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Annexin IV (Xanx-4) has a functional role in the formation of pronephric tubules

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SUMMARY

Vertebrate kidney organogenesis is characterised by the successive formation of the pronephros, the mesonephros and the metanephros. The pronephros is the first to form and is the functional embryonic kidney of lower vertebrates; although it is vestigial in higher vertebrates, it is a necessary precursor for the other kidney types. The *Xenopus* pronephros is a simple paired organ; each nephron consists of a single large glomus, one set of tubules and a single duct. The simple organisation of the pronephros and the amenability of *Xenopus laevis* embryos to manipulation make the *Xenopus* pronephros an attractive system in which to study organogenesis. It has been shown that pronephric tubules can be induced to form in presumptive ectodermal tissue by treatment with RA and activin. We have used this system in a subtractive hybridisation screen that resulted in the cloning of *Xenopus laevis annexin IV* (*Xanx-4*). *Xanx-4* transcripts are specifically located to the developing pronephric tubules, and the protein to the luminal surface of these tubules. Temporal expression shows zygotic transcription is upregulated at the time of pronephric tubule specification and persists throughout pronephric development. The temporal and spatial expression pattern of *Xanx-4* suggests it may have a role in pronephric tubule development. Overexpression of *Xanx-4* yields no apparent phenotype, but *Xanx-4* depletion, using morpholinos, produces a shortened, enlarged tubule phenotype. The phenotype observed can be rescued by co-injection of *Xanx-4* mRNA. Although the function of annexins is not yet clear, studies have suggested a role for annexins in a number of cellular processes. Annexin IV has been shown to have an inhibitory role in the regulation of epithelial calcium-activated chloride ion conductance. The enlarged pronephric tubule phenotype observed may be attributed to incorrect modulation of exocytosis, membrane plasticity or ion channels and/or water homeostasis. In this study, we demonstrate an in vivo role for annexin IV in the development of the pronephric tubules in *Xenopus laevis*.

Key words: Annexin, Pronephros, Kidney, *Xenopus*, Morpholino

INTRODUCTION

Vertebrate kidney morphogenesis involves the progressive development of three kidney forms the pronephros, mesonephros and the metanephros (Saxén, 1987). Each kidney type arises from the intermediate mesoderm by an inductive process that is dependent for its formation on the previous temporal form. In amphibia, the pronephros is the functional larval kidney and is the first to form. It is a paired organ that consists of a single non-integrated nephron found in a lateral position of the embryo (Saxén, 1987; Vize et al., 1995; Brändli, 1999). There are three identifiable components that together form the functional pronephros; the glomus, the pronephric tubules and the collecting duct. The glomus filters waste into the coelom, where it is collected into the coiled tubules via the ciliated nephrostomes. Water balance is controlled as the waste passes down the tubules, along the duct and to the exterior via the cloaca. The simplicity of this organ coupled with the fact that it displays the same basic organisation and function as the more complex mesonephros and metanephros make this an attractive model to study the earliest events in vertebrate kidney morphogenesis (Vize et al., 1997).

We, and others, have started to establish some of the biological parameters that control pronephros formation. The temporal specification of all three components of the kidney have been established and all occur between stages 12.5 and 14 (Brennan et al., 1998; Brennan et al., 1999). A molecular map of many of the early markers of the pronephric field and pronephric anlagen is also being built up in order to unravel the molecular control of early kidney induction and patterning. In previous studies, more than 30 genes have been shown to be expressed in the pronephros and their temporal and spatial patterns of expression established (http://golgi.ana.ed.ac.uk/kidhome.html) (Wallingford et al., 1999; Carroll and Vize, 1999; Carroll et al., 1999a; Carroll et al., 1999b; Brändli, 1999; Sato et al., 2000; Onuma et al., 2000; McLaughlin et al., 2000). Many of these genes have also been shown to be expressed in mesonephric and metanephric kidneys in higher vertebrates. Evidence from knockout mutant mice has shown that *Lim1*, *Pax2* and *Wt1* are essential for pronephros development. The role of known growth factors in the process has also been investigated (Moriya et al., 1993; Uochi and Asashima, 1996; Brennan et al., 1999).

However, the initial events in kidney organogenesis are not yet fully understood and *Xenopus* provides an ideal vertebrate system to identify and establish the functional role of genes involved in early kidney development. It is relatively easy to establish the domains of expression of novel genes expressed in particular regions of interest, and then it is possible, by overexpression, antisense depletion or morpholino treatment to perturb normal expression pattern and get a developmental handle on function (Zhang et al., 1998; Summerton and Weller, 1997; Heasman et al., 2000). Furthermore, direct expression cloning is proving an invaluable tool in identifying novel genes with defined roles in developmental patterning and organogenesis (Smith and Harland, 1991; Smith and Harland, 1992; Smith et al., 1995; Hsu et al., 1998; Grammer et al., 2000).

In order to identify novel genes that may be involved in pronephros development, we have adopted a subtractive hybridisation strategy that increases the levels of those genes expressed early in kidney development. This approach is based on the observations that animal caps treated with a combination of retinoic acid and activin develop in vitro into differentiated kidney tubules. Recent studies in our laboratory have also shown that glomus can be induced in animal caps by treatment with retinoic acid and activin or retinoic acid and FGF (Brennan et al., 1999). No combinations of RA, activin or bFGF have been found to induce pronephric duct at high frequency (E. A. J., unpublished). Evidence from histological studies indicates that these tubules have normal morphology (Moriya et al., 1993) (H. C. Brennan and E. A. J., unpublished). Furthermore, these tubules express differentiation markers characteristic of the correct developmental stage and in the correct developmental sequence (Uochi and Asashima, 1996). The kidney tubules formed have been reported to rescue pronephric function in tadpoles in which the pronephros has been extirpated by dissection. During the course of our experiments a similar strategy has been used by others to successfully clone the pronephros specific genes *XCIRP* (Uochi and Asashima, 1998) and *XSMP-30* (Sato et al., 2000). In vitro induction followed by differential display has recently resulted in the isolation of *Xsal-3* that is also expressed in the pronephros (Onuma et al., 2000).

We describe the isolation of *Xenopus annexin IV* (*Xanx-4*) via a subtractive hybridisation strategy designed to increase the levels of tubule-specific genes, which are expressed specifically and at high levels in the pronephric tubules. We have established the temporal and spatial expression patterns both of mRNA and protein in embryos. We have established the mRNA expression pattern by northern analysis in the adult frog. Finally, we have used morpholino oligonucleotides to specifically inhibit the translation of *Xanx-4* and show that a tubule phenotype results, which can be rescued by the addition of wild-type message. The tubules appear less coiled and have a diameter that is significantly greater than that seen in control embryos. These results indicate that *Xanx-4* plays an important role in morphogenesis of the pronephric tubules.

MATERIALS AND METHODS

Preparation of subtracted probe

Animal caps were isolated from stage 9 *Xenopus laevis* embryos by manual dissection and divided into three groups each containing approximately 400 animal caps. The first group was incubated in Barth X media containing 1/40 dilution of WEHI factor, a kind gift from Professor J. Slack (Bath) (source of activin A at ~10 ng/ml) and 10^{-5} M retinoic acid, RA. The second group was incubated in media containing WEHI factor alone and the third group in media containing 10^{-5} M RA. Each group of caps was harvested at stage 20 and total RNA prepared as described by Barnett et al. (Barnett et al., 1998). A small part of each sample was used for spectrophotometric quantification. PolyA+ RNA was prepared from total RNA using an Oligotex mRNA Kit (Hybaid) according to the manufacturer's protocol.

Tester cDNA was prepared from 2 μg of group one polyA+ RNA. The polyA+ RNA from groups two and three were pooled and Driver cDNA was prepared from 2 μg of this pool. The subtracted probe was prepared by subtracting Driver cDNA from Tester cDNA, according to manufacturer's protocol using Clontech PCR-Select cDNA Subtraction Kit (K1804-1).

