The specification and growth factor inducibility of the pronephric glomus in *Xenopus laevis*

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**SUMMARY**

We report a study on the specification of the glomus, the filtration device of the amphibian pronephric kidney, using an explant culturing strategy in *Xenopus laevis*. Explants of presumptive pronephric mesoderm were dissected from embryos of mid-gastra to swimming tadpole stages. These explants were cultured within ectodermal wraps and analysed by RT-PCR for the presence of the *Wilm’s Tumour-1 gene*, xWT1, a marker specific for the glomus at the stages analysed, together with other mesodermal markers. We show that the glomus is specified at stage 12.5, the same stage at which pronephric tubules are specified. We have previously shown that pronephric duct is specified somewhat later, at stage 14.

Furthermore, we have analysed the growth factor inducibility of the glomus in the presence or absence of retinoic acid (RA) by RT-PCR. We define for the first time the conditions under which these growth factors induce glomus tissue in animal cap tissue. Activin together with high concentrations of RA can induce glomus tissue from animal cap ectoderm. Unlike the pronephric tubules, the glomus can also be induced by FGF and RA.

Key words: Pronephros, Glomus, Specification, *Xenopus*

**INTRODUCTION**

Mesoderm induction and patterning occurs in the equatorial region of the amphibian blastula through an inductive interaction between the vegetal pole cells and the overlying ectodermal, animal pole cells (Nieuwkoop, 1969). Members of the TGFβ family, such as activin and Vg1, are candidate signalling molecules which induce dorsal tissue types, members of the FGF family can induce the more ventral types (reviewed in Sive, 1993; Klein and Melton, 1994). Further regionalisation of the mesoderm then occurs during gastrulation, the Spemann organizer dorsalising adjacent, more ventral mesoderm, such that the whole mesodermal range of tissues are generated (Slack and Forman, 1980).

The regionalisation normally displayed by mesodermal tissues within the whole embryo can also be generated by the treatment of disaggregated animal cap cells with a concentration gradient of activin, each mesodermal cell type has been shown to be induced by a narrow range of concentrations (Green and Smith, 1990, 1991; Wilson and Melton, 1994). Furthermore, FGF can modify the action of activins, broadening the threshold-bounded dose windows (Green et al., 1992). Thus the whole spectrum of mesodermal tissues could be generated by concentration gradients of activin and FGF in vitro. Gurdon and colleagues have shown that changes in cell fate can also occur in explanted tissues in a spatially ordered way, which is consistent with the idea that activin could form a gradient in vivo to which cells respond (Gurdon et al., 1994).

Recent experiments have indicated that cells respond to morphogen gradients by activating genes in a concentration-dependent manner (Gurdon et al., 1994; Reilly and Melton, 1996; Jones et al., 1996). It has been demonstrated that an artificial source of radiolabelled activin protein can form an activin gradient by diffusing over distances of approximately 120 μm, equivalent to about 7 cell diameters (McDowell et al., 1997). It has also been shown that increasing occupancy of a single receptor type is sufficient to cause cells to switch patterns of gene expression (Dyson and Gurdon, 1997).

The functioning embryonic kidney in *Xenopus laevis*, the pronephros, is derived from the mesodermal germ layer. This organ consists of three components, the glomus, which is the filtration device equivalent to the higher vertebrate glomerulus, the pronephric tubules, which collect waste filtered by the glomus into the coelom, and the pronephric duct, which carries the waste to the exterior via the cloaca. Although these three components form a functional unit, they in fact have different embryonic origins; the tubules and duct are formed from the intermediate mesoderm of the lateral plate, while the glomus is formed from the splanic mesoderm on the other side of the coelom (Saxén, 1987; Vize et al., 1997).

In adult Amphibia, the pronephros is not retained, but this larval kidney form initiates the induction of the mesonephros, which is retained by the adult as the functional kidney. In higher
vertebrates the mesonephros, the functional embryonic kidney, induces the metanephros, which is the functioning adult kidney. Studies aimed at understanding the development and induction of the pronephros are consequently of considerable interest since, without pronephric development, whether this is functional as in the case of the lower vertebrates, or non-functional as is the case for higher vertebrates, the sequential inductions necessary to generate the functional adult kidney forms cannot occur (Carroll et al., 1998).

Retinoic acid (RA) is a vitamin A metabolite that has profound effects on growth differentiation and development. RA has been implicated as an important signalling molecule in vertebrate development, particularly with respect to axial patterning, where it has been shown to be able to alter positional identities along the anteroposterior axis (reviewed in Conlon, 1995; Lumsden and Krumlauf, 1996; Blumberg, 1997). Treatment of embryos with RA results in characteristic concentration-dependent anterior truncations, which have been interpreted as posterior transformations (Durston et al., 1989). Retinoic acid is also able to modify the development of mesodermal tissue. In vitro treatment of mesodermal explants and whole embryos with RA has also been shown to modulate mesodermal patterning (Ruiu i Altaba and Jessel, 1991). Endogenous retinoids have been identified within Xenopus embryos; 4-oxo-trans-retinoic acid has been shown to be present at biologically active concentrations within the embryo (reviewed in Blumberg, 1997). Modulation of retinoid signalling results in changes in gene expression elicited by the ligand-dependent transcription factors, RA receptors (RARs) and 9-cis RA receptors (RXRs) (Blumberg, 1997). This ligand activated gene expression is therefore presumed to be responsible for the observed phenotypes.

