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Developmental expression of *Pod 1* in *Xenopus laevis*

SUBREENA SIMRICK, KARINE MASSÉ and ELIZABETH A. JONES*

Molecular Physiology, Department of Biological Sciences, Warwick University, Coventry, U.K.

ABSTRACT The basic helix-loop-helix transcription factor, *Pod 1*, has been shown to be expressed in the mesenchyme of many developing mouse organs, including the heart, lungs and gut. In the kidneys of developing mice, *Pod 1* is highly expressed in the condensing metanephric mesenchyme, differentiating and late stromal cells and in developing podocytes. We have obtained an EST (CF270487) which contains the *Xenopus laevis Pod 1* sequence. Conceptual translation of the *Xenopus laevis Pod 1* sequence shows approximately 85% similarity to other vertebrate homologues. RT-PCR indicates that expression is initiated at stage 13 and increases differentially in the developing pronephros compared to the whole embryo. RT-PCR of a kidney dissection at stage 42 shows higher expression in the glomus than in the tubule or duct. *In situ* hybridisation analysis at tail bud stages shows the anterior-most branchial arch and pronephric glomus are intensely stained. At stage 40, staining persists in the glomus and in the epicardium region of the heart. Adult organ analysis shows expression is highest in the rectum and the spleen, with significant expression in the duodenum, heart, kidney, lungs, pancreas, skin, liver and muscle.

KEY WORDS: *Pod 1, epicardin, capsulin, transcription factor 21, glomus*

During development, basic helix-loop-helix transcription factors are involved in cell lineage commitment and organogenesis. They bind to E box regions (CANNTG) (Murre *et al.*, 1989) and are classified into two groups, ubiquitous class A and tissue specific class B (Lassar *et al.*, 1991). *Pod 1*, also known as *Epicardin*, *Capsulin*, *Transcription factor 21*, is a class B basic helix-loop-helix transcription factor (Hidai *et al.*, 1998, Quaggin *et al.*, 1998, Robb *et al.*, 1998; Lu *et al.,* 1998). In murine development *Pod 1* is first expressed at 9.5 days post coitus (dpc) with high levels in the presumptive epicardium and the mesenchymal cells surrounding the gut, lung and kidney (Hidai, *et al.*, 1998). Expression then extends to the mesenchyme of the lung, metanephros, gonads and the gut at 13.5 dpc (Lu *et al.*, 1998, Robb *et al.*, 1998).

In the adult mouse, RT-PCR reveals expression in the lung, ovary, spleen, kidney, intestine and uterus (Hidai *et al.*, 1998). *In situ* hybridisation analysis, however, indicates that expression is restricted to the podocytes lining the glomerulus, lungs and gut (Lu *et al.*, 1998).

Pod 1 has been shown to have multiple roles in vertebrate development. The *Pod 1* null mouse dies from multiple organ failure. The kidneys show severe branching defects, lacking mature glomeruli and the lungs are deficient in alveoli (Quaggin *et al.*, 1999). Chimeric null *Pod 1* mice show that although *Pod 1* is not required for the specification of glomeruli, podocytes and stromal cells, it is required for their terminal differentiation and branching morphogenesis (Cui *et al.*, 2003). *Pod 1* is also required for capillary remodelling, chimeric null *Pod 1* mice having large, dilated and poorly organised vascular structures (Cui *et al.*, 2003).

Pod 1 appears to have a role in regulating differentiation and proliferation of a variety of tissues. *Pod 1* is required for the initial differentiation and proliferation of the splenic lineage, where the absence of *Pod 1* results in apoptotic cell death (Lu *et al.*, 2000). In skeletal muscle, exogenous expression of *Pod 1* inhibits the terminal differentiation of C2C12 myoblasts (Funato *et al.*, 2003). *Pod 1* knock-down using antisense oligonucleotides, inhibits the differentiation of stratified cells to differentiated epithelia in stomach tissue cultures in the presence of 1 μM hydrocortisone (Andersson *et al.*, 2001). Studies using *Pod 1* null/GFP chimeric mice reveal that the loss of *Pod 1* results in an increase of metanephric condensing mesenchyme and a failure of stromal cells to differentiate (Cui *et al.*, 2003). *Pod 1* is also involved in gonadogenesis. *Pod 1* transcriptionally represses steroidogenic factor 1, a regulator of sexual differentiation (Tamura *et al.*, 2001, Cui *et al.*, 2004).