Library screening and clone sequencing

Whole embryo stage 13 *Xenopus laevis* λgt 11 cDNA library (gift from I. Dawid) was plated at a density of 2.5×10^5 plaques on 25 cm^2 plates and duplicate plaque lifts taken on Hybond N+ (Amersham) nylon filters. The subtracted probe was hybridised at 62°C (0.1%SDS, 5×SSC, 0.1% BSA, 0.1% poly vinyl-pyrollidone, 0.1% Ficoll, type 400). The first wash was performed at 62°C (2×SSC, 0.1% SDS), followed by two further washes (1×SSC/ 0.1% SDS, 0.5×SSC/0.1% SDS and 0.2×SSC/ 0.1% SDS). The 1131 bp K2 (*annexin IV*) cDNA insert was subcloned into pGEM-T Easy (Promega) and both strands were sequenced using an Applied Biosystems 373A instrument.

Embryo culture and dissection

Embryos were obtained by in vitro fertilisation of hormonally stimulated *Xenopus laevis* and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Standard embryological procedures were used as described by Jones and Woodland (Jones and Woodland, 1986). Embryos were dejellied in 2% cysteine hydrochloride pH 8 and cultured in 1/10 BarthX. Dissected animal caps were cultured in BarthX and staged using whole embryo controls.

Expression clones, mRNA Synthesis and micro-injection

The full-length *Xanx-4* cDNA was removed by digestion with *Eco*RI from pGEM-T Easy plasmid and cloned into the RN3 pBluescript vector (a gift from J. B. Gurdon, Cambridge) at the *Eco*RI sites. *Xanx-4* mRNA was synthesised from *Xanx-4*/RN3 plasmid template, linearised with *Sfi*I, using the mMessage mMachine (T3 RNA polymerase, Ambion). For the Myc-tagged *Xanx-4* expression construct, a PCR cloning approach was used. Primers were designed to contain *Bam*HI restriction sites (U-gccgggatcccatggcagcactc, D-cggcggatccgtcttcccctccg) and the resulting product cloned into pCS3+MT vector (a gift from H. Benisek, Michigan). Myc-tagged *Xanx-4* mRNA was synthesised by linearising the *Xanx-4*/MT3 construct with *Eco*RI and transcribed using SP6 RNA polymerase mMessage mMachine Kit. The *Sox17β* mRNA and Myc-tagged constructs were a gift from D. Clements (Warwick). Approximately 0.5 ng of mRNA was injected into dejellied embryos at the one-cell stage, alone or in combination with MOs (as specified in the text), under 3% Ficoll in BarthX.

Morpholinos

Xanx-4 and *Xsox17β* (gift from D. Clements) morpholinos (MO) were designed and supplied by GeneTools, LLC (Corrallis, OR):

Xanx-4: 5′-acccttagttccgagtgctgccatg-3′
XSox17β: 5′-cctcttacctcagttacaatttata-3′
control: 5′-cctcttacctcagttacaatttata-3′.

The MOs were dissolved in double distilled H_2O to a stock concentration of 10 μg/ml and were injected into one-cell stage embryos (5 ng, 10 ng or 20 ng) alone or in combination with mRNA as specified in the text.

RT-PCR

Total RNA from whole embryos was isolated and used for RT-PCR as described by Barnett et al. (Barnett et al., 1998). Primers used in this study are as follows.

Xanx-4: U-AGCAGGCACGATGAAGATG and D-TCATTCAC-GGTGCTGCTCTG (this work)

Xlim-1: U-GAAGGATGAGACCACTGGTGG and D-CACTGCC-GTTTCGTTCATTTC (Witta and Sato, 1997)

XPax-2I: U-TCGGAAGAAGAGTGGTCTAC (this work) and D-GGTATTCATATTCCGCATTC (Accession Number, AF179300)

XPax-8: U-CCAACAGCAGCATCAGATC (this work) and D-CAATGACACCTGGCCGGATA (Accession Number, AF179301)

XWnt-4: U-GAGTGGAATGCAAGTGTC (this work) and D-TACACTGCCGACCAGTTG (Accession Number, U13183)

XWnt-11: U-GAAGTCAAGCAAGTCTGCTGG and D-GCAG-TAGTCAGGGGAACTAACCAG (http://www.lifesci.ucla.edu/hhmi/derobertis/index.html)

xWT1: U-CACACGCACGGGGTCT and D-TGCATGTTGTG-ATGACG (Carroll and Vize, 1996)

ODC: U-GGAGCTGCAAGTTTGGAGA and D-TCAGTTGCC-AGTGTGGTC (Bassez et al., 1990)

EF1α: U-CAGATTGGTGCTGGATATGC and D-CACTGCCTT-GATGACTCCTA (Mohun et al., 1989)

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was carried out as described elsewhere (Harland, 1991). The embryos were fixed in MEMFA (0.5 M MOPS, pH 7.4, 100 mM EGTA, 1 mM $MgSO_4$, 4% formaldehyde) and hybridised with RNA probes produced from cDNA clones. The *Xanx-4* antisense probe was transcribed with SP6 RNA polymerase from the full-length *Xanx-4* in pGEMT-Easy. The *XPax-8*, *Xlim-1* and *xWT1* were kind gifts from T. Carroll (Texas). Probes were synthesised and labelled using a DIG labelling kit (Boehringer) and visualised using anti-DIG-alkaline phosphatase secondary and NBT/BCIP for the colour reaction according to manufacturer's recommendations (Boehringer).

Immunohistochemistry

Whole-mount immunohistochemistry was performed using standard methods on MEMFA fixed embryos. The primary antibodies used were pronephric tubule-specific monoclonal antibody 3G8 and pronephric duct specific monoclonal antibody 4A6 (Vize et al., 1995), and an anti-annexin IV monoclonal antibody BL7B1, a kind gift from D. Massey-Harroche, Marseille (Massey et al., 1991). The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse (Sigma) and FITC-conjugated goat anti-mouse (Sigma). BCIP/NBT (Boehringer) or Fast Red TR/Napthol AS/MX (Sigma) was used for the colour reaction, according to manufacturer's recommendations.

Nuclear staining was carried out using Hoechst stain (33258) at a concentration of 1 μg/ml on acrylamide embedded sections, mounted in glycerol and viewed on Nikon microscope and using a u.v. filter.

Acrylamide embedding and cryostat sectioning

Embryos were fixed in MEMFA, rinsed in phosphate-buffered saline (PBS) and incubated at 4°C for 5 hours in embedding acrylamide (8.4 g acrylamide, 13.4 mg bis-acrylamide, 700 μl TEMED to 100 ml in PBS). The embryos were embedded in acrylamide using 5 μl/ml 10% ammonium persulphate to polymerise overnight at 4°C. The acrylamide blocks were frozen in iso-pentane over liquid nitrogen for 5 minutes. The blocks were then allowed to warm to –20°C and sectioned on a cryostat at 12 μm. The sections were lifted onto 0.1% gelatin subbed slides (300 bloom), fixed in acetone and mounted in 50% PBS/glycerol.

A

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Xenopus      1) maalgtkgtikpypnfnaaddvqklrnamkgagtdedavidvianrtlsqrqeiktaykt
Human        1) ..matkg..v.aasg...me.a.t..k....l......i.s.l.y.nta.....r....s
Rat          1) .etk.--..v.aasg...te.a.v..k....l......i.g.l.c.nta.....r....s
Mouse        1) .e.k.--..v.aasg...te.a.t..k....l......i.gil.y.nta.....rs...s
Cow          1) ...k.--..v.aasg....e.a.t..k....l......i.n.l.y.sta.....r.....
Medaka       1) ...i.nr..vteasg..pd..a....e.........a.i.k.l.h..ia...r..l...q

            (61) tvgkdldddlkseltgnfekvilglitsstlydveelkkamkgagtdegclieilasrsa
            (61) .i.r..i.......s....q..v.mm.ptv....q..rr...................tp
            (59) .i.r..le......ss...q....mm.ptv....q..rr...................np
            (59) .i.r..ie......ss...q.....m.ptv....q..rr...................tp
            (59) .i.r..m.......s....q....mm.ptv....q.vr....................tp
            (61) s.....ae..s...s.h.qs.v...lmpapv..ay...a.......e.a...d......n

           (121) eeikninitykikygksleddicsdtsfmfqrvlvslaaggrdqsstvnedlakqdandl
           (121) ...rr.sq..qqq..r......r..............s.....egnyldda.vr...q..
           (119) ...rr..q..qqq..r...e.................t.....egnyldda.vr...q..
           (119) ...rr..q..qqq..r...e...............f.s.a...egnyldda.m....qe.
           (119) ...rr..q..qlq..r......r..............s.....e.nyldda.mr...q..
           (121) s.mna..ev..ke...t...av.g...g.........lta...e.dk.d.aq.vk..k.i

           (181) yeagekkwgtdevkfltilcsrnrnhllkvfeeykkiakkdleasiksemsghledslla
           (181) .................v..........h..d...r.sq..i.q.....t..sf..a...
           (179) ......r.........s...........h..d...r.sq..i.q.....t..sf..a...
           (179) ......r.........s...........h..d...r.sq..i.q.....t..sf..a...
           (179) .................v..........h..dk..r..q..i.q.....t..sf..a...
           (181) f....ar..........v..v.......r..d.....s.r.i.d...r....s...vf..