The growth factor induction of pronephric tissues has been analysed in vitro using an animal cap model. Moriya et al. (1993) first showed that the treatment of animal cap ectoderm with the growth factor activin and the modifier RA caused high frequency induction of kidney tubules, as identified by histology, over a range of concentrations of both substances. This result was confirmed and extended by Uochi and Asashima (1996), who analysed the timing of expression of Xlim1 and Xlcaax-1, markers of the pronephros, in induced explants. However, it should be noted that these markers are not specific for the pronephric lineage at the stages tested, nor do they distinguish between the presence of duct or tubule. Neither of these markers are expressed in the glomus.

We have recently reported studies on the specification of two of the components of the pronephros, the pronephric tubules and duct (Brennan et al., 1998). We have shown that isolated presumptive pronephric material cultured in epidermal wraps is specified for pronephric tubule development at stage 12.5, whereas the duct is not specified until stage 14. In this paper, we report the specification analysis of the third component of the pronephros, the glomus using an identical culturing strategy. We have analysed the cultured wraps by RT-PCR for the presence of the Wilms’ Tumour-1 gene, xWT1, a marker specific to the glomus at the stage analysed together with other markers of mesodermal differentiation (Carroll and Vize, 1996).

Furthermore, we have analysed the growth factor inducibility of the glomus in response to activin and FGF gradients applied both alone and in combination. We have also analysed the effect of retinoic acid treatment in combination with these growth factors. The results have been analysed by RT-PCR analysis. We establish, for the first time, the growth factor inducibility of the glomus as defined by xWT1 expression. xWT1 is expressed in animal caps treated at high concentrations of RA (10^{-4} M) in combination with activin, in contrast to tubule markers that are expressed after treatment with lower, more physiological levels of RA (10^{-5} M) and activin (Moriya et al., 1993; Uochi and Asashima, 1996). Unlike the other components of the pronephros, the glomus is also inducible by FGF and RA. Retinoic acid alone does not induce the glomus. These studies clearly define different conditions of growth factor inducibility for the glomus and establish these as distinct from those conditions that induce tubules.

**MATERIALS AND METHODS**

**Embryo collection and cultivating**

*Xenopus laevis* embryos were obtained by in vitro fertilisation of eggs from females primed with 80 i.u. serum gonadotrophin (Intervet UK Ltd) 1-5 days previously and injected with 600 i.u. choricron gonadotrophin B (Intervet UK Ltd) 8-16 hours prior to the desired laying time. Eggs were collected into Barth’s saline (88 mM NaCl; 1 mM KCl; 24 mM NaHCO3; 15 mM Tris-HCl: 0.33 mM Ca(NO3)2: 0.41 mM CaCl2; 0.8 mM MgSO4 pH 7.5) and then fertilised by the addition of crushed testis. Fertilised eggs were dejellied in 2% cysteine-HCl pH 8.0 (BDH Laboratory Supplies). Embryos were then washed thoroughly and cultured in 10% Barth’s medium at appropriate temperatures to obtain the stages required (13°C, 18°C or 23°C). Embryos were staged according to Nieuwkoop and Faber (1994).

**Microdissection and explant culture**

All manipulations were performed in full-strength Barth’s saline containing 10 μg ml^{-1} gentamycin and 5 μg ml^{-1} penicillin and streptomycin, on a cushion of 1% agarose in full-strength Barth’s saline. Embryos were manipulated and dissected with watchmaker’s forceps and an eyebrow hair knife. An explant of developing glomus/glomus anlagen/presumptive glomus mesoderm was removed from the donor embryo and placed between two animal caps removed from stage 9 embryos (Fig. 1). The precise region excised from the donor embryo depended on the stage from which it was isolated and was related to the extent of development of the glomus at that stage. In all cases, the initial step was to peel away the ectoderm overlying the region of interest. The desired mesodermal explant was then removed.

For donor embryos of stage 29 and above, the developing pronephric tubules and duct that overlay the developing glomus were removed and discarded. The developing glomus only was then separated from the underlying lateral plate mesoderm. For donor embryos between stages 14 and 20, a region of presumptive pronephric mesoderm was removed from the lateral plate. As shown in Fig. 1, this region is just caudal of the anterior part of the neural plate, and just ventral of the middle part of the neural plate. This is the area in which cells condense to form the pronephric and glomus anlagen and later the pronephros and glomus (Hausen and Riebesill, 1991). For donor embryos of stages 12-13, three regions of lateral plate mesoderm were explanted. The dorsal limit of the mesoderm removed was along a line 35° from the dorsal midline, approximately along the border of the presumptive neural plate. The ventral limit of the mesoderm removed was approximately 15° below this at the anterior end (see Fig. 1). The lateral plate mesoderm was separated from the underlying endoderm and subsequently split into anterior, middle and posterior segments, each to be cultured within its own ectodermal wrap. When explants were removed from donor embryos at stage 11, the dorsal limit of the mesoderm removed was approximately 40° from the dorsal midline, the ventral edge 15° below this. The whole explant was placed within
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an ectodermal wrap at this stage. After placing the desired explant between two stage 9 animal caps, the ectodermal wrap was allowed to heal for a minimum of 15 minutes at room temperature before transfer to 50% Barth’s saline in poly-HEME-coated dishes (2% poly-HEME (Sigma) in 1:1: acetone:ethanol) and cultured until equivalent to stage 33/34, at which point RNA extractions were performed.

