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Development of the *Xenopus* Pronephric System

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The pronephros serves as the embryonic kidney of the lower vertebrates. In this report we describe the development of the pronephric system of *Xenopus laevis* utilizing scanning electron microscopy and novel monoclonal antibodies that specifically recognize different parts of the pronephros. Antibody 3G8 recognizes the tubules and nephrostomes of the pronephroi only and does not react with the duct. Antibody 4A6 stains only the duct and the nephrostomes. These antibodies thus allow the positive identification of these two intermediate mesoderm derivatives. Both reagents detect antigens expressed some time after the pronephric structures first form and probably represent markers of terminal differentiation. When the tubules and duct first form they are separate structures that can easily be distinguished; the connective tubules have a distinctive organization, the collecting (or common) tubule is broader than other tubules, and the narrow pronephric duct has a specific shape and position. In later stages the collecting tubule and the rostral portion of the duct undergo a considerable amount of convolution, and both contribute to the final coiled tubular body of the pronephros. The ability of 3G8 and 4A6 to distinguish these two elements of the nephric system was used to reexplore classical experiments on the interaction between these two structures during development of the pronephric system. The use of whole-mount analysis has allowed us to examine large numbers of embryos from different stages and dissected in a variety of planes. These experiments demonstrate the dynamic nature of the intermediate mesoderm and indicate that although the pronephros may be specified by mid-neurula stages, patterning is not complete until tailbud stages.

INTRODUCTION

The developing kidney is an unusual organ in that embryos use a succession of different nephric systems to dispose of wastes and control water balance during their development. The duct of the earlier forms is required for the induction of the later forms, and the early kidneys degenerate or reorganize into other structures once their function is no longer required. In amphibians and fish the pronephros is the first kidney and is essential to the survival of the early embryo (Howland, 1921). In amphibians the pronephros is superseded by the mesonephros which takes over disposal of wastes around the time of metamorphosis and will eventually form the adult kidney (Nieuwkoop and Faber, 1994). In mammals only a rudimentary pronephros is present, and the major embryonic kidney is the mesonephros. The mesonephric duct will in turn induce the adult kidney which we are more familiar with, the metanephros (see Burns, 1955; Fox, 1963; and Saxén, 1987 for reviews).

Both amphibian forms of the kidney are derived from the intermediate mesoderm, which is located between the somites and the lateral plate of postgastrula stages. The first histological indication of pronephric development in *Xenopus* is observable at around stage 21 when cells begin to condense away from the intermediate mesoderm below somites 3 to 5 (head somites 3 and 4 and trunk somite 1). These cells will form the main body of the pronephros. At around the same time a similar condensation is occurring slightly more caudally, below somites 5 to 7, which will give rise to the pronephric duct. The primordia of these two structures then fuse and the body of the pronephros forms a lumen and begins to extend and coil while the duct migrates caudally, where it will fuse with the rectal diverticula and thus open into the cloaca (see Nieuwkoop and Faber, 1994, for a full description).

In this report we present observations on the development of the pronephros obtained using novel monoclonal antibodies, one of which recognizes the tubular component of the pronephros and one of which specifically stains the pronephric duct. These reagents have been used to assemble a description of pronephric development in *Xenopus laevis* up until feeding stages and to investigate the influence these different structures have on each other's development.

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The pronephric/Wolffian duct has been demonstrated to be involved in the induction of both the mesonephros and the metanephros, or to have the capacity to induce these structures, in a number of organisms ranging from amphibia to mammals (see Burns, 1955; and Saxén, 1987). The interdependence of the tubules and duct during development of the amphibian pronephros was explored by researchers investigating the caudal migration and origins of the pronephric duct. O'Connor (1940) removed the portion of the pronephric rudiment lying below somites 3 and 4, which normally gives rise to the pronephric tubules, and examined the ability to form the pronephric duct. This operation was performed on tailbud embryos when the pronephric rudiment had already formed. In three different urodeles, *Pleurodeles, Triton,* and *Ambystoma,* he observed that this operation did not disrupt the formation of the pronephric duct, its caudal migration, or its fusion with the cloaca (O'Connor, 1940). The number of embryos of each species examined is not discussed. Holtfreter (1944) performed a related series of experiments using another urodele, *Triturus alpestris.* In these experiments 25 neurulae, shortly before closure of the neural folds, were cut transversely into anterior and posterior portions, and the separate portions raised and examined for the nephric structures in each piece. The results from these experiments indicated that subdivision of the pronephric rudiment into two fragments resulted in correspondingly proportioned pronephroi, if the anterior segment was one-third of the normal size and the posterior segment was two-thirds of the normal size. There was no capacity to compensate for the loss of a portion of the pronephric anlage. Similar observations on this lack of regulative capacity have been made by Howland (1921), Machemer (1929), and O'Connor (1940). Holtfreter (1944) also observed that when only a portion of the pronephros was present in the anterior segment it was often associated with a small amount of duct tissue. This was interpreted to mean that the pronephric rudiment is capable of transformation into pronephric duct. A final interesting observation was that division of the embryo posterior to the tubule rudiment resulted in normal development of pronephric duct in the posterior explant, as long as the dissection was made between somites 5 and 7.