As a group B basic helix loop helix transcription factor, *Pod 1* is expected to either form homodimers or heterodimerise with class A basic helix-loop-helix transcription factors. Although a number of studies have been carried out, it is unclear which is the case *in vivo*.

Abbreviations used in this paper: BLAST, basic local alignment search tool; RT-PCR, reverse transcription-polymerase chain reaction; UF, unfertilised.

***Address correspondence to:** Dr. Elizabeth A. Jones. Molecular Physiology Department of Biological Sciences, Warwick University, Coventry, CV4 7AL, U.K. Fax: +44-24-7652-3701. e-mail: Elizabeth.Jones@warwick.ac.uk

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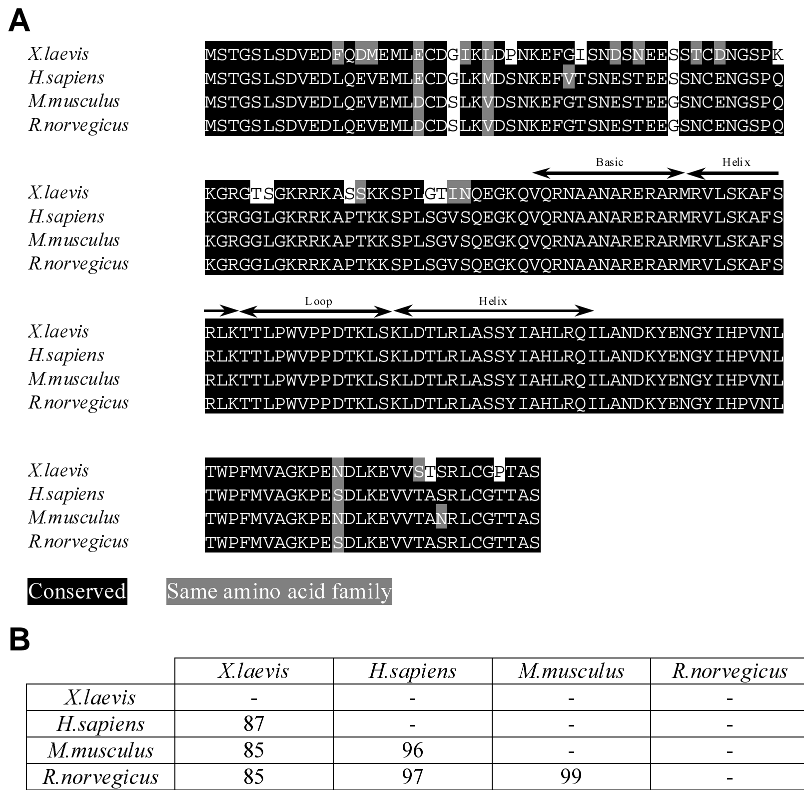

	X.laevis	H.sapiens	M.musculus	R.norvegicus
X.laevis	-	-	-	-
H.sapiens	87	-	-	-
M.musculus	85	96	-	-
R.norvegicus	85	97	99	-

Fig. 1. Alignment of related Pod 1 amino acid sequences. (A) *Alignment of the predicted* X. laevis *amino acid sequence (conceptually translated from CF270487),* H. sapiens *Pod1 (NP_003197),* M. musculus *Pod 1 (NP_035675) and potential* R. norvegicus *homologue Cor1 (XP_341738). Black shading indicates identical amino acids, whereas the gray shading specifies the same amino acid family.* **(B)** *Percentage identity (%). The table indicates paired percentage identity as given by BLAST (Basic Local Alignment Search Tool) (Altschul* et al.*, 1990).*

Gel mobility shift assays using E-box sequences as the probe have shown that Pod 1 alone does not bind to DNA, but a heterodimer of Pod 1 and E12 does bind to DNA (Lu *et al.*, 1998). Yeast two-hybrid screens have identified other possible Pod 1 heterodimer complexes, HEB, HEB-s and ITF-2 (Miyagishi *et al.*, 2000a, Miyagishi, *et al.*, 2000b). However, sequence analysis and *in vitro* experiments have shown that Pod 1 alone is capable of binding cognate E-box consensus sequences and activating transcription (Hidai *et al.*, 1998).