For each series of experiments, control wraps were set up that contained no inserted material. These were consistently negative for the mesodermal markers assayed (see Fig. 3 for an example).

**Growth factor and retinoic acid treatments**

Animal caps were removed from stage 9 *Xenopus* embryos and treated with differing concentrations of human recombinant activin A (A. F. Schuetzdeller), human recombinant bFGF (GibcoBRL) or retinoic acid (RA) alone or in combination, in 1 ml of Barth’s saline. Each growth factor treatment was applied to 10 animal caps, and each treatment was repeated in at least two independent experiments. Animal caps were cultured in the presence of growth factors until stage 12.5 equivalent, then transferred to 50% Barth’s saline and cultured until equivalent to stage 33/34, if to be assayed for the presence of glomus by RT-PCR, at which point RNA extractions were performed.

**RT-PCR analysis and primers**

Total RNA was extracted using the protocol detailed below. Volumes given apply to extraction from 10 animal caps, extractions from 10 explants within ectodermal wraps or from 2 *Xenopus* embryos, required 1½ times these volumes.

Each group of 10 animal caps was homogenized in 300 µl XT buffer (300 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS) and digested at 37°C with proteinase K. Following phenol extraction and addition of 10 µg glycogen (as carrier), samples were ethanol precipitated at –20°C. Dried pellets were resuspended in 60 µl of transcription buffer containing DNase I and placental RNase inhibitor and incubated for 15 minutes at 37°C. 240 µl XT buffer was added and digestion at 37°C with proteinase K was performed. After phenol and phenol/chloroform extraction, the RNA sample was ethanol precipitated at room temperature and resuspended in 20 µl water.

0.5 ng of total RNA was used to generate first-strand cDNA in the presence of Superscript II reverse transcriptase (Gibco) using random hexamers as primers. Control syntheses were also carried out in the presence of Superscript II reverse transcriptase (Gibco) or RNA. The cDNA was then used for PCR analysis using the primers shown in Table 1. The conditions were as follows: an initial cycle of 94°C for 3 minutes (denaturation step); 55°C for 1 minute (annealing step); 72°C for 1 minute (extension step). The required number of cycles, as shown in Table 1, were carried out as for the initial cycle but with 30 seconds for the 94°C denaturation step. The final cycle was 72°C for 5 minutes. These conditions were followed for all primers with the exception of those for *cardiac actin*, which required an annealing temperature of 62°C. PCR products were analysed on 6% non-denaturing polyacrylamide gels and exposed on X-ray film, or on a phosphorimagner screen. All RT-PCR experiments were carried out at least twice with independently made RNA samples.

**RESULTS**

**Glomus specification occurs at the late gastrula stage**

In order to identify the stage at which the glomus compartment of the pronephros was specified during the early development of *X. laevis*, we employed an explant strategy similar to that described in Brennan et al. (1998). Briefly, presumptive glomus mesoderm, the glomus anlagen or the fully differentiated glomus was microdissected from progressively earlier embryonic stages starting from stage 29, at which time the glomus has begun to differentiate (Nieuwkoop and Faber, 1994), down to stage 11 (see Materials and Methods and Fig. 1 for descriptions of the exact dissections). The explanted region was sandwiched between two stage 9 animal caps, which provided a neutral environment within which the explant may be cultured. The ectodermal wraps produced were then incubated until control, undissected embryos were stage 33/34 equivalent, at which time the pronephros has clearly differentiated and is potentially functional (Nieuwkoop and Faber, 1994). The wraps were then harvested and RNA prepared. The presence or absence of glomus material was identified by RT-PCR analysis for *Xenopus Wilms’ tumour 1* gene, *xWT1*, and also additional markers of intermediate mesoderm were analysed, shown in Fig. 2. The mRNA of *xWT1* has previously been shown to be restricted to the developing glomus between stages 18 and stage 38 and thus can be used as a definitive marker of glomus differentiation (Carroll and Vize, 1996). We chose to analyse the wraps at stage 33/34, well
within this period. After stage 38, expression is also seen in the developing heart. Adult expression of xWTI persists in the mesonephric kidney.

Fig. 3 shows the results of semiquantitative RT-PCR analysis used to identify xWTI transcripts in the explant specification assay with wraps containing glomus, presumptive glomus or intermediate mesoderm explanted from embryos at stages 29 to stage 11. A positive signal is observed from all wraps containing explants explanted from stages 29 to 16, the signal slightly increasing with intensity as the donor embryo increases in age. Since the cDNA input to the RT-PCR has been equalised with respect to the EF1α signal, this variation in intensity reflects broadly the amount of glomus material within the wraps at the end of the incubation period. Presumptive pronephric mesoderm from stages 12-13 was divided into three sections prior to placing it within the ectodermal wraps. At both stage 12.5 and 13, the anterior and middle sections expressed xWTI transcripts, the anterior section being a much weaker signal. Wraps containing the posterior section were completely negative. At stage 12, no signal was observed in wraps containing any of the three explants. Stage 11 explants, which consisted of the whole lateral mesoderm from the right side of the embryo, were also negative. The presence of signal in wraps derived from stage 12.5 donors and above indicate that glomus specification, as defined in this explant assay, occurs between stages 12 and 12.5. This dissection data indicates that the pronephric field is restricted to the anterior and middle sections of the lateral plate at this stage.