We chose to reexplore the interaction between anlage of the tubules and duct utilizing the tubule and duct-specific antibodies to unambiguously identify these structures. As the experiments described above do not explore the timing of pronephros/pronephric duct specification, it is quite possible that the interaction between anlage was being tested after the different cell types had been specified. This is in fact probably the case, as Fales (1935) has demonstrated that pronephroi are specified by Harrison stage 15 in *Ambystoma.*

Our data indicate that the inability to compensate for subdivision of the pronephric field also holds true for *Xenopus.* However, the more extensive data generated using molecular reagents indicate that the presence of duct material in anterior explants reported by Holtfreter (1944) is probably due to the spatial relationship of the tubule and duct primordia, rather than transformation from the former to the latter. Furthermore, we find that in *Xenopus* duct is rarely found in posterior explants without some associated tubule. Examples of explants containing duct alone were observed, but they were never found in dissections separating anterior and posterior segments prior to stage 20. A novel observation is that separation of the dorsal portion of the pronephric anlage results in duct formation without associated tubules in ventral portions in explants of all tested stages.

These data indicate that the primordia of these two structures are organized differently at different stages of development and that the specification of primordia as either tubule or duct changes during neurula stages. The data are consistent with those obtained in urodeles (O'Connor, 1940; Holtfreter, 1944), except that only late stages were examined in these early experiments and both the ventral nature of the duct primordia and the dynamic patterning of the pronephric anlage have not previously been observed.

MATERIALS AND METHODS

Monoclonal antibodies were raised by standard procedures (Kohler and Milstein, 1976; Galfré *et al.,* 1977). Kidney extracts from adult and larval *Xenopus laevis* were homogenized in PBS and used to immunize 6-week-old Balb/c females intraperitoneally, in combination with Freund's adjuvant. Immunization was continued until an antibody titre could be detected from test bleeds. Splenocytes were fused to P3-NS1-Ag4-1 (NS1) in the presence of PEG 1500. Supernatants from hybrid clones were screened by indirect immunofluorescence on cryostat sections through the pronephric region of stage 47 tadpoles after fixation in 2% TCA (Jones and Woodland, 1986). Positive cultures were cloned by the limiting dilution method.

Western blotting was performed using standard protocols (Harlow and Lane, 1988). Pronephroi were manually isolated from stage 47 *Xenopus* embryos and homogenized directly in SDS sample buffer. Samples were spun in a microfuge for 1 min prior to loading onto a 12.5% polyacrylamide gel. Equivalent numbers of heads and tails from the same stage embryos were loaded as negative controls. Following electrophoresis, electroblotting, and incubation with primary antibody, antibody binding was visualized using a peroxidase-conjugated rabbit anti-mouse secondary antibody and diaminobenzidine as the histochemical substrate. Histological sections were prepared from plastic (3G8)- or wax (4A6)-embedded embryos that had been previously been subjected to whole-mount immunohistochemistry. Plastic sections were generated using the *Drosophila* eye disc protocol of Tomlinson and Ready (1987) and cut at a thickness of 2 μm. Wax sections were cut from paraplast-embedded embryos at a thickness of 6 μm.

Embryos were generated and manipulated by standard procedures (Vize *et al.,* 1991). Dissections were performed in 0.5× MMR at various stages and explants cultured to

stage 41 or 42 in the same media plus 1× antibiotic/antimycotic (Sigma).