           (241) ivkciksrpayfaerlyksmkglgtddktlirvmvsrceidmleircefkkmygkslhsf
           (241) ....mrnks.....k...........n.........a.....d..ah..rl.....y..
           (239) ....mrnk..................s.........p.....d.pan..rv.....y..
           (239) ....mr.k.s................n.........a.....d..as..rl.....y..
           (239) ....mrnks.................d.........a.....d..an..rl.....y..
           (241) ....lr.k..f.............t.sv...i....a.....d.keh.l.t........

           (301) ikgdcsgdyrkvllklcgged
           (301) ....t.........v....d.
           (299) ....t.........i....d.
           (299) ....t.........i....d.
           (299) ....t.........i....d.
           (301) ....t......i..e.....-
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B

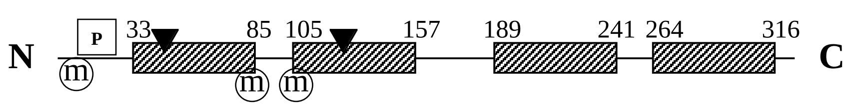


Fig. 1. Amino acid sequence and motif diagram of Xanx-4A. (A) Predicted amino acid sequence of Xanx-4 compared with human (72%), rat (72%), mouse (71%), cow (74%) and medaka (67%). '.' indicate identical amino acids. (B) Xanx-4 motif diagram showing N-terminal PKC phosphorylation site (P), myristoylation site (m) and C-terminal core domain of four annexin repeats (shaded boxes). which contain calcium-binding sites.

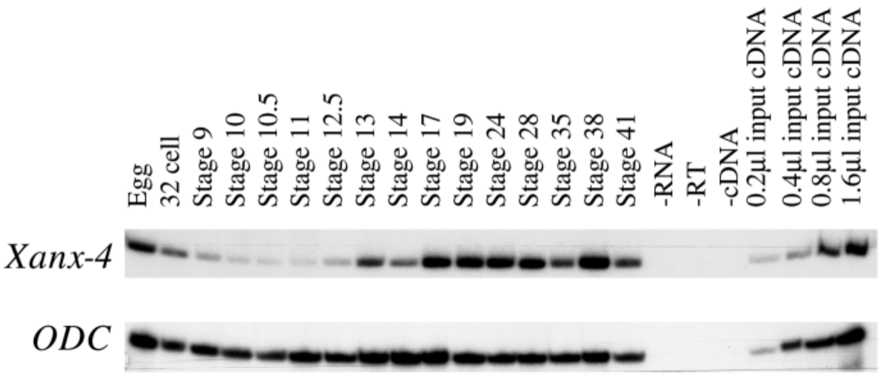


Fig. 2. Temporal expression profile of *Xanx-4*; RT-PCR analysis showing the expression pattern of *Xanx-4* transcripts in *Xenopus laevis* unfertilised egg and embryo stages. Maternal expression is detected in the egg that is much reduced by the 32-cell stage. Zygotic expression is detected at low levels between stages 9 and 12.5, and at a significantly increased level at stage 13, which is maintained through to stage 41.

In vitro and in vivo translation of Myc-tagged construct mRNA

mRNA (10 ng) was translated in vitro in the Rabbit Reticulocyte Lysate System (Promega) using the according to manufacturer's protocol. Reactions (5 µl) were denatured at 95°C in 2×SDS loading buffer (Harlow and Lane, 1988) and subjected to western immunoblot analysis.

For in vivo analysis, 0.5 ng of mRNA was microinjected into one-cell stage embryos, which were then cultured to the appropriate stage. Groups of five embryos were homogenised in 50 µl of homogenisation buffer (0.1 M NaCl, 1% Triton X-100, 1 mM PMSF, 20 mM Tris-Cl pH 7.6) at 4°C and centrifuged in a bench top microcentrifuge at 4°C for 10 minutes at 10,000 ***g***. The cytosolic layer was removed, 5 µl of each sample was denatured at 95°C in 2×SDS loading buffer and subjected to western analysis.

Western analysis

SDS-PAGE was performed on 12% (w/v) resolving gel using a vertical minigel apparatus for 1 hour at 20 mA. The proteins were transferred to nitrocellulose membrane (Amersham) according to Harlow and Lane (Harlow and Lane, 1988) for 2 hours at 350 mA or overnight at 50 mA. After transfer the nitrocellulose was incubated for 1 hour at room temperature in TBS-Tween (0.15 M NaCl, 10 mM Tris-Cl, pH 7.4, 0.1% Tween-20) containing 3% (w/v) powdered milk. The blots were then incubated in 1:1000 anti-Myc monoclonal antibody (gift from D. Stott, Warwick) in TBS-Tween overnight at room temperature. After washing, the blots were incubated in 1:2000 alkaline phosphatase-conjugated goat anti-mouse (Sigma) in TBS-Tween for 1 hour at room temperature. An Immun-star Detection Kit (BioRad) was used for detection according to manufacturer's recommendations.

Preparation of adult tissue RNA samples and northern blot analysis

Total RNA was extracted from different adult tissues using Trizol (Gibco-BRL Life Technologies) following the manufacturer's protocol. Samples of denatured RNA (30 µg per lane) were fractionated by electrophoresis though 1.2% agarose, 2.2 M formaldehyde gels in MOPS buffer (50 mM MOPS pH7; 1 mM EDTA; 20 mM sodium acetate), blotted for 48 hours in 10×SSC onto a Hybond-N membrane (Amersham) and fixed by baking for 2 hours at 80°C. The filters were hybridised overnight at 42°C with [^{32}P]-labelled *Xanx-4* specific probe in 0.5M phosphate buffer pH 7.2 in the presence of 7% SDS and 5mM EDTA. The probe was prepared from a 3′ specific fragment, by PCR amplification (Primers U-gcataaagagcaggccagcc, D-cgattggttatgtgttcaat). After hybridisation, the filters were washed at room temperature in 2×SSC, 0.1% SDS, twice for 15 minutes at 42°C, and then for 15 minutes at 65°C in 1×SSC, 0.1%SDS. Autoradiographs were obtained by exposure of Fuji super RX films with intensifying screens at –80°C.

RESULTS

Cloning and sequence analysis of *Xenopus laevis annexin IV* (*Xanx-4*)

In order to identify novel molecules involved in the early events of pronephric tubule development, we have used a subtractive hybridisation approach. We have generated a subtracted probe based upon the results reported in other experiments (Moriya et al., 1993) and our own data (H. C. Brennan and E. A. J., unpublished). It has been shown that treatment of animal cap ectoderm, dissected from blastula stage *Xenopus* embryos, with 5 ng/ml activin A and 10^{-5} M retinoic acid (RA) results in the formation of pronephric tubules at high frequency, whereas, treatment of animal caps with activin A or RA alone does not. cDNA synthesised from poly A+ mRNA extracted from animal caps treated with RA and activin was therefore subtracted with cDNA synthesised from polyA+ mRNA synthesised from animal caps treated with RA alone pooled with that prepared from activin alone (see Materials and Methods). This allowed the production of a probe enriched for molecules expressed at the time of induction and specification of pronephric tubules. Our laboratory has shown that pronephric tubules are specified at stage 12.5 (Brennan et al., 1998), therefore we used the subtracted probe to screen a *Xenopus laevis* stage 13 cDNA library in order to identify genes expressed early in pronephric development. K2 was one of the clones isolated in this screen.