Expression of xWTI transcripts has been shown to occur in the heart of stage 38 tadpoles by whole-mount in situ hybridisation (WISH). The increased sensitivity of the RT-PCR technique over WISH may identify low, previously undetectable, levels of xWTI transcripts potentially expressed in the heart at earlier stages. In order to exclude the possibility that the transcripts of xWTI expressed in the wraps were due to the differentiation of heart mesoderm, the expression of the heart marker, Xenopus myosin heavy chain α (xMHCα), was analysed in the specification wraps. xMHCα is expressed in the embryo from stage 28 onwards (Logan and Mohun, 1993 and Fig. 2). Fig. 3 clearly shows that none of the explants contain differentiated heart mesoderm, confirming that the expression of xWTI transcripts observed was not due to the wraps containing heart tissue, and therefore marked the presence of glomus in the explanted material.

To additionally confirm that the regions explanted contained pronephric material, the same samples were analysed for Xlim-1 transcripts. Xlim-1 is a marker of three lineages in the Xenopus embryo; the dorsal mesoderm of the Spemann organiser, a defined population of cells of the nervous system and the pronephros. It is first expressed at stage 10.5 in the organiser region but, from stage 13, is also found in the pronephric lineage (Taira et al., 1994; Fig. 2). Although it is not possible to confirm in which of these lineages the observed Xlim-1 expression occurs, it is highly likely that, since lateral mesoderm is explanted in these experiments, the majority of the expression of Xlim-1 transcripts is from pronephric lineages, in particular duct and tubule. Fig. 3 shows the expression of Xlim-1 in the

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**Table 1. Primers for RT-PCR analysis**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequence (5′-3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>xWTI (27 cycles)</td>
<td>U-CACACGCACGGGGGTCT D-TGTGATGATGATGACG</td>
<td>Carroll, T. J. and Vize, P. D., 1996</td>
</tr>
<tr>
<td>Cardiac actin (21 cycles)</td>
<td>U-TCTGAAAGCAGACCAAGCCACATA D-ATGCTGTAATTATTTAATTGTC</td>
<td>Mohun et al., 1986</td>
</tr>
<tr>
<td>xMHCα (24 cycles)</td>
<td>U-CAGATTGGTGGATGATGC D-CACGTCCCTTGAGTACCTC</td>
<td>Fu, Y. and Izumo, S., 1995</td>
</tr>
<tr>
<td>EF1α (24 cycles)</td>
<td>U-CAGATTGGTGGATGATGC D-CACGTCCCTTGAGTACCTC</td>
<td>Mohun et al., 1989</td>
</tr>
</tbody>
</table>

U, Upstream; D, Downstream; RT-PCR, Reverse transcription polymerase chain reaction.
Fig. 3. RT-PCR analysis of wraps shows that glomus specification occurs at stage 12.5. Explants removed from progressively earlier stages of embryo from stage 29 down to stage 11 were cultured within ectodermal wraps and assayed at stage 33/34 equivalent for the presence of the glomus marker xWT1. The presence of this glomus marker, and therefore specified glomus tissue, can be seen in explants removed from all stages from 29 to 12.5. At stages 13 and 12.5, anterior (A) and middle (M) regions of mesoderm explants contain xWT1 transcripts; however, the posterior (P) explants do not, showing that in this region of mesoderm the glomus is not specified. No expression of xWT1 is observed in explants dissected from stage 12 or stage 11. This data clearly shows that glomus is specified between stages 12 and 12.5. Absence of the expression of xMHC, a marker of heart tissue, in explants removed from any stage confirms that the tissue expressing xWT1 is indeed glomus tissue. xWT1 expression, which marks three lineages including the pronephros, is seen in explants of all stages from 20 to 12.5. Expression of Xlim-1 is absent from stage 29. At this stage, it is possible to remove the developing pronephric tubules and duct before explanting the glomus. The absence of Xlim-1 transcripts therefore confirms that this dissection was performed accurately. Explants excised from stage 11 or 12 embryos did not express Xlim-1. This data concurs with previous data that showed pronephric tubules are specified at stage 12.5 (Brennan et al., 1998). Cardiac actin is expressed in explants removed at stages 11 and 12, thus proving that these ectodermal wraps did contain viable mesodermal tissue including somite tissue. The absence of xWT1 expression in explants removed at these stages is not therefore due to the lack of viable explant within the ectodermal wraps, but to the absence of specified glomus tissue. A no insert control was included consisting only of the ectodermal wrap. This sample was negative for all markers with the exception of EF-1α, which is present within the animal caps that are used in the ectodermal wraps. Stage 33/34 embryos were used as a positive control. Other controls are as detailed for Fig. 2 above. For each sample, total RNA was extracted from 10 ectodermal wraps (or 2 control embryos) and subjected to RT-PCR analysis as detailed in the Materials and Methods above.