Scanning electron microscopy was performed on stage 26 and 35 embryos fixed in modified Karnovsky's as described by Kelley *et al.* (1991). The epidermis was dissected using tungsten needles and forceps to expose the pronephros. Embryos were then dehydrated with ethanol and infiltrated with amylacetate. They were then critical point-dried, sputter-coated with a 2 n*M* gold layer, and examined using a Phillips 515 scanning electron microscope.

Whole-mount immunohistochemistry and immunofluorescence were performed by standard methods on formalin-fixed embryos. Albino embryos were widely used, but both 3G8 and 4A6 antibodies also work well on bleached pigmented embryos (Klymkowsky and Hanken, 1991). Staining was performed using undiluted hybridoma supernatant. Alkaline phosphatase blue color reaction products were generated using BCIP/NBT and red with naphthol phosphate/new fuchsin (Kirkegaard and Perry Laboratories). Both of these stains are stable in benzyl alcohol-based clearing agents. Double staining was performed by first-round staining utilizing the darker colored substrate and fixation in formalin for 60 min and then second-round staining with the new primary and detection using the lighter colored substrate. Documentation of staining was performed by video capture using an Optronics LX450 and a NeXTDimension system.

RESULTS

Production of Monoclonal Antibodies Against Pronephric Antigens

Initial attempts to raise antibodies against the pronephric kidney followed two distinct methods. The first, utilizing adult mesonephric kidney as the immunogen, generated antibodies which reacted solely with the adult kidney and so were not useful for studying pronephric structure or development. A second immunization strategy utilized adult kidney as the primary immunogen, followed by secondary boosts of manually dissected pronephric kidney from stage 47 tadpoles. This strategy yielded monoclonal markers of pronephros differentiation in a single fusion from which approximately 200 hybridomas were analyzed. Both antibodies are of the IgG subclass. Antibody 3G8 fails to detect an antigen on Western blots despite a loading of 30 pronephroi per lane (not shown), and the molecular nature of the antigen recognized by this antibody is currently unknown. 3G8 stains only the apical surface of tubule epithelia (Fig. 1A). Antibody 4A6 recognizes a 50×10^3 M_r protein present on Western blots containing extracts of manually isolated stage 47 pronephroi (Fig. 1B). This antibody stains the entire cell surface (Fig. 1C).

Development of the Pronephros and the Molecular Marker 3G8

The pronephros begins to form in the early tailbud stages from mesenchyme derived from the intermediate mesoderm. By stage 25 the main body of the pronephros appears as a solid mass of cells immediately below somites 3 and 4, and the pronephric, or Wolffian, duct has begun its extension posteriorly toward the cloaca. The pronephros begins to develop a lumen and branch into the pronephric tubules at around stage 30, which is also about the time at which the nephrostomes first form. The nephrostomes are ciliated funnels that connect the pronephric tubules to the coelomic chamber. Once the pronephros becomes functional at stage 38, wastes are swept from the coelom by the nephrostomes, where they travel to the collecting duct, from there to the pronephric duct, and from there to the cloaca (Nieuwkoop and Faber, 1994).

Antibody 3G8 recognizes the pronephric tubules from stage 31 onward, with the only other detectable reactivity being to the border of the otic vesicle (Fig. 2A). The staining of the pronephros initiates at the time when the tip of the growing tail first elongates beyond the extended endoderm of the future gut. Staining begins with a small patch within the pronephros which expands rapidly over the next 2 hr of development (Fig. 2). The beginning of staining is not strictly coordinated in the one individual, as the pronephroi on different sides of an individual can appear quite different (not shown). The initiation and expansion of the 3G8 staining pattern corresponds to the time at which the lumen of the pronephros forms and expands. This observation, along with the localization of the 3G8 epitope to the apical surface of tubule cells, indicates that the staining pattern visualized with this antibody reflects the shape of the lumen, not the entire pronephros. This distinction is only important in the early stages of pronephric morphogenesis when the lumen is still forming. By stage 34 the entire tubular structure is strongly stained on both sides of the embryo. The right hand side tubules resemble the Greek letter τ until stage 38, at which time they begin to extend and coil, and by the feeding tadpole stage they are quite convoluted (Fig. 2G). In strongly stained embryos the canaliculi of the nephrostomes are also visible connecting the tubules to the coelom (see Fig. 3A). In these early stages of development the pronephroi of *Xenopus* closely resemble those of other anurans and differ from urodeles only in that three, rather than two, nephrostomes and associated tubules are formed (Field, 1891; Mangold, 1923 (see Spemann, 1938, p. 292)). The anterior portion of the upper arm, or connective tubule, is branched and connects to two nephrostomes, while the posterior portion connects with a single nephrostome. The position of the anterior pair of nephrostomes varies between embryos (e.g., compare Figs. 3A and 3B) and is not strictly related to specific positions relative to somitic segments. The tubules connected to the nephrostomes join a single broad tubule which has been called the collecting or common tubule, corresponding to the vertical arm of the letter τ. The entire tubular network (connecting and common tubules) stains with a similar intensity.