Sequencing revealed K2 to be a clone of 1131 nucleotides, containing an in-frame coding sequence corresponding to *Xenopus laevis annexin IV* (*Xanx-4*) of 963 bases, including a 50 base 5′UTR and a 118 base 3′UTR (Accession Number, AY039235). Conceptual translation of *Xanx-4* yielded a

Fig. 3. *Xanx-4* mRNA is localised to the pronephric tubules. (A)Whole-mount (stage 36) and (B) section (stage 42) in situ hybridisation of an *Xanx-4* DIG-labelled antisense RNA probe. Expression of *Xanx-4* is restricted to the pronephric tubules. The control sense probe showed no staining pattern (data not shown).

Fig. 4. Xanx-4 protein is localised to the luminal surface of the pronephric tubules. Whole-mount (A) and section (B) stage 40 embryos stained with anti-Anx-4 antibody show that Xanx-4 protein is specifically localised to the pronephric tubules and in section to the apical surface of the pronephric tubule epithelium.

predicted amino acid sequence of 321 amino acids that displays identity with annexin IV in other vertebrate species of between 67-74% (Fig. 1A).

Analysis of the predicted amino acid sequence (Prosite) indicated that Xanx-4 contains the archetypal conserved annexin motifs, the four-fold repeat containing calcium and phospholipid binding sites, myristoylation sites, N-glycosylation sites, and a PKC phosphorylation site (Thr6) (Fig. 1B).

Temporal expression of *Xanx-4*

The temporal expression profile of *Xanx-4* transcripts was revealed by RT-PCR (Fig. 2). Maternal expression of *Xanx-4* was detected in the egg but subsequently declined rapidly and was substantially reduced by the 32-cell stage. Zygotic expression was detected at a very low level between stages 9-12.5 and at a significantly increased level at stage 13. This coincides with the time of pronephric tubule specification (Brennan et al., 1998). Expression then continues at a similar level up to and beyond the stage when the pronephros becomes functional (stage 37). ODC was used as a loading control.

Expression of *Xanx-4* transcripts and protein is restricted to the pronephric tubules in embryos and to specific organs in the adult

Whole-mount in situ hybridisation using a DIG labelled *Xanx-4* antisense probe in albino embryos detected transcripts in the pronephric anlagen from stage 26 onwards and more specifically in pronephric tubules as they continue to develop through to stage 42 (Fig. 3A,B, arrows). Low levels of *Xanx-4* transcript were also detected in the otic vesicle at stage 26 (data not shown). A sense *Xanx-4* control probe showed no staining pattern in embryos at any stage (data not shown).

The monoclonal anti-annexin IV antibody BL7B1, raised against rabbit annexin IV, was used in whole-mount immunohistology to establish the protein distribution of Xanx-4. This antibody has been shown to react specifically with rabbit annexin IV (Massey et al., 1991), and crossreacts with the Xanx-4 protein. The staining observed indicates that Xanx-4 protein is specifically localised to the pronephric tubules of *Xenopus laevis* embryos from stage 38 onwards (Fig. 4A). Anti-annexin IV staining of transverse sections of stage 40 embryos reveals Xanx-4 is located on the luminal side of the tubule and is confined to the apical membrane of the pronephric tubule epithelium (Fig. 4B).

Northern blot analysis of mRNAs isolated from dissected adult organs was carried out (Fig. 5). Transcripts of approximately 1.6 kb were detected at high levels in the gall bladder and intestine, at lower levels in tadpole, lung, kidney, ovary, testis, stomach, bladder, spleen and pancreas. No transcripts were detected in neural tissues, heart, liver, muscle or skin. All the organs in which transcripts were detected contain significant quantities of polarised epithelial tissue, thus correlating with the observed polarised expression of the Xanx-4 protein.

Perturbation of the expression of *Xanx-4* using morpholino technology

Recently, morpholino technology has been used to knock out endogenous gene expression in *Xenopus* and other organisms by blocking translation of specific genes (Heasmann et al., 2000; Nasevicius and Ekker, 2000; Yang et al., 2001). An MO complementary to *Xanx-4* was designed and tested in vitro and in vivo for its ability to specifically perturb *Xanx-4* translation. In order to control for the effects of the MO, a number of anti-annexin IV antibodies were tested by western blotting and immunoprecipitation for their ability to detect Xanx-4. Unfortunately, none of these reagents was able to detect Xanx-4 by western analysis, although the antibodies did perform in immunohistochemistry. Therefore, a *Xanx-4*-Myc-tagged expression construct was prepared (see Materials and Methods). Myc-tagged *Xanx-4* mRNA was synthesised and used in the following assays to demonstrate the effectiveness and specificity of the MO.

Xanx-4 MO depletion of the translation of *Xanx-4* mRNA *in vitro*

Initially the ability of the *Xanx-4* MO to deplete *Xanx-4* translation was established in vitro. *Myc*-tagged *Sox17β* mRNA (a kind gift from D. Clements, Warwick) was used as a control to test for the *Xanx-4* MO specificity. A combination of 10 ng of *Myc*-tagged *Sox17β* mRNA and 10 ng of *Myc*-tagged *Xanx-4* mRNA was incubated either alone

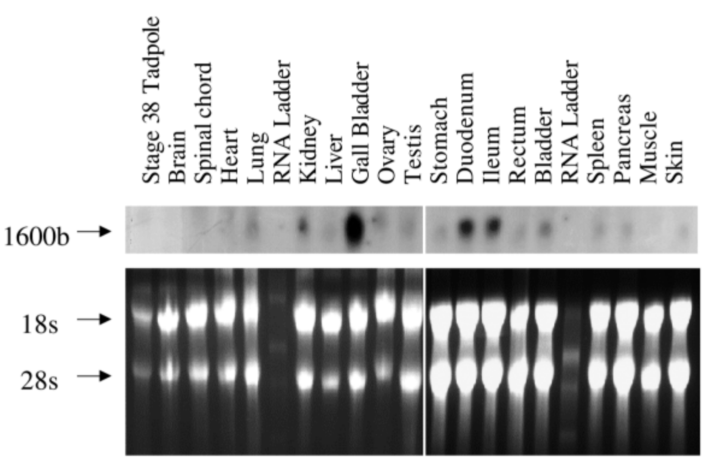


Fig. 5. Northern blot analysis of RNA isolated from dissected adult organs. (A) *Xanx-4* transcripts were detected at high levels in the gall bladder and intestine, and at lower levels in tadpole, lung, kidney, ovary, testis, stomach, bladder, spleen and pancreas. No transcripts were detected in neural tissues, heart, liver, muscle or skin. (B) Ethidium bromide stained agarose gel showing RNA loading for different adult tissue types prior to transfer.