Specification wraps. At all stages from 12.5 to 20 and in all dissected fragments of lateral mesoderm, Xlim-1 transcripts can be detected. At stage 29, explanted glomus can be dissected free of contaminating duct and tubule. There is no Xlim-1 expression in this sample, indicating that the glomus does not express this marker. This confirms the accuracy of these later dissections. The Xlim-1 expression pattern is in agreement with our previously published data, which defined the time point at which the specification of pronephric tubules occurred as between stage 12 and 12.5 (Brennan et al., 1998). No expression of Xlim-1 was detected in wraps with explants taken from stages 11 and 12. Interestingly the domain of expression of Xlim-1 in the anterior, middle and posterior explants at stages 12.5 and 13 is broader than that observed for tubule specification, the latter occurring in middle and posterior sections only and not in the anterior section. The expression of Xlim-1 in these samples also demonstrates that the ectodermal wraps did not extrude the grafted mesoderm but still contained lateral mesoderm at the end of the culturing period.

In order to demonstrate that the stage 11 and 12.5 explanted material had been retained in the wraps, these samples were analysed for cardiac actin mRNA. The results shown in Fig. 3 show that in these early wraps mesodermal material is clearly present throughout the culturing period. To confirm that the correct region was being explanted in those gastrula and early neurula stages that were negative for glomus specification, embryos from a parallel series of experiments were dissected unilaterally and then allowed to heal. These embryos were allowed to develop until stage 38 and then whole-mount immunostained with the tubule-specific monoclonal antibody 3G8 to check that the pronephric rudiment had been removed by the grafting procedure. These embryos showed either partial or complete loss of the pronephros from the dissected side, demonstrating that the correct region had been excised. In addition, lineage-labelled (FLDX) donor embryos were dissected at stage 13 into the three intermediate mesodermal explants. The middle explant was then grafted into an equivalent position on a recipient embryo and the embryo allowed to develop. Fig. 4 shows that the grafted material is making a major contribution to the developing pronephros.

Fig. 4. Lineage-labelled orthotopic grafts from the intermediate mesoderm in the presumptive pronephric region contribute to the developing pronephros. Embryos were injected with the lineage label FLDX at the 1-cell stage and allowed to develop to stage 12.5. Grafts were taken from the middle intermediate mesoderm explant from stage 12.5 embryos, as described in Fig. 1, and inserted into host embryos of the same stage at an equivalent position. The grafted embryos were allowed to develop until stage 28 and then observed under a Leica MZFLIII UV dissecting microscope. Images were captured using a Leica DC100 video camera.
Ectodermal wraps made by sandwiching two stage 9 animal caps together, as a negative control, failed to express xWT1, xMHCα, Xlim-1 or cardiac actin mRNAs, as expected.

Growth factor induction of pronephric components

It has been demonstrated that it is possible to induce the formation of pronephric tubules in Xenopus animal cap tissue at high frequency by treatment with a combination of activin A and retinoic acid (RA) (Moriya et al., 1993). In these studies the presence of tube was identified by histological procedures identifying eosphinophilic columnar or cuboidal epithelia, characteristic of pronephric tubules. We have extended these observations, analysing explants for the third component of the pronephros, the glomus, after induction of animal caps with combinations of activin A and retinoic acid. Glomus was identified by the presence or absence of xWT1 expression (Carroll and Vize, 1996).

The induction of glomus by activin and retinoic acid

We have investigated the induction of the glomus by activin and FGF with and without RA. Animal caps taken at stage 9 were treated with a concentration of activin from 0.1 ng ml⁻¹ to 20 ng ml⁻¹. These caps were incubated until stage 33/34 equivalent and then analysed by RT-PCR for the presence of the glomus

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Fig. 5. RT-PCR analysis shows that glomus tissue can be induced in Xenopus animal caps by treatment with activin A plus retinoic acid. Animal caps removed at stage 9 were treated with either activin A alone or activin A together with RA, until stage 33/34 equivalent and then analysed for expression of the markers shown.