While this antibody does not stain the majority of the pronephric duct, some reactivity is observed with a few cells of the duct in the area immediately adjacent (caudal)

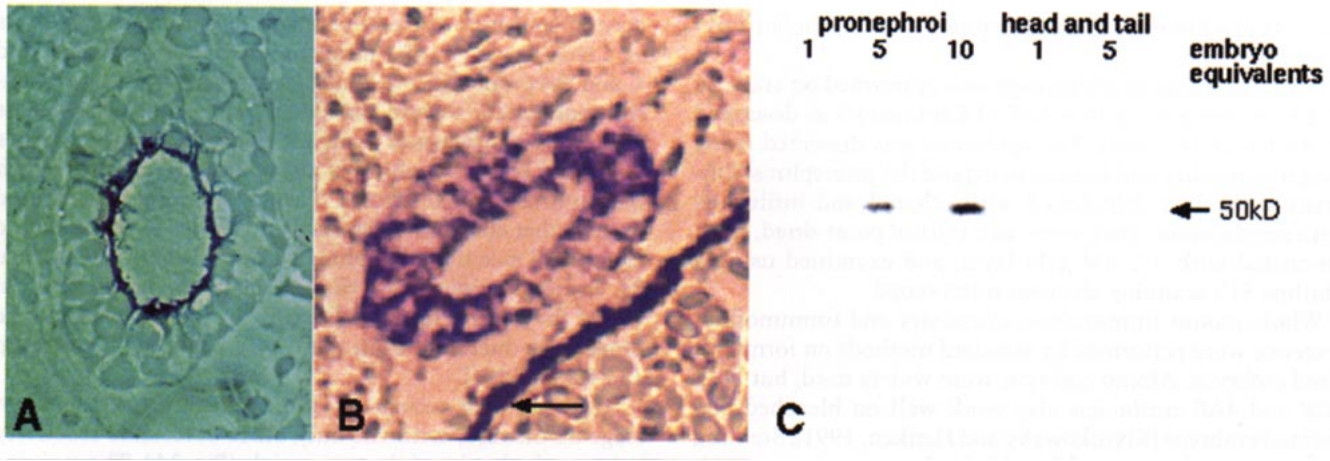


FIG. 1. Characterization of monoclonal antibodies. (A) Transverse section through a pronephric tubule stained with antibody 3G8 and developed with a blue substrate. Staining is localized to the apical surface of the tubule. (B) Transverse section through the pronephric duct stained with antibody 4A6 and developed with a blue substrate; staining is intense around the entire cell surface of the pronephric duct epithelia. The arrow indicates the layer of melanocytes lining the gut, which are distinguishable from antibody staining using phase contrast (not shown). (C) Western blot of stage 47 pronephric extract developed with antibody 4A6. The number of embryo equivalents of pronephroi extract loaded per lane is indicated above each track. Extracts of head and tail from stage 47 embryos serve as negative controls. A single band of 50×10^3 M_r is detected in pronephric extracts only.

to the collecting tubule (Figs. 2E and 2F). This staining is very light and easy to distinguish from the strong reaction observed with cells of the common tubule. The specificity of the antibody can be seen in Fig. 2A and by comparing the scanning electron micrograph in Fig. 2C and staining of a similar stage with 3G8 (Fig. 2E).

3G8 also recognizes the pronephric tubules of another anuran, *Bombina orientalis*, but does not cross-react with those of the urodele *Ambystoma mexicanum* (not shown).

