Nco* I and SP6.

Wax Embedding and Sectioning of Whole Mount* In Situ *Hybridised Embryos

Wholemount embryos were selected for sectioning on the basis of staining quality. Embryos were dehydrated gradually into absolute ethanol and cleared with Histoclear II (Lamb). Samples were then transferred into 1:1 Histoclear II:Paraplast X-tra (Sigma) for 30 minutes at 60°C. This was replaced with molten Paraplast X-tra for overnight incubation. One embryo was then embedded on a cushion of Paraplast X-tra per watchglass and allowed to set. The blocks were sectioned on a Bright microtome to give 10μm sections that were lifted onto Superfrost slides (BDH Laboratory Supplies) and dried. Slides were cleared with Histoclear II followed by Xylene washes and mounted with coverslips using DePex mountant (BDH Laboratory Supplies).

Northern Blot Analysis

RNA was extracted with Trizol® reagent (Life Technologies) according to manufacturers instructions. 30 μg of total RNA in 100 μl of H_20 was precipitated with 2.5 volumes of ethanol and $^1/_{10}$ volume of sodium acetate (NaOAc) overnight at -20°C. This was centrifuged at 13,000 rpm for 30 minutes at 4°C. The pellet was dried at 65°C and resuspended in 8 μl loading dye (80% formamide, 10 mM EDTA pH 8, 1 mg/ml Xylene cyanol FF, 1 mg/ml bromophenol blue) with 1 μl of ethidium bromide and 1 μl of 10x MOPS EDTA (0.2 M MOPS pH 7, 0.01 M EDTA pH 8, 0.02 M NaOAc pH 5). 3 μl of RNA kb ladder was treated in the same way. The samples were denatured for 10 minutes at 65°C and then loaded onto a 1.2% denaturing agarose gel made up with 1x MOPS EDTA and 6.6% formaldehyde and run at 80 V until the RNA kb ladder had resolved sufficiently. The gel was washed twice with H_20 for 10 minutes and once with 2x SSC and blotted over 2-3 nights. The blot was set up after Southern (1975). A PCR product from a non-conserved 5' coding region of *Xlmx1b* was labelled with αdGTP^{32} and used to hybridise overnight at 42°C. Membranes were then washed twice with 2x SSC, 0.1% SDS at 42°C and once with 1x SSC, 0.1% SDS at 65°C before being exposed to a phosphorimager (Molecular Dynamics).

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