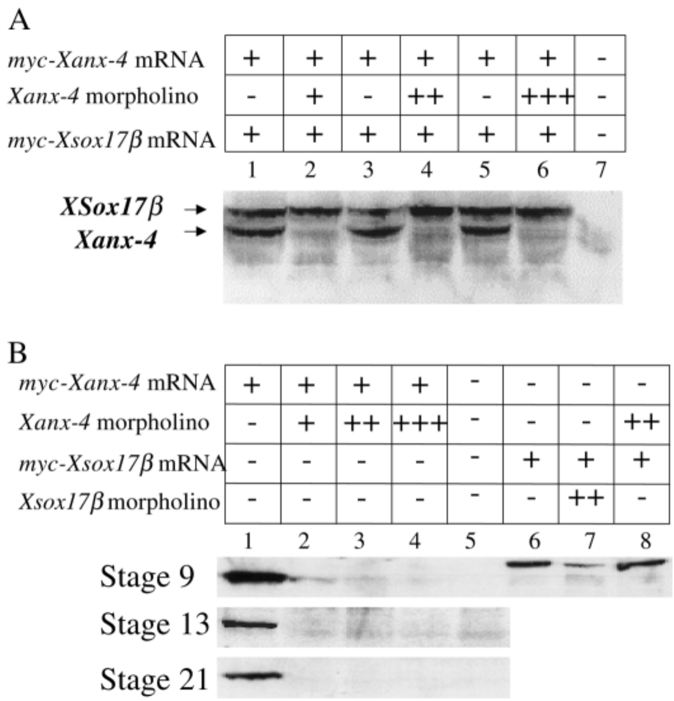


Fig. 6. (A) *Xanx-4* MO specificity in vitro; western blot analysis showing that *Xanx-4* MO interferes with in vitro translation of Myc-tagged *Xanx-4* mRNA (Myc-*Xanx-4*) and not of Myc-tagged *Xsox17β* mRNA (Myc-*Xsox17β*). Lane 1: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA. Lane 2: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA and 1 μg *Xanx-4* MO. Lane 3: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA. Lane 4: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA, and 5 μg *Xanx-4* MO. Lane 5: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA. Lane 6: 10 ng Myc-*Xanx-4* and Myc-*Xsox17β* mRNA and 10 μg *Xanx-4* MO. Lane 7: no input mRNA or MO. (B) *Xanx-4* MO specificity in vivo; western blot analysis using anti-Myc antibody showing that the Xanx-4 MO interferes with in vivo translation in *Xenopus* embryos of Myc-tagged *Xanx-4* mRNA (Myc-*Xanx-4*), lanes 2-4, and not of Myc-tagged *Xsox17β* mRNA (Myc-*Xsox17β*), lane 8. One-cell stage *Xenopus* embryos were injected with Myc-*Xanx-4* mRNA, Myc-*Xanx-4* mRNA and *Xanx-4* MO, Myc-*Xsox17β* mRNA, Myc-*Xsox17β* mRNA and *Xsox17β* MO, and cultured to stage 9, 13 and 21. Lane 1: 0.5 ng Myc-*Xanx-4* mRNA. Lane 2: 0.5 ng Myc-*Xanx-4* mRNA and 5 ng *Xanx-4* MO. Lane 3: 0.5 ng Myc-*Xanx-4* mRNA and 10 ng *Xanx-4* MO. Lane 4: 0.5 ng Myc-*Xanx-4* mRNA and 20 ng *Xanx-4* MO. Lane 5: uninjected control. Lane 6: 0.5 ng Myc-*Xsox17β* mRNA. Lane 7: 0.5 ng Myc-*Xsox17β* mRNA and 10 ng *Xsox17β* MO. Lane 8: 0.5 ng Myc-*Xsox17β* mRNA and 10 ng *Xanx-4* MO.

or in combination with 1 μg, 5 μg or10 μg of *Xanx-4* MO in the reticulocyte lysate system. The lysates were subjected to SDS-PAGE and western blotting using anti-Myc antibody (Fig. 6A). Both *Myc*-tagged *Sox17β* mRNA and *Myc*-tagged *Xanx-4* mRNA were successfully translated at similar levels (lanes 1, 3 and 5) but when incubated with 1 μg, 5 μg or 10 μg *Xanx-4* MO, *Xanx-4* translation was blocked, whereas *Sox17β* translation was not (lanes 2, 4 and 6). In a similar experiment, the *Xanx-4* MO was also shown to have no effect on the translation of *XEZ* (Barnett et al., 2001) mRNA (data not shown). This shows that the *Xanx-4* MO preferentially depletes *Xanx-4* mRNA in vitro.

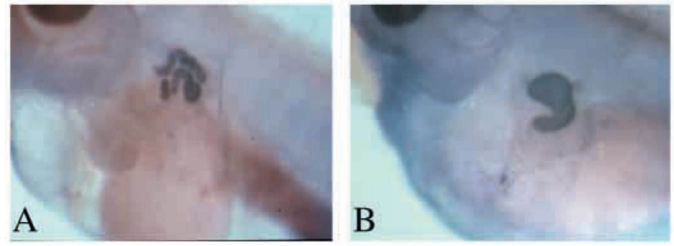


Fig. 7. Whole-mount antibody staining of stage 40 *Xanx-4* MO-injected embryos identifies a tubule phenotype. One-cell stage *Xenopus* embryos were injected with 10 ng of Xanx-4 MO, cultured to stage 40 and subjected to whole-mount antibody staining with pronephric tubule specific antibody 3G8. (A) Normal uninjected control embryo. (B) Embryo injected with *Xanx-4* MO shows shortened, enlarged tubule phenotype.

Xanx-4 MO depletion of the translation of *Xanx-4* mRNA *in vivo*

Embryos were injected at the one-cell stage with 0.5 ng *Myc*-tagged *Xanx-4* mRNA alone or in combination with *Xanx-4* MO and cultured to stage 9, 13 or 21. The embryos were homogenised, fractionated and the cytosolic fraction subjected to SDS-PAGE and western blotting using anti-Myc antibody. As specificity controls, 0.5 ng Myc-tagged *Sox17β* mRNA was also injected alone or in combination with *Sox17β* MO or *Xanx-4* MO. The results (Fig. 6B) show that *Myc*-tagged *Xanx-4* is translated in vivo (lane 1) and that its translation is blocked by 5 ng (lane 2), 10 ng (lane 3) or 20 ng (lane 4) *Xanx-4* MO at all stages examined (stage 9, 13 and 21). For the specificity control, injected *Myc*-tagged *Sox17β* alone is translated (lane 6) and translation is depleted by *Sox17β* MO (lane 7). However, the expression of *Myc*-tagged *Sox17β* is not affected by the *Xanx-4* MO (lane 8). Uninjected, control embryos do not react with the anti-Myc antibody at any of the stages tested (lane 5). Therefore, we show that the *Xanx-4* MO preferentially depletes *Myc*-tagged *Xanx-4* mRNA in vivo. *Myc*-tagged *Sox17β* mRNA injected embryos could not be analysed at later stages, owing to lethality at late gastrula/early neurula stages of this level of message.

Xanx-4 depletion using *Xanx-4* MO produces pronephric tubules with an enlarged diameter

In order to examine the activity of *Xanx-4* in vivo, we have used overexpression and depletion to perturb endogenous expression of *Xanx-4*. Embryos were injected at the one-cell stage with 10 ng control MO, 10 ng *Xanx-4* MO or 0.5 ng *Xanx-4* mRNA. The injected embryos were cultured to stage 40 and whole-mount antibody stained with 3G8 and 4A6, which are tubule- and duct-specific monoclonal antibodies, respectively. Although no obvious effect on pronephric tubule or duct morphology was observed after overexpression of *Xanx-4* mRNA or on injection of control MO (two experiments, counting each pronephros individually, total number of animals scored n=116, n=102), a clear effect on tubule morphology was observed after *Xanx-4* MO injection. On whole-mount inspection, the normal coiled tubular structure of the pronephric tubules appeared completely disrupted in MO-injected embryos. This led to the formation of apparently shortened and enlarged tubules (24/56, 38/60 in

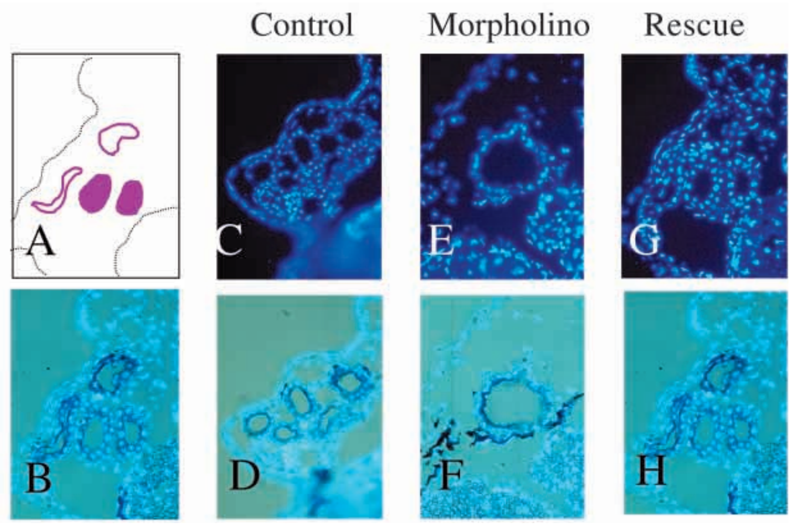


Fig. 8. Cryostat transverse sections of stage 40 *Xenopus* pronephroi stained with tubule-specific antibody 3G8 and counterstained with Hoechst. One-cell stage embryos were injected with 10 ng *Xanx-4* MO, 10 ng *Xanx-4* MO and 0.5 ng *Xanx-4* mRNA, cultured to stage 40 and subjected to whole-mount antibody 3G8 staining. The embryos were acrylamide embedded, cryostat sectioned at 12 μm and lifted on to subbed slides. The slides were counterstained with Hoechst, inspected under light and u.v. illumination. (A) A schematic representation of B showing pronephric tubule sections illustrating true transverse sections, which were scored (filled) and partial longitudinal sections which were not counted (unfilled). (C,D) Normal uninjected control embryo. (E,F) Embryo injected with 10 ng *Xanx-4* MO showing enlarged pronephric tubule phenotype. (G,H) embryo injected with 0.5 ng *Xanx-4* mRNA and 10 ng *Xanx-4* MO showing partial rescue of pronephric tubule phenotype. C,E,G are viewed under UV illumination to identify Hoechst nuclei staining. B,D,F,H are viewed under partial white light and u.v. illumination to identify 3G8 pronephric tubule staining and Hoechst nuclei staining.