Pronephric Duct Development and the Molecular Marker 4A6

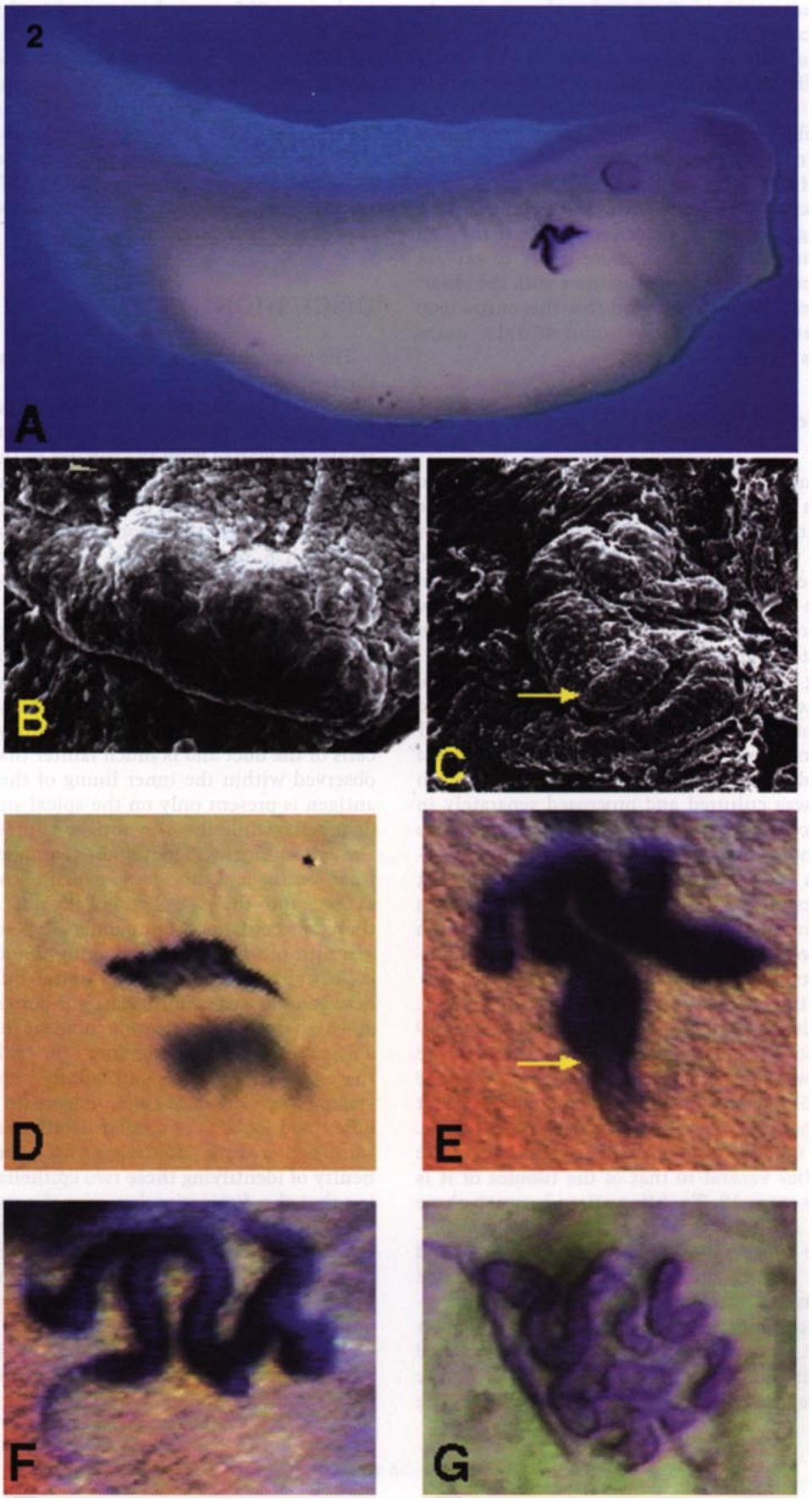
The duct primordium forms at the same time as the main body of the pronephros. It migrates caudally (Fox and Hamilton, 1964; Lynch and Fraser, 1990) and reaches the rectal diverticula and becomes contiguous with the cloaca by stage 38. The antigen detected by antibody 4A6 is first detected in whole-mount staining at around stage 38.