(A) (i) Activin A does not induce xWT1 in animal caps. Activin alone was applied at a range of concentrations from 0.1 ng ml⁻¹ to 20 ng ml⁻¹. No induction of xWT1 expression was observed, showing that glomus tissue was not induced. Xlim-1 expression was induced in animal caps treated with all concentrations of activin A between 0.5 ng ml⁻¹ and 20 ng ml⁻¹, a higher level of expression observed at lower concentrations. Expression of cardiac actin was induced in animal cap tissue by all but the lowest concentration of activin A (0.1 ng ml⁻¹). This expression shows that somite tissue, and therefore mesoderm, was induced as expected. (ii) Animal caps treated with high concentrations of activin A can induce the heart marker xMHCα. (B) Treatment with activin A plus RA can induce glomus. Application of activin A at all concentrations above 5 ng ml⁻¹, plus 10⁻⁴ M RA, induces the expression of xWT1 and therefore glomus tissue. This semiquantitative RT-PCR shows that the expression of xWT1, and thus the amount of glomus tissue induced, increases with higher concentrations of activin A present. No induction of xWT1 was observed when lower concentrations of RA (10⁻⁶ M or 10⁻⁵ M) were applied together with activin A, or when activin A was applied alone. The absence of xMHCα expression, and therefore heart tissue, in animal caps treated with any combination of activin A and/or RA confirms that the xWT1 expression observed is within glomus tissue. Xlim-1 expression is induced within animal caps when treated with activin alone or activin plus RA, however no induction is observed following treatment with RA only. The presence of cardiac actin transcripts, and therefore somite tissue, in all samples treated with activin A shows clearly that mesoderm induction has occurred. The level of cardiac actin expression induced by each concentration of activin A decreases with increasing concentration of RA applied; a consequence of the RA ventralising the mesoderm produced and thus inducing less somite tissue. In all samples, EF-1α was used as a control to ensure equalised loading. For each treatment, total RNA was prepared from 10 caps (or 2 embryos for stage 33/34 control embryo) and subjected to RT-PCR analysis as detailed in the Materials and Methods. Stage 33/34 embryos were used as a positive control. Other controls are as detailed in Fig. 2 above.
in all samples, RA on the mesoderm to the ventralizing effects of activin is being induced within these explants. Analysis of the activin gradient for including somite, is being induced within these explants. The induction of cardiac actin at all but the lowest level of activin confirms that mesodermal tissue, as previously reported (Taira et al., 1992). If the expression of cardiac actin is induced at concentrations higher than 0.25 ng ml\(^{-1}\) of activin, Xlim-1 is induced at all concentrations of activin used. However, Xlim-1 is induced at concentrations higher than 0.25 ng ml\(^{-1}\) of activin, consistent with the previously reported observation that this gene is activin inducible (Taira et al., 1992). If the expression of Xlim-1 is in the pronephric lineage these tissues are clearly not able to develop sufficiently to express the later markers of pronephric development (for example, 3G8 and 4A6, see previous section). The induction of cardiac actin at all but the lowest level of activin confirms that mesodermal tissue, including somite, is being induced within these explants. Analysis of the activin gradient for Xlim-1 transcripts shows that, as previously reported, high levels of activin can induce these heart-specific transcripts (Logan and Mohun, 1993).

Animal caps were also cultured with a combination of activin and RA. Concentrations of activin in the range 1-20 ng ml\(^{-1}\) were used in combination with RA concentrations of 10\(^{-6}\) - 10\(^{-4}\) M. Fig. 5B shows that xWT1 is induced at all concentrations of activin (weak at 1 ng ml\(^{-1}\)) in the presence of 10\(^{-4}\) M RA, the strength of the signal increasing with activin concentration. Xenopus myosin heavy chain \(\alpha\) is not expressed under these conditions suggesting that the xWT1 expression is not due to the induction of heart tissue, but is instead due to its domain of expression in the glomus. The ventralising effect of RA on the dorsal mesoderm induced by activin is shown at the higher concentrations of RA by the reduction in the cardiac actin signal, with increasing concentrations of RA applied. xWT1 was also induced in all animal caps treated with activin, but not by RA alone at any of the concentrations tested. This contrasts with the results of Taira et al. (1992). However, it is not possible to make a direct comparison with these data since Taira et al. (1992) analysed RA-treated explants at stage 10.5 whereas our experiments analysed stage 34. At this time, the RA induction of Xlim-1 may no longer be apparent. These data show that, as with tubule induction (Taira et al., 1992; Uochi and Asashima, 1996; our unpublished observations), a combination of activin and RA at precise concentrations are necessary to induce glomus tissue from animal cap ectoderm.

**FGF in combination with retinoic acid does induce glomus tissue in animal caps**

Initially animal caps were incubated in FGF from 6.25 ng ml\(^{-1}\) to 100 ng ml\(^{-1}\), concentrations, which have been shown previously to induce the full range of ventral and intermediate mesodermal tissues (Slack et al., 1987). The presence of glomus tissue was assayed by RT-PCR as described earlier, the results are shown in Fig. 6A. None of the FGF concentrations used induced glomus as indicated by the lack of detectable xWT1 transcripts. Cardiac actin transcripts were identified in all the FGF-treated samples but not in the control untreated caps, both confirming the quality of the animal cap dissections and acting as a positive control for the activity of the FGF, the signal increasing with the concentration of FGF.

Addition of RA to the FGF inductions resulted in the...
factors. The lack of expression of cardiac actin transcripts in caps treated with activin alone, or a combination of both growth factors. It is perhaps unlikely that this level of expression is due to the neural expression profile of this gene, induced by secondary inductions from dorsal mesoderm within the explants.

**Activin in combination with FGF does not induce glomus tissue**

Animal caps were treated with 10 ng ml\(^{-1}\) activin together with a range of FGF concentrations from 6.25 ng ml\(^{-1}\) to 50 ng ml\(^{-1}\) (Fig. 7). All of the combinations of activin and FGF tested failed to induce \(xWT1\) in the animal cap tissue, showing that glomus tissue was not induced under these conditions. Cardiac actin is induced with all concentrations of FGF, but the explants are posteriorised by the RA treatment with the resultant loss of the actin signal in all but the lowest RA treatments. Caps treated with RA alone do not express \(xWT1\), \(xMHC\alpha\) or cardiac actin.