two independent experiments; Fig. 7). In some embryos, the tubules were reduced in overall size and in some cases missing completely (8/56, 12/60 in the same independent experiments). In order to investigate the phenotype, a random sample of six immunostained embryos from each treatment group were acrylamide embedded, frozen and cryostat-sectioned (12 μm). The slides were counterstained stained with Hoechst 33258, mounted and compared with uninjected controls. Analysis of the sections revealed that MO-treated embryos had tubules that were substantially wider than control uninjected, control MO injected or *Xanx-4* mRNA injected embryos.

In order to quantify the extent of the MO phenotype, complete serial sections of the pronephroi from each of the six samples for each treatment were inspected. Approximately 24 sections were counted from each pronephros scored. Tubules were positively identified by the light microscopic identification of 3G8-positive immunostain, and nuclei counted under u.v. illumination to identify Hoechst staining (Fig. 8A,B). Owing to the coiled nature of the pronephric tubules, many of the tubule sections could be seen as elongated or oval shaped cross-sections. An example of a section with true transverse (counted) and a partial longitudinal section (not counted) is shown in Fig. 8A,B. Any section of a pronephric tubule considered not to be a true transverse section on the basis of such cross-sectional shape was discounted. All cells of each sectioned pronephric tubule, on both sides of the embryo and in each section were counted. The mean cell count, together with the standard deviation for each treatment was calculated. The numbers of cells contributing to normal control tubules were remarkably consistent, averaging nine cells and with a range of 6-15 cells. Inspection of the sections of the *Xanx-4* MO-injected embryos for the number of cells making up a tubule, however, revealed that the pronephric tubules had an enlarged lumen, but appeared normal in all other respects. The cells of the tubules retained normal cell integrity and shape, the organisation of the tubule epithelium was one cell in diameter, as in normal controls. The average number of cells contributing to the tubule, however, was 25 and the range was 6-48. The wide range observed reflects the observation that the tubule width at the start and the finish of each tubule domain was of normal size. This represents a significant difference from either uninjected control values or from control MO-injected values. Injection of either *Xanx-4* mRNA or a control MO had no effect on the phenotype of the tubules. Representative results are presented in Fig. 8C-F and numerical data shown in Fig. 9A. Duct staining was carried out using specific monoclonal antibody 4A6 (Vize et al., 1995); no effect on duct morphology was observed.

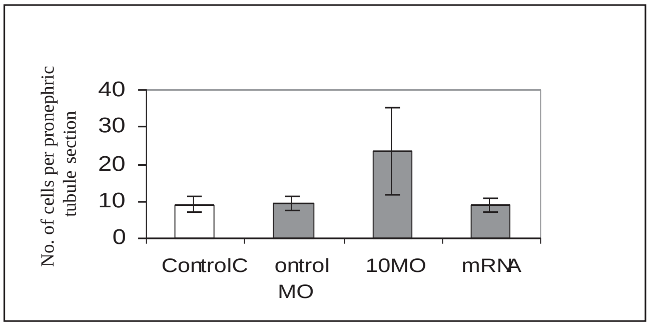
Xanx-4 depleted phenotype can be rescued by co-injection with *Xanx-4* mRNA

We have used *Xanx-4* mRNA co-injected with *Xanx-4* MO, in order to rescue the phenotype caused by injection of the *Xanx-4* MO. Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA alone or in combination with 5 ng, 10 ng or 20 ng of *Xanx-4* MO. Equal volumes were injected into all embryos. The embryos were cultured to stage 40 and whole-mount antibody immunostained with tubule-specific 3G8. Embryos injected with *Xanx-4* MO were compared with the co-injected and to the uninjected controls. The embryos were examined initially as whole-mount specimens (data not shown).

As previously described, pronephric tubules of the embryos injected with the *Xanx-4* MO appeared in general to be wider, shorter and less coiled than those of the normal control embryos. It was also observed that the most severe phenotype occurred in those embryos injected with the higher concentration of MO. Apart from the aberrant tubule phenotype, the embryos appeared to be of normal morphology. Those embryos co-injected with 5 ng *Xanx-4* MO and *Xanx-4* mRNA appeared almost completely rescued and had almost normal tubules (Fig. 8, compare C-F with G,H). The 10 ng and 20 ng *Xanx-4* MO embryos co-injected with *Xanx-4* mRNA showed significant, although incomplete, rescue. The tubules appeared somewhat less normal and displaying some larger and less coiled tubules. Seemingly the rescue was not complete at the higher concentrations of MO. Other than the pronephric tubule phenotype described above, the gross phenotype of the embryos in all groups appeared normal.

In order to quantify this phenotype, a random sample of six

A



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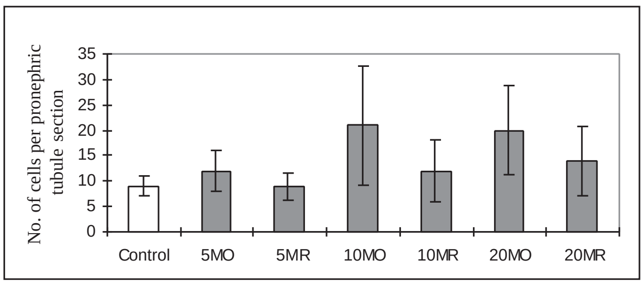

Fig. 9. Graphical representation of data collected from cell counts derived from serial sections of pronephric tubules from uninjected control embryos and embryos injected with *Xanx-4* MO and/or *Xanx-4* mRNA. (A) One-cell stage embryos were injected with 10 ng control MO (control M), 10 ng *Xanx-4* MO (10M), 0.5 ng *Xanx-4* mRNA (mRNA), cultured to stage 40 and subjected to whole-mount antibody 3G8 staining. The embryos were acrylamide embedded, cryostat sectioned at 12 μm and lifted on to subbed slides. The slides were counterstained with Hoechst, inspected under light and u.v. illumination and cell counts per pronephric tubule were obtained. The mean number of cells per tubules section in the control, control MO and *Xanx-4* mRNA injected embryos were not significantly different, whereas those embryos injected with 10 ng *Xanx-4* MO displayed an enlarged pronephric tubule phenotype. (B) The enlarged pronephric tubule phenotype produced by injection of Xanx-4 MO can be rescued by co-injection with *Xanx-4* mRNA. One-cell stage embryos were injected with 5 ng *Xanx-4* MO (5M), 5 ng *Xanx-4* MO and 0.5 ng *Xanx-4* mRNA (5MR), 10 ng *Xanx-4* MO (10M), 10 ng *Xanx-4* MO and 0.5 ng *Xanx-4* mRNA (10MR), 20 ng *Xanx-4* MO (20M), 20 ng *Xanx-4* MO and 0.5 ng *Xanx-4* mRNA (20MR), cultured to stage 40 and analysed as before. The columns represent the arithmetic mean of each sample set of counted cross sections. Error bars indicate1 standard deviation either side of the mean. MO, morpholino; MR, morpholino and mRNA rescue.

embryos from each group were acrylamide embedded, frozen and cryostat-sectioned as described previously. The mean number of cells in the circumference of the pronephric tubules, of the embryos co-injected with 5 ng *Xanx-4* MO and 0.5 ng *Xanx-4* mRNA (mean=9, range 5-21), was not significantly different (P<0.01) from control normal embryos (mean=9, range 5-15) (Fig. 9B). It appears therefore, that 0.5 ng *Xanx-4* mRNA was able to rescue 5 ng *Xanx-4* MO completely. Those injected with 10 ng (mean=20, range=6-52) or 20 ng (mean=21, range=6-44) of MO, were partially rescued by 0.5 ng *Xanx-4* mRNA (10 ng/mRNA rescue mean=12, range 6-38, 20 ng/mRNA rescue mean=14, range 6-38). Embryos injected with 10 ng of MO were rescued to a 5 ng MO phenotype (mean=12, range=6-24) and 20 ng was rescued to an intermediate phenotype between 10 ng and 20 ng. The data collected from the serial sections of each of six randomly chosen embryos from each group is shown as a graphical representation in Fig. 9B. Similar results were obtained from a repeat experiment (data not shown).