Sequencing and cloning

Using the BLAST programme (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) and the mouse *Pod 1* homologue, O35437, we have identified an EST, Expressed Sequence Tag, containing the full *Xenopus laevis Pod 1* sequence. This EST CF270487, was obtained from the IMAGE consortium (http://image.llnl.gov, IMAGE No. 5512805) and used in this study. The published NCBI nucleotide sequence (http://mgc.nci.nih.gov/) of this clone fails to identify a translational start site. The open reading frame runs from 23bp to 560bp, encoding a 179 amino acid protein that is approximately 25kDa when separated on a 10% SDS polyacrylamide gel (data not shown). In house sequencing corrected the sequencing error by identification of the translational start site at an equivalent point to that of the mouse sequence (AF029753).

Conceptual translation of the *Xenopus laevis Pod 1* sequence showed 100% conserved identity in the basic helix-loop-helix region between the vertebrate Pod 1 homologues (Fig. 1A). A similarity of approximately 85% among the Pod 1 vertebrate homologues was identified over the whole length of the protein (Fig. 1B).

Temporal expression of Xenopus laevis Pod 1

RT-PCR was performed on unfertilised *Xenopus laevis* eggs and whole *Xenopus laevis* embryos at stages 9, 13, 16.5, 21, 26.5, 30 and 35. This temporal RT-PCR analysis indicated that *Pod 1* expression was initiated by stage 13 after which there was a gradual increase in *Pod 1* expression from stages 16.5 to 26.5 and maintenance at this concentration through the stages 30 and 35 (Figs. 2,3A). The input cDNA was approximately equalised using the ubiquitously expressed gene *ODC* and a linearity using doubling dilutions of stage 35 cDNA was performed. The *Xenopus laevis* temporal expression pattern is comparable to that observed in mouse embryos. Detection of *Pod 1* in mouse embryos by Northern blot analysis shows first expression at 8.5 dpc in the branchial arches, after which there is an increase to 15.5 dpc where it peaks at and persists to 17.5 dpc (Hidai *et al.*, 1998). By comparing the developmental stages at the start of neurulation, stage 13 in *Xenopus laevis*, 8-9 dpc in mouse, the onset of *Pod 1* expression is temporally similar.

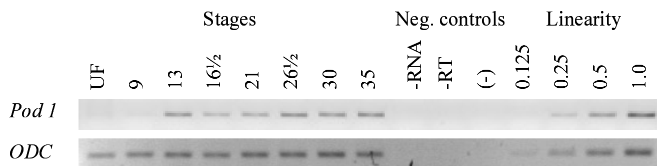

Fig. 2. Temporal expression pattern of *Pod 1* in *X. laevis*. *RT-PCR was performed on unfertilised* Xenopus laevis *eggs (UF) and whole* Xenopus laevis *embryos at stages 9, 13, 16.5, 21, 26.5, 30 and 35, using* Pod 1 *primers. Equalisation was carried out using* ODC *as a loading control and linearity was performed with doubling dilutions of input cDNA from stage 35 embryos to reduce the risk of a PCR plateau. Negative controls were carried out as described in the methods. RT-PCR analysis shows that* Pod 1 *expression is not initiated until the beginning of neurulation, stage 13 followed by a gradual increase in expression until stage 35.*