4A6 wholemounts indicate that initially staining is solid in the anterior-most portion of the duct in the small coil underlying the pronephric tubules, with more patchy expression leading back along the length of the nephric duct (Fig. 2A). Individual cells stain strongly, but fewer cells distal to the pronephros stain. The duct is still somewhat extended at this stage, and more solid staining patterns are correlated with the retraction of the duct as the cloaca and endoderm move anteriorly as the gut coils between stages 38 and 41. It is important to note that the pronephric duct is complete and functional by this stage, and 4A6 is recognizing only some of the cells. The 4A6 duct staining pattern is quite solid by stage 43, but this may be due to condensation of the duct masking some unstained cells.

The rostral limit of 4A6 staining overlaps slightly with the point at which 3G8 staining ceases. The rostral border of the region recognized by 4A6 corresponds to the point at which the pronephric duct meets the broad collecting, or common, tubule (Figs. 2C, 2E, and 2F). 3G8 staining extends caudally slightly beyond this border while 4A6 staining is restricted to cells caudal of this point.

By stage 38 the right side duct can be seen to be in a coiled sideways "S" shape joining the common tubule to a

FIG. 2. Formation of the *Xenopus* pronephric tubules. Immunostaining with antibody 3G8 compared to scanning electron micrographs of the developing pronephros. In all figures except G anterior is to the right and dorsal is up. The scale is similar in B through C. (A) Antibody 3G8 recognizes antigens present in the pronephros and the otic vesicle of stage 35/36 embryos. (B) SEM of stage 26 pronephros. (C) SEM of stage 34–35 pronephros. The arrow marks the junction between the collecting tubule and the pronephric duct. (D) 3G8 staining of stage 31–32 pronephros. The stain covers only a portion of the pronephric condensation. (E) 3G8 stain of stage 34–35 pronephros. Note the similarity to the SEM of a similar stage in C. The arrow marks the junction between the collecting tubule and the pronephric duct. The stain extends a short distance into the duct. (F) Stage 40 pronephros stained with 3G8. (G) Dorsal view of stage 44–45 pronephros stained with 3G8. Anterior is facing upward. Magnification is similar in B through G.


2
A
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segment of the duct running just ventral to the somites. As development proceeds the anterior region of the duct coils underneath the convoluted pronephric tubules and finally would become difficult to distinguish from the main tubule network without the aid of these molecular markers. Although it is possible that at these later stages distribution of the 4A6 antigen spreads rostrally partway into the main tubule network of the pronephros, we feel that as the anterior end of the duct is already coiled to some extent, further convolution along with a reorientation such that the coil lies flat underneath the tubules is more likely to explain this pattern of expression. This is consistent with the observations of Howland (1921), who found that this entire loop undergoes extensive coiling in *Ambystoma*. 4A6 also stains the nephrostomes (Fig. 3B).

Experimental Separation of Tubule and Duct Primordia

In order to examine the interdependence of tubule and duct specification in *Xenopus*, embryos ranging from stage 13 to 21 were cut transversely into anterior and posterior parts which were then cultured separately to stage 41. The plane of this dissection was positioned through the region predicted to form the pronephros, which is located below future somites 3 to 6 (Fig. 4). Some variability in the position of the cut was intentionally allowed to increase the likelihood of obtaining a clean segregation of the two primordia. The explants generated in this manner were then assayed for the presence of differentiated tubule or duct at stage 41 using double staining with antibody 3G8 developed with a blue substrate and antibody 4A6 with a red substrate. Each pair of explants was cultured and processed separately in the well of a microtitre tray and then scored for the presence of tubules immunoreactive with 3G8 or duct immunoreactive with 4A6 on each side of each explant. Both the left and the right sides of explants were scored separately, as a slight deviation in the transverse plane of the dissection often resulted in very different patterns of pronephric differentiation on the two sides of an explant.