Analysis of the expression of pronephric molecular markers in *Xanx-4* over-expression and *Xanx-4* MO treated embryos

In order to assess whether either the observed phenotype or other unidentified earlier events was related to other genes known to be expressed in the pronephros, the following experiments were carried out. Both semi-quantitative RT-PCR and in situ hybridisation were performed for a selection of genes whose mRNAs have been shown to play an early role in kidney development. Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO, cultured to stage 25. Groups of five embryos (in duplicate) were analysed by RT-PCR (Barnett et al., 1998) using primers designed against a selection of genes known to be expressed in the pronephros (see Materials and Methods). EF1α is used as a loading control. No effect on expression of any of the marker genes was observed (Fig. 10). Embryos injected with 0.5ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO were cultured to stages 26-28 and subjected to in situ hybridisation using RNA probes prepared from cDNA clones of pronephric marker genes *XPax-8*, *Xlim-1* and *xWT1*. The expression pattern of each marker gene RNA in the embryos injected with *Xanx-4* mRNA and *Xanx-4* MO1 were compared with uninjected control embryos (Fig. 11). No apparent change in *XPax-8* or *Xlim-1* expression pattern was observed in either group with perturbed *Xanx-4* expression, compared with uninjected control. However, a reduced field of *xWT1* expression was observed in 27% (n=12/44) of the embryos that overexpressed *Xanx-4*, compared with controls. This apparent reduction may have been due to a more dispersed *xWT1* expression domain, as no detectable reduction in *xWT1* expression was observed by RT-PCR. No altered *xWT1* field of expression was seen in the embryos injected with *Xanx-4* MO1. It seems perturbation of expression of *Xanx-4* is not linked to any of the tubule markers analysed. We also assume from these results that the *Xanx-4* depletion phenotype is not associated with more or less tissue being specified to become pronephric tubule in character.

DISCUSSION

The aim of this work was to use a subtractive hybridisation strategy to identify novel molecules involved in the early events of pronephric tubule development in *Xenopus laevis* larval kidney. We report the cloning and characterisation of the *Xenopus* orthologue of *annexin IV* (*Xanx-4*), a pronephric tubule specific gene with an important role in the morphogenesis of the pronephric tubules in *Xenopus laevis*.

Cloning of *Xanx-4*

A gradient of the TGFβ superfamily member activin A will induce different kinds of mesodermal tissues in dissociated cells (Green and Smith, 1990; Green and Smith, 1991; Green

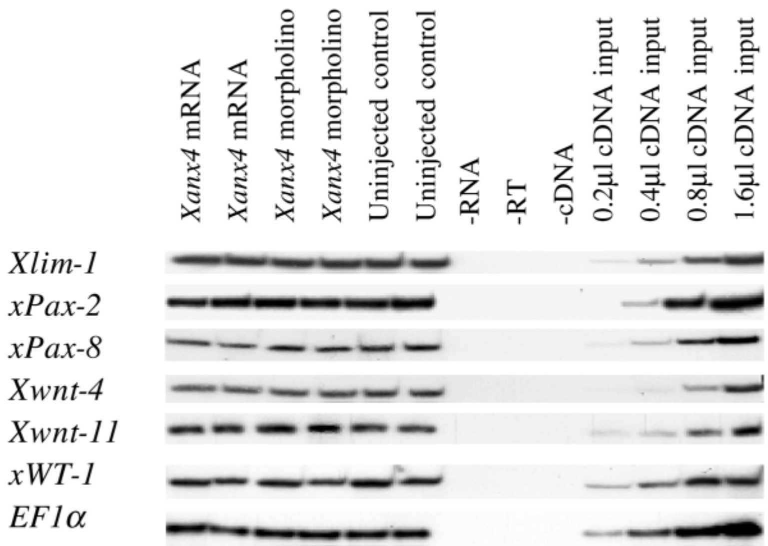

Fig. 10. Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO and cultured to stage 25. Groups of five embryos were then subjected to RT-PCR using primers designed against a range of pronephric molecular markers. No effect on expression of any of the markers was observed. *EF1α* was used as a loading control.

et al., 1992) and in embryonic explants in culture (Gurdon et al., 1994; Moriya et al., 1993). As pronephric tubules can be induced at high frequency by treatment of animal caps with a combination of activin and RA (Moriya et al., 1993), this provides a very attractive method with which to produce large amounts of specific tissues for use in a variety of cloning strategies or screens. We have successfully used this approach to produce a high frequency of pronephric tubules in *Xenopus* explants as a source of mRNA enriched with pronephric transcripts.

As caps treated with RA or activin alone do not induce pronephric tubules with high frequency, our approach of preparing a subtracted probe allowed the elimination of molecules that were upregulated by RA or activin alone. Thus, we have created a probe specific for molecules upregulated only by the combination of activin and retinoic acid, which would include those expressed at the time of induction and specification of pronephric tubules. In our study, we chose to screen an early *Xenopus* cDNA library, stage 13, based on the work carried out in our laboratory that demonstrated that the tubules were specified at stage 12.5 (Brennan et al., 1998). In choosing to screen an early library, we were able to select for genes not only specific for pronephric tubule specification, but also genes that are involved in early events of pronephric differentiation, patterning and development.

The zygotic upregulation of *Xanx-4* occurs at stage 12.5-13. RT-PCR carried out on dissected stage 13 embryos (dorsal, lateral and ventral domains) revealed that *Xanx-4* is expressed in the lateral domain at this stage (data not shown). *Xanx-4* expression is maintained through all the stages of pronephric tubule development to and beyond the time when the pronephros becomes functional. This implies an important and specific role for *Xanx-4* not only in the development, but also perhaps in the function of the pronephros. In this work, we have described the highly restricted distribution of both *Xanx-4* mRNA and protein during embryogenesis. The expression patterns of *Xenopus annexin II* and *annexin VII* are less restricted, being expressed in various neural and mesodermal tissues (Izant and Bryson, 1991; Srivastava et al., 1996).

We have shown by northern blot analysis that the expression pattern of *Xanx-4* in the adult frog is restricted to epithelial tissues. In agreement with this result, *annexin IV* displays polarised expression in adult epithelial tissues of other animal species: lungs (Sohma et al., 1995), intestines and pancreas (Massey et al., 1991), liver (Boustead et al., 1993) and kidney (Massey-Harroche et al., 1995; Kojima et al., 1994). It appears that the common theme for localisation of annexin IV expression in adult tissues is that of polarised epithelial tissue.