Temporal expression of Xenopus laevis Pod 1 *in the pronephros*

Pronephric tissue or presumptive pronephric tissue was dissected from *Xenopus laevis* embryos at stages 12.5, 15, 20, 28 and 35 (Brennan *et al.*, 1998) and RT-PCR was performed. This temporal RT-PCR of the whole embryo and kidney dissections revealed an increased expression of *Pod 1* in the developing pronephros compared to the whole embryo (Fig. 3A). No expression was seen in stage 12.5 whole embryos or dissected pronephros, thus confirming the initiation of *Pod 1* expression at stage 13, the start of neurulation. The input cDNA was approximately equalised

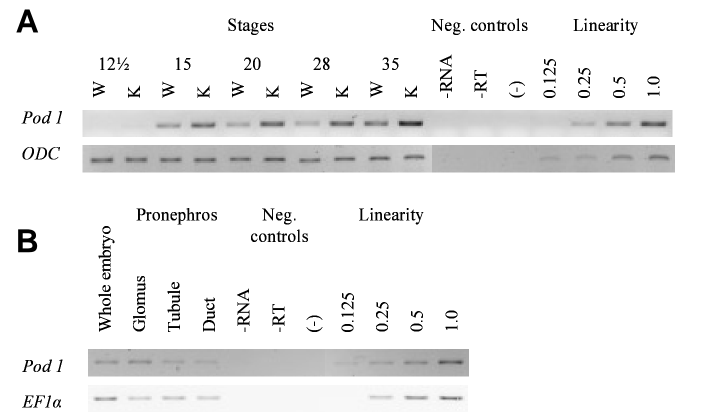

Fig. 3. Whole embryo and dissected kidney temporal expression of *Pod 1* in *X. laevis.* (A) *Pronephric tissue or presumptive pronephric tissue was dissected from* Xenopus laevis *embryos at stages 12.5, 15, 20, 28 and 35 (Brennan,* et al.*, 1998) and RT-PCR was performed using specific* Pod 1 *primers. The RT-PCR was equalised using* ODC *and linearity and negative controls carried out as described in Experimental Procedures. Expression of* Pod 1 *in whole embryos (W) was compared with that of the kidney or kidney primordium dissections (K). The whole embryo temporal expression pattern confirms the profile shown in Fig 2. The* Pod 1 *expression in the kidney dissections, however, is proportionally greater than that of the whole embryo, first appearing during neurulation, stage 15 and increasing as the embryo develops.* **(B)** *The pronephros from a* Xenopus laevis *stage 42 embryo was dissected into the glomus, tubules and duct regions and RT-PCR using* Pod 1 *primers was carried out. Equalisation was carried out using* EF1α *and linearity and negative controls carried out as described in Experimental Procedures. A fine dissection of the pronephros of a stage 42* X. laevis *tadpole reveals the spatial expression of* Pod1 *within the pronephros.* Pod 1 *appears to be most expressed in the glomus, less in the tubules and weakest in the duct.*

using ODC and a linearity using doubling dilutions of stage 35 cDNA was performed. This increased expression in the kidney compared to the whole embryo was also observed in the mouse, where Northern blot analysis at 15.5 dpc revealed higher expression in the kidney, lung and intestine than in the whole embryo (Hidai, *et al.*, 1998).

The pronephros from a *Xenopus laevis* stage 42 embryo was dissected into the glomus, tubules and duct regions and RT-PCR was performed on mRNA extracted from the dissected pieces. The pronephric dissection RT-PCR suggested a greater expression of *Pod 1* in the glomus compared to the tubules and duct (Fig. 3B). This is comparable to mouse kidney expression, where *Pod 1* is expressed in several differentiated cell types including podocytes in the glomerulus, peritubular interstitial cells, pericytes in the renal vessels and adventitial cells in the blood vessels (Cui *et al.*, 2003).