The results of this analysis are summarized in Table 1. In the 149 examples separated transversely prior to stage 19 not one instance of an explant with only duct tissue in the posterior sample was observed. In samples separated at stage 19 or later 5/86 (6%) cases were found that contained duct tissue in the posterior explant with no detectable tubule. This observation indicated that either the primordia of the pronephric duct lies ventral to that of the tubules or it is formed only after stage 18. To differentiate between these two possibilities a further series of dissections was performed, this time using frontal cuts to separate dorsal and ventral explants. In this instance cuts were positioned slightly below somites 3 to 5, aimed at segregating the bottom portion of the pronephric field into the ventral explant (Figs. 4C and 4D). Once again the explants were developed to stage 41 in pairs and then processed and scored for the presence of tubule, duct, or both on each side (Table 1). The analysis of 225 pronephroi scored in this manner revealed that at all stages tested, from 12.5 to 21, ventral explants containing duct with no associated tubule were observed. The frequency of obtaining such explants drops over time, with the highest being 2/15 (13%) at stage 12.5 and 5/46 (11%) at stage 13–14 and the lowest 3/84 (4%) at stage 19–21. This difference indicates that the area specified as duct either moves or gets smaller over time. The former possibility is more consistent with the anterior–posterior separations described above.

DISCUSSION

The pronephros of *Xenopus laevis* is a simple organ with three main components, a glomus that filters wastes from the blood into the coelom, a tubular system that joins to the coelom via thin ciliated nephrostomes, and a duct that transports wastes from the tubules to the cloaca. This simple organization makes it an excellent system in which to study organogenesis. In this report we present reagents that specifically detect the second two of these components. Antibody 3G8 recognizes the pronephric tubules, nephrostomes, and the otic vesicle while antibody 4A6 stains the pronephric duct and the nephrostomes.

The staining patterns of the two antibodies in the nephric system overlap slightly; a very small region below the common tubule and the nephrostomes is stained by both reagents. The region of overlap in the collecting tubule is caused by an extension of 3G8 staining into the first few cells of the duct and is much fainter than the intense stain observed within the inner lining of the tubules. The 3G8 antigen is present only on the apical surface of tubule and duct cells, while the 4A6 antigen is present around the entire cell surface. 3G8 may stain a component of the extracellular matrix, and this would explain how some of the stain extends into the rostral end of the duct. The border region therefore contains a faint inner 3G8 stain surrounded by a sheath of 4A6 staining, making the distinction between collecting tubule and duct quite straightforward. The border between the two staining patterns corresponds to the point at which the broad collecting tubule is fused to the considerably narrower pronephric duct. The ability to differentiate these two structures is particularly important during later stages, when the duct coils underneath the pronephric tubules, and looks very similar. Holtfreter (1944), discussing some earlier work (Holtfreter, 1938), comments on the difficulty of identifying these two epithelia by histology, stating that the distinction between the two structures could "not always be established," and probably relied on the presence of cilia or cell shape to indicate tubular identity. In urodeles the tubules connect directly to a common tubule which is the same width as the duct, as opposed to anurans which, at least in early stages, have a broad common or collecting tubule (Fig. 2E; Field, 1891; Howland, 1921). In addition, the cells of the collecting tubule and the duct have a similar low cuboidal shape, while those of the dorsal

tubules have a high cuboidal aspect (Jaffee, 1952). The plane of section can make a distinction on the basis of cell shape even more difficult. The two antibodies described here circumvent these difficulties and allow the positive identification of both pronephric components in anurans. Unfortunately, antibody 3G8 does not cross-react with urodele tubules under any of the conditions tested to date (unpublished observations), so a reevaluation of this data is not possible using the reagents presented here.