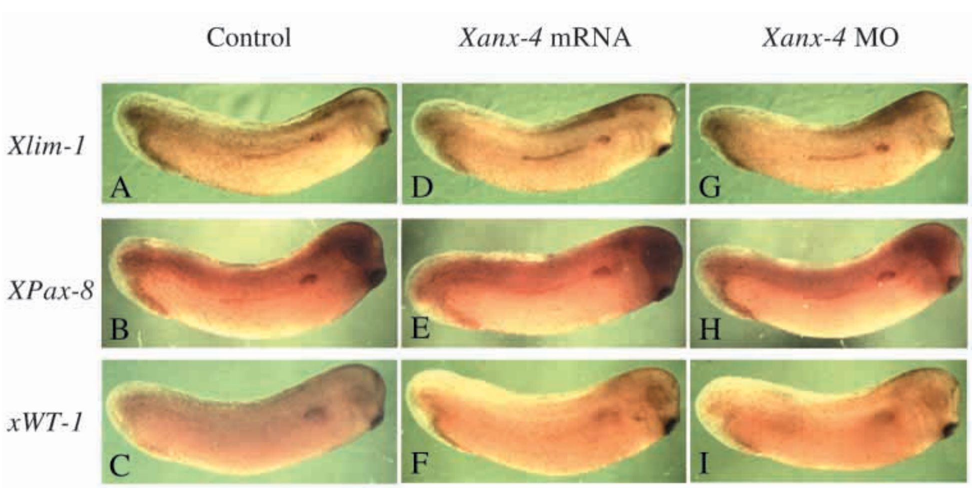

Fig. 11. Analysis of the effects of *Xanx-4* depletion or overexpression on the expression of pronephric marker genes by in situ hybridisation. Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO and cultured to stage 27. The embryos were then subjected to in situ hybridisation using specific probes prepared from pronephric molecular markers *Xlim-1* (A,D,G), *XPax-8* (B,E,H) and *xWT1* (C,F,I). No effect on expression of *XPax-8* or *Xlim-1* was observed; however, a reduced field of *xWT1* expression was observed in the embryos injected with *Xanx-4* mRNA (compare C with F).

The effects of altered expression of *Xanx-4*

We have shown that *Xanx-4* is expressed at the right time in the right place to have a functional role in pronephric tubule development. To investigate this, we have perturbed the expression of *Xanx-4* in *Xenopus* tadpoles and identified a clear phenotype on pronephric tubule morphology in whole-mount and in section.

Depletion of *Xanx-4* protein by

MO causes an enlarged, shortened and uncoiled pronephric tubule phenotype. The effect of *Xanx-4* MO can be rescued by co-injection of *Xanx-4* mRNA. Longevity of the MO is suggested to be considerable (Summerton and Weller, 1997; Nutt et al., 2001), and may be still acting at much later times that that of the ectopic mRNA. The rescue of the MO phenotype observed may be due to action of the *Xanx-4* mRNA antagonising the action of the *Xanx-4* MO on co-injection. We have shown that 0.5 ng of injected message will completely rescue the tubule phenotype seen in 5 ng *Xanx-4* MO embryos, whereas at higher concentrations of *Xanx-4* MO, the phenotype is only partially rescued. Interestingly *Xanx-4* mRNA injected alone does not produce a phenotype. This enables a rescue experiment to be performed that will return the MO phenotype to the normal range, rather than to producing an overexpression phenotype. The lack of overexpression phenotype may be due to the relatively late requirement of *Xanx-4* in kidney morphogenesis and the inability of injected mRNA to survive to this point. We are currently investigating the potential of transgenics to overcome this problem.

The pronephric tubules of the *Xanx-4* MO-injected embryos, although enlarged in diameter, were constructed and organised in the normal way. The tubules consisted of a lumen, albeit enlarged, and the walls were constructed of one epithelial cell layer, though they contained more cells in their circumference. The pronephroi of *Xanx-4*-depleted embryos consisted of the normal complement of pronephric components, including capsule, nephrostomes, tubules and duct, as shown by the expression of marker genes (Fig. 11). It appears that the pronephroi were functional, maintaining body water and electrolyte homeostasis, as none of the components appeared cystic and no general oedema was observed. Recent work (Drummond et al., 1998) has isolated 18 independent recessive mutations that affect pronephric development from a large scale ENU mutagenesis screen (Driever et al., 1996). A common theme for the phenotypes of these mutants was the appearance of fluid-filled cysts in the pronephric region followed by general oedema. The authors suggest that the phenotypes observed are the consequence of pronephric failure and altered osmoregulation. Histological analysis of the *double bubble* mutant showed that the glomerulus was loose and distorted, swelling was apparent, and the cells appeared flattened. The glomerulus overall architecture was disorganised and the basement membrane was severely distorted (Drummond et al., 1998). No such tissue disorganisation, cell shape distortion or fluid filled cysts were observed in any of the *Xanx-4*-depleted embryos.

In counting all cells in serial sections, we have shown that the number of cells making up the circumference of the pronephric tubules in normal controls and *Xanx-4* MO-injected embryos differs. A transverse section of the tubules does not, in general, provide perfect circles. By using cell counting as a measure of the phenotype observed, we have taken a measure of the size of tubule sections, thereby removing errors that could complicate the analysis and introduce errors.

The pronephric tubule markers tested were not affected by perturbation of *Xanx-4* expression. We suggest that the phenotype observed is not due to a change in the amount of pronephric tubule tissue specified but is due to an alteration in the morphological process during tubulogenesis. Preliminary studies of the pronephric tubule morphology of *Xanx-4* MO-depleted embryos at earlier stages (35-36) revealed no aberrant tubule phenotype (data not shown). It appears that the enlarged tubule phenotype, caused by depletion of Xanx-4, may only manifest itself at later stages of tubulogenesis during tubule maturation and elongation.

Previous studies have shown that co-expression of either *XPax-2* or *XPax-8* with *Xlim-1* results in a synergistic effect producing increased pronephric tubule complexity, enlarged tubules and ectopic tubules, while expression of either *XPax-2* or *XPax-8* alone has a moderate effect (Carroll and Vize, 1999). We did not see complex or ectopic tubules under any of the conditions tested. We have also shown that perturbation of *Xanx-4* expression does not affect the expression of either *XPax-8* or *Xlim-1*. Ectopic expression of *xWT1*, which is required for metanephric development in vivo, inhibits pronephric tubule development, probably by repressing tubule specific gene expression in the region of the pronephros fated to become tubules (Wallingford et al., 1999). Depletion of *Xanx-4* does not effect the expression of *xWT1*. The effect of *Xanx-4* overexpression on *xWT1* was unexpected, as no *Xanx-4* overexpression phenotype was previously observed. The fact that RT-PCR analysis gave similar levels of *xWT1* expression in controls and *Xanx-4* overexpressing embryos suggests that the effect of *Xanx-4* overexpression results in more diffuse expression of *xWT1*. This is consistent with the results observed in the *xWT1* in situ hybridisation of embryos overexpressing *Xanx-4*, where in some embryos the *xWT1* expression domain was more dispersed. These results place *Xanx-4* downstream of *Xlim-1/XPax-2/8* and *xWT1* in a molecular pathway of tubulogenesis. Further experiments will be required to confirm the relative positions of these pronephric genes.

It is thought that annexins participate in calcium homeostasis, regulation of ion channel activities and membrane traffic events. Annexins have been well characterised with regard to their binding activities. They have been shown to bind various proteins, including proteoglycans, F-actin and collagen, in addition to calcium and phospholipids (Lecat and Lafont, 1999; Seaton and Dedman, 1998). Annexins have been shown to bind phospholipid membranes in ordered arrays, and it has been suggested that annexin complexes may function to modify membrane structure (Oling et al., 1999). A role for annexin IV in epithelial membrane integrity, adhesion or plasticity could account for the aberrant tubule phenotype observed. Depletion of Xanx-4 in the pronephric tubules may cause a remodelling of tubule morphology during development. Annexin IV has been shown to be involved in calcium-activated cellular signal transduction events (Raynal et al., 1996), exocytosis (Sohma et al., 2001) and in the regulation of calcium-activated epithelial chloride ion channel activity (Kaetzel et al., 1994; Chan et al., 1994; Jorgensen et al., 1997). The primary function of the pronephric tubules is the transport of ions, water and other molecules, and as the site of *Xanx-4* localisation (apical) in the pronephric tubules is in agreement with a possible role in these events, further studies will be directed at ascertaining its function directly. Although, as previously discussed, a role in osmoregulation seems unlikely, the enlarged tubule phenotype observed could well be attributed to incorrect modulation of exocytosis, ion channel activity or other calcium signalling events. Direct investigations of these processes in MO-treated embryos will form the basis of future work. This study represents a study of the role of *Xanx-4* in the formation of pronephric tubules in *Xenopus laevis*.

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