Spatial expression of Xenopus laevis Pod 1

In situ hybridisation analysis was performed on albino *Xenopus laevis* embryos from stage 22 through to stage 40.5. The *in situ* hybridisation analysis gave little staining at stage 22 with either the anti-sense probe or the control sense probe, even when visualised after clearing the embryos (Fig. 4A-B and data not shown). The first indication of specific staining is seen initially in the anterior branchial arch at stage 26 (data not shown) and in the branchial arch and glomus at stage 28 (Fig C-D). Between stages 28 to 40.5 the anterior branchial arch, followed by the remaining arches, are

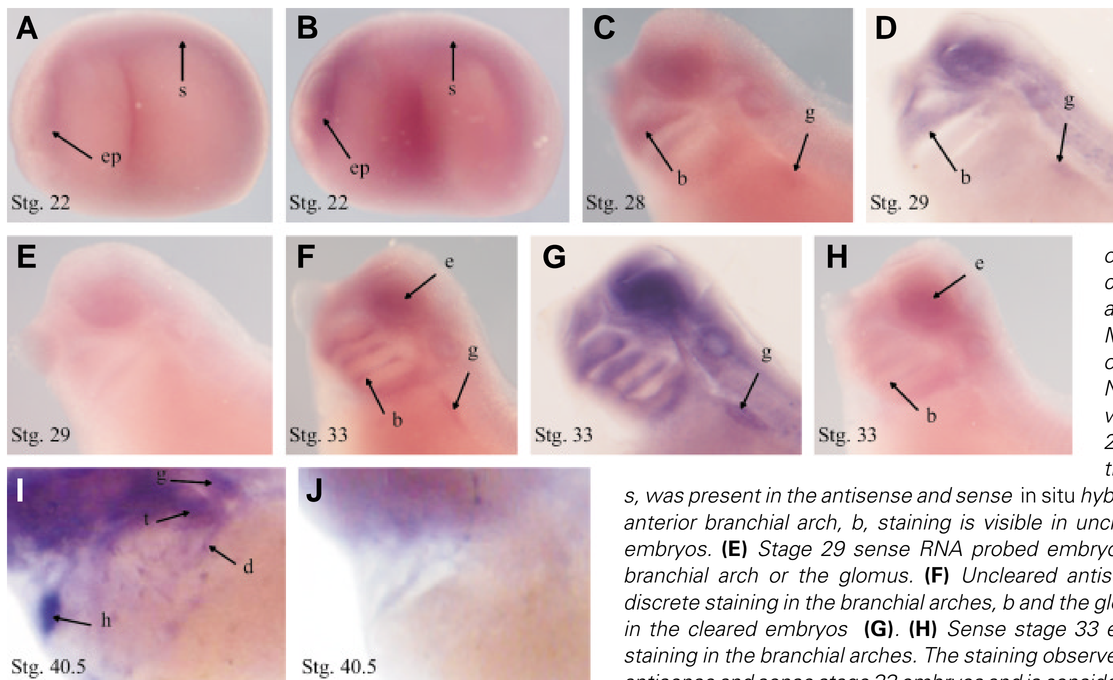

Fig. 4. Whole mount *in situ* hybridisation of *Pod 1* in *X. laevis.* *Wholemount* in situ *hybridisation with a* Pod 1 *DIG-labelled antisense (A,C,D,F,G and I) and sense (B,E,H and J) RNA probe was performed on embryos at the stage indicated. Embryos in panels D, G, I and J have been cleared in Murray's solution to facilitate the observation of internal staining. No staining above background was present in embryos at stage 22* **(A,B)** *as staining observed in the eye placode, ep, and somites, s, was present in the antisense and sense* in situ *hybridisations.* **(C,D)** *Glomus, g and anterior branchial arch, b, staining is visible in uncleared and cleared stage 28-29 embryos.* **(E)** *Stage 29 sense RNA probed embryos do not show staining in the branchial arch or the glomus.* **(F)** *Uncleared antisense stage 33 embryos show discrete staining in the branchial arches, b and the glomus, g, which is also observed in the cleared embryos* **(G)**. **(H)** *Sense stage 33 embryos also have some weak staining in the branchial arches. The staining observed in the eye is observed in both antisense and sense stage 33 embryos and is considered to be background (F,H). The late tailbud embryos are stained in the epicardium, h, of the heart and in all three components of the pronephros, the glomus, g, tubules, t and duct, d* **(I)**. *Non-specific staining is seen in the anterior region of late stage embryos in both sense and antisense hybridised embryos* **(J)**.