**Fig. 7.** RT-PCR analysis shows that induction of glomus tissue does not occur when animal caps are treated with bFGF plus activin A. Animal caps were treated with 10 ng ml\(^{-1}\) activin A plus a range of concentrations of bFGF, activin A alone or with no growth factors. Induction of \(xWT1\) expression was not seen in any of the animal caps treated. Therefore induction of glomus did not occur. \(xMHC\alpha\) and \(xWT1\) were induced in all treatments where activin A was present. In all samples, EF-1\(\alpha\) was used as a loading control. For each treatment, total RNA was prepared from 10 caps (or 2 embryos for stage 33/34 control embryos) and subjected to RT-PCR analysis as detailed in the Materials and methods. Stage 33/34 embryos were used as a positive control. Additional controls were performed as detailed in Fig. 2 above.

**DISCUSSION**

The aim of this work was to identify the timing of specification for the pronephric glomus and to establish growth factors that may induce the formation of glomus tissue and then compare these conditions with those that induce duct and tubule. This has been successfully addressed using explant culture techniques.

**Specification of the glomus**

Our explant culture system clearly shows that the glomus of *Xenopus laevis* is specified by stage 12.5. The specification of the pronephric tubules has also been shown to occur at this late gastrula stage (Brennan et al., 1998) although duct specification occurs somewhat later. We also show that *Xlim-1* transcripts, which are found in the normal pronephros (Taira et al., 1994) and induced pronephric tubules (Uochi and Asashima, 1996), are present in presumptive pronephric explants removed from donor embryos at stage 12.5 but not 12, confirming our previously published immunohistological data defining the time point of specification of the pronephric tubules. Earlier studies by Fales (1935) in *Ambystoma* have put the time point for specification of the pronephros somewhat later than this at stage 15. Fales carried out orthotopic or heterotopic transplants of presumptive pronephros together with the overlying ectoderm. Pronephric tubules developed when such grafts were made from stage 15 or later, but not prior to stage 15. However, it is hard to compare these results directly with ours since not only was a different organism used, but also the study was at the level of determination rather than specification since the grafts were not placed into a neutral environment, but were inserted into the flank of the developing tadpole where both positive and negative influences could affect the experimental outcome. Fales did not provide any specific information on glomus specification in *Ambystoma*.

The pronephric tubules and glomus are therefore specified at the same time, but may or may not be specified by the same signals. *Xlim-1* is first expressed in the presumptive pronephros at approximately stage 12.5 and is potentially responsible for the specification of the pronephric tubules. However, its expression is not seen in the glomus and it therefore seems unlikely that expression of *Xlim-1* is able to specify this tissue. The presence of glomus tissue was assessed by the presence of *xWT1* transcripts in explants cultured to stage 33/34. It has been suggested that *xWT1* expression may have a role in determining whether intermediate mesoderm will form glomus or pronephric tubule, expression of this molecule rendering those cells competent to form glomus tissue on receipt of subsequent signals (Carroll and Vize, 1996). However, the molecular mechanisms by which the glomus and pronephric tubules are specified are not yet known. It is likely that both form as a result of inductive interactions between adjacent tissues in the mesoderm, for example the somitic mesoderm, and either the...
ectoderm and/or endoderm (Nieuwkoop and Faber, 1994). Preliminary data from exogastrulated embryos show that tissue interactions during gastrulation are not necessary for the formation of glomus, although they are essential for the formation of duct and tubule epithelia (data not shown). This is also consistent with Seuver et al. (1999) who provide evidence that the somites can directly induce otherwise unspecified intermediate mesoderm to form kidney tubules and, furthermore, that the most extreme UV ventralised embryos, which do not form pronephroi, can be induced to do so in recombination experiments with embryo explants containing anterior somites.

In more complex kidney forms (i.e. the mesonephros and metanephros), a series of inductive interactions between individual components occur during the development of the kidney (Saxén 1987). For example, as the mammalian metanephros forms, a reciprocal inductive interaction takes place between the ureteric bud and the metameric mesenchyme. This interaction is essential for the correct development of both components (Saxén, 1987). Both pronephric tubules and glomus are specified at stage 12.5, suggesting that a reciprocal interaction might occur between these two tissues during development. However, it is unlikely that such inductive interactions are essential for the formation of glomus or pronephric tubule tissue; as our data show, it is possible to induce the formation of one tissue in the absence of the other in Xenopus animal caps (discussed further below). It has been suggested that as the development of the mesonephric and metameric mesenchyme into kidney tubules can be initiated in the complete absence of the normal inducer (i.e. in the presence of heterologous inducers), this mesenchyme must be at least partially specified (reviewed by Vize et al., 1995). As the molecules expressed during the development of the three kidney forms appear to be conserved (Vize et al., 1995; Lechner and Dressler, 1997; Carroll et al., 1998), it is plausible that the initial signal specifying these kidneys is external to the developing kidney and is conserved between kidney forms.

**Growth factor induction of pronephric tubule or duct tissue within animal cap explants**

Moriya and colleagues (1993) examined the induction of pronephric tubule tissue within Xenopus animal caps by treatment with activin A plus RA, and demonstrated that this led to high frequency induction of pronephric tubule tissue, as analysed by histology. We have confirmed that high frequency induction of pronephric tubule tissue is only seen following treatment of animal caps with activin A plus RA, and not after treatment with either molecule alone (Moriya et al., 1993, H. C. B. and E. A. J., unpublished observations; Table 2). In addition, we have established that pronephric duct is not induced at high frequency by either molecule, alone or in combination (data not shown).