intensely stained (Fig. 4C, D, F, G and I). As embryos progress through the somite forming stages, non-specific staining is observed in the head region of both sense and anti-sense probe hybridised embryos (Fig. 4E, H and J). Hybridisation of anti-sense probe to the developing glomus is first observed from stage 28 (Fig. 4C, D, F, G and I) and persists to stage 40.5, the last stage tested. No hybridisation to the glomus is observed in any of the negative control, sense hybridised embryos (Fig. 4E, H and J). At stage 40.5 the presumptive epicardium and all components of the pronephros show intense *Pod 1* expression (Fig. 4I). At these late stages non-specific staining with both sense and antisense probe is observed in the anterior head region (Fig. 4J). In murine development *in situ* hybridisation has revealed expression from 9.5dpc with high levels in the presumptive epicardium and the mesenchymal cells surrounding the gut, lung and kidney (Hidai *et al.*, 1998). This may be comparable to the staining pattern in the *Xenopus laevis* stage 40.5 embryos where expression is observed in the heart and kidney, although anterior branchial arch expression is identified earlier.

Expression of Xenopus laevis Pod 1 *in adult organs*

RT-PCR was performed on mRNA isolated from the organs of an adult *Xenopus laevis*. The input cDNA was approximately equalised using *EF1α* and a linearity using doubling dilutions of kidney cDNA was performed. The RT-PCR indicated that *Pod 1* expression is highest in the rectum and the spleen, with significant expression in the duodenum, heart, kidney, lungs, pancreas, skin, liver and muscle (Fig. 5). A similar adult expression pattern is observed in the mouse. Northern blot experiments have shown *Pod 1* expression in the lung, kidney, heart liver, spleen and testis (Lu *et al.*, 1998 and Miyagishi *et al.*, 2000). However, unlike the *Pod 1* expression in *Xenopus laevis*, there is no expression in the muscle (Lu, *et al.*, 1998 and Miyagishi *et al.*, 2000).

We have identified the *Xenopus laevis Pod 1* homologue within the EST CF270487 in the NCBI database (http://mgc.nci.nih.gov/) and have analysed the temporal and spatial expression patterns of this gene. In summary, there is a high percentage of protein sequence similarity of the *Xenopus laevis Pod 1* homologue with other vertebrates, especially within the basic helix-loop-helix region suggesting a conserved DNA binding function. Temporal expression pattern shows that *Pod 1* expression is initiated at stage 13 and is found to be higher in the kidney, specifically in the glomus, compared to the whole embryo. Spatial expression analysis identifies expression domains in the glomus, heart and branchial arches. The adult organs show high expression in the rectum, spleen, duodenum, heart, kidney, lungs, pancreas, skin, liver and muscle.

This paper, therefore describes the distribution of a basic helix-loop-helix transcription factor expressed very early in the development of the pronephric glomus. *Pod 1* is one of the few transcription expressed at this early stage and now the functional role of this gene in transcription hierarchy of glomus development can be established. *Xenopus laevis* is particularly amenable to functional analysis by mRNA over expression and morpholino oligonucleotide knock down. Preliminary data shows that *Pod 1* plays a major role at these early stages which cannot be easily analysed in the mouse.

Experimental Procedures

Bioinformatics

The *Xenopus laevis Pod 1* sequence was identified by a BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) search using the murine *Pod 1* homologue protein sequence, GenBank Acc. O35437. The EST containing the *Xenopus laevis Pod 1* sequence, GenBank Acc. CF270487, was obtained from the IMAGE Consortium (http://image.llnl.gov, IMAGE No. 5512805). Alignment has been carried out with CLUSTAL (Thompson *et al.*, 1994).

Xenopus *embryo handling*

Xenopus laevis females were induced to lay by hormone injections of follicle stimulating hormone and human chorionic gonadotrophin. The eggs were fertilized *in vitro* using dissected testes. The embryos were dejellied in 2% cysteine pH 8, washed and incubated until the required stage in 1/10 Barth's X at 12ºC. Embryos were staged according to Nieuwkoop and Faber (1994).

The whole embryo and kidney dissections were prepared as detailed in Brennan *et al.,* (1998). For the glomus, pronephric tubule and duct dissection, the pronephros was first removed from stage 42 embryos and the glomus and the posterior pronephric duct dissected from the tubules. This resulted in the anterior duct being included in the tubule preparation. mRNA from the adult organ tissues was extracted using TRIzol® (INVITROGEN) reagents in accordance to the manufacturers instructions.

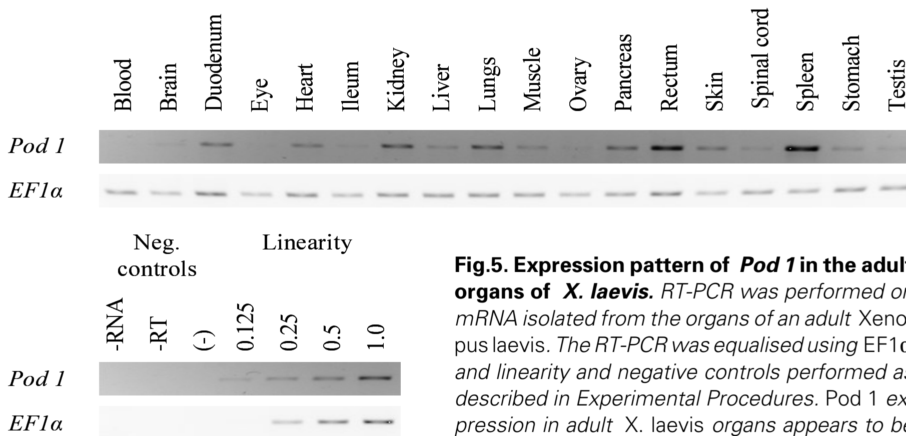

Fig.5. Expression pattern of *Pod 1* in the adult organs of *X. laevis.* *RT-PCR was performed on mRNA isolated from the organs of an adult* Xenopus laevis. *The RT-PCR was equalised using* EF1α *and linearity and negative controls performed as described in Experimental Procedures.* Pod 1 *expression in adult* X. laevis *organs appears to be greatest in the spleen and rectum. Other, more moderate expression is seen in the duodenum, heart, kidney, lungs, pancreas and skin. There are also low levels in the liver, muscle, spinal cord and stomach.*

RT-PCR

The tissues were homogenised, total RNA was extracted and cDNA synthesis was performed as described in Barnett *et al.*, (1998). The cDNA was equalised using *EF1α* (Mohun *et al.*, 1989) (organs) or *ODC* (whole embryos) (Bassez *et al.*, 1990) as loading controls. Linearities were included to ensure that the PCR signal fell within a linear range using doubling dilutions of input cDNA from an appropriate stage or organ. Negative controls were carried out where no mRNA (-RNA), no reverse transcriptase (-RT), or no cDNA (-) was added to the PCR.

Pod 1 PCR was carried out using the *Pod 1* forward primer, 5' TCT CAG TGA TGT GGA GGA CTT 3' and the reverse primer, 5' TGA CGC AGG TGA GCT ATG TAA 3', giving a product of 384 bp.

The annealing temperature was 57ºC and the PCR was run for 22 cycles in total.

In situ *hybridisation*

The *in situ* hybridisation was carried out on albino embryos previously fixed in MEMFA and was performed using protocols adapted from Hemmati-Brivanlou *et al.*, (1990) and Harland (1991). The probes were prepared using a Roche digoxygenin (DIG) labelling kit. The IMAGE *Pod 1* open reading frame was cloned into pCS2+ using *Eco RI* and *Xho I* (INVITROGEN). This clone was then used to make an antisense probe by linearising the IMAGE clone with *Cla I* (INVITROGEN) and transcribing with T7 RNA polymerase (TAGN). Similarly, the sense *in situ* probe was made by linearising with *Xba I* (INVITROGEN) and transcribing with SP6 RNA polymerase (TAGN).

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