Pronephric tubule tissue is rarely induced in animal cap tissue when treated with purified activin alone at any concentration. The concentrations of activin A used in published studies would induce a range of mesodermal tissues, ventral at low concentrations and dorsal types at high concentrations (Moriya et al., 1993). The ventralising effects of RA are well documented in both whole embryos and explants (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991). However, the induction of pronephric tubules on treatment of animal caps with activin and RA cannot be explained by the induction of more ventral mesoderm alone, since neither low concentrations of FGF nor activin treatments, in isolation, induce tubule tissue at high frequency. Furthermore, the application of RA together with bFGF induces more ventral mesoderm than that obtained by treatment with bFGF alone (Ruiz i Altaba and Jessell, 1991). These data viewed as a whole suggest that the induction of pronephric tubules observed when RA is applied together with activin A is not simply a result of ventralizing the mesoderm induced, but rather due to a more specific effect resulting from the upregulation of an RA responsive gene.

**Xlim-1** is expressed in pronephric tissue and is induced in animal caps, assayed at stage 10.5, by treatment with activin A alone or activin A together with retinoic acid (Taira et al., 1992; Uochi and Asashima, 1996). It has been speculated that Xlim-1 induced by activin alone corresponds to expression in dorsal mesoderm and notochord, whereas induction by activin A plus RA results in expression in the pronephric region and neuroectoderm (Taira et al., 1994). RA treatment of embryos results in a caudally expanded domain of Xlim-1 expression and, subsequently, a larger pronephros, potentially due to a recruitment of cells into the pronephric lineage (Taira et al., 1994). Therefore in animal caps treated with activin A plus RA, RA may be acting to induce the expression of Xlim-1, resulting in the formation of pronephric tubule tissue. However, this induction of Xlim-1 must be mediated via other RA-inducible genes, as it is eliminated by addition of the protein synthesis inhibitor cyclohexamide (Tadano et al., 1993). In addition, it is not known if the effect of RA is physiological. High frequency induction of pronephric tubules in animal caps is observed following treatment with 10 ng ml−1 activin A plus 10−5 M RA (Moriya et al., 1993). The total concentration of endogenous bioactive retinoids within embryos has been calculated at the much lower concentration of 0.92×10−7 M (Blumberg, 1997). However, the distribution of these retinoids within the embryo is not known, it is possible that local high concentrations exist, capable of effecting these developmental switches. Retinoic acid receptors have been found that are expressed during these early stages of embryonic development in Xenopus (Ellinger-Ziegelbauer and Dreyer, 1991); however, the expression of these receptor molecules has not yet been noted in the pronephros.

The pronephric duct is not induced at high frequency, by any of the combinations of activin A, bFGF or RA used in these experiments (H. C. B. and E. A. J., unpublished). Therefore, the pronephric duct requires different signals for its induction than the pronephric tubules. This is in agreement with data that show pronephric duct specification occurs at a later stage than tubule

**Table 2. Summary of the growth factor inducibility of the three components of the pronephros in Xenopus laevis**

<table>
<thead>
<tr>
<th>Growth factor combination</th>
<th>Glomus (xWT1)</th>
<th>Pronephric tubule (3G8)</th>
<th>Pronephric duct (4A6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activin A</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Activin A + RA</td>
<td>41</td>
<td>42</td>
<td>–</td>
</tr>
<tr>
<td>FGF</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FGF + RA</td>
<td>43</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Activin A + FGF</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

1 Positive at RA concentration 10−4 M.
2 Positive at RA concentration 10−5 M (Moriya et al., 1993, Brennan and Jones, unpublished).
3 Positive at RA concentration 10−4 M and 10−5 M.
4 Brennan and Jones, unpublished.
Glomus tissue can be induced in animal caps by activin A or bFGF together with retinoic acid

Glomus tissue, as previously shown for pronephric tubules (Moriya et al., 1993; Uochi and Asashima, 1996) may be induced by treatment of animal caps with activin A plus retinoic acid. However, the concentration of retinoic acid required for this induction is 10-fold higher (10^{-4} M) than the minimum required for tubule induction (10^{-5} M). Induction of glomus tissue occurs when this concentration of RA is applied together with any of the concentrations of activin A tested. Glomus tissue also develops within animal caps treated with bFGF plus RA (10^{-4} M and 10^{-5} M), but not with FGF alone. Therefore, unlike the induction of pronephric tubules, glomus induction can occur when bFGF is the mesodermal inducer. Application of activin A plus a range of concentrations of bFGF failed to induce the formation of glomus tissue (summarised in Table 2).

These data clearly show that, in vitro at least, glomus induction occurs via a different set of signals than those required for pronephric tubule induction. However, as for pronephric tubules, it appears as though RA is necessary, in addition to a mesodermal inducer, to get induction of glomus tissue within animal caps. As discussed above for pronephric tubules, it is not known whether the RA is an endogenous inducer of glomus. As glomus tissue is not induced under the same range of conditions as those required for pronephric tubule induction, formation of the two tissues can occur independently. Since both pronephric tubules and glomus are specified at the same time, it seems unlikely that an inductive interaction between these two tissues is required for in vivo development of these tissues.

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