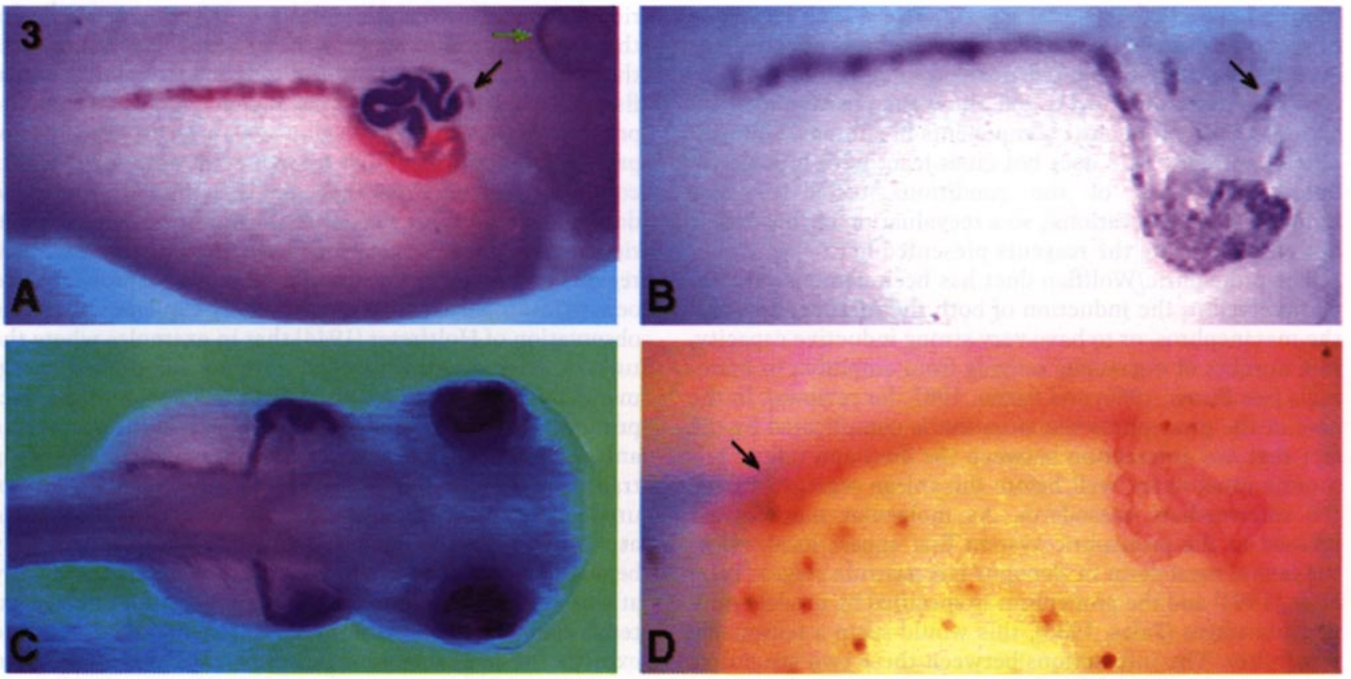
The pronephric/Wolffian duct has been demonstrated to be involved in the induction of both the mesonephros and the metanephros, or to have very strong inductive capacity, in a number of organisms ranging from amphibia to mammals (see Burns, 1955; and Saxén, 1987, for reviews). In the case of the pronephros the situation is complicated by the fact that any interaction between the duct and tubule primordia must occur well before the anlage segregates from the intermediate mesoderm. As molecular markers expressed in the pronephric system first appear localized to the lateral mesoderm in *Xenopus* late gastrula stages (Taira *et al.*, 1994) and the pronephros is specified by urodele mid-neurula stages (Fales, 1935), this would seem a reasonable possibility. The interactions between these two structures was investigated by dissecting embryos into two portions, in a variety of planes close to the predicted border between these two structures, and at a number of different developmental stages. In contrast to previous experiments (Holtfreter, 1944) we find that separation of neurulae into anterior and posterior segments through the pronephric primordia does not generate explants with only duct tissue.

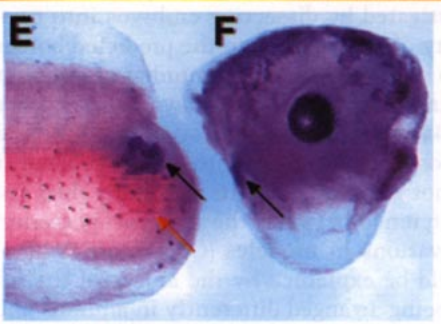
The difference between the anterior–posterior data and the observations in urodeles (O'Connor, 1940; Holtfreter, 1944) could be explained by the anlage of the tubules and the duct being arranged differently in anurans and urodeles or to differences in the timing of the induction of these two structures. In urodeles the duct anlage may be positioned directly caudal to the tubule anlage, while in anurans our data indicate that it must be positioned ventral to the posterior margin of the tubule anlage. In descriptive studies of the *Rana pipiens* pronephroi Jaffee (1952, 1954) indicates that the pronephric primordium is divided by a longitudinal fissure separating a mediodorsal region which will form the pronephros and a lateral region which forms the anterior part of the duct. The high frequency of anterior explants containing tubule alone indicates that the duct primordia does not extend along the entire ventral side of the pronephric anlage; it must be positioned ventral to the most caudal regions only (Fig. 5). This spatial relationship probably also occurs in urodeles and is a more likely explanation for the observation of Holtfreter (1944) that in examples where the tubules were divided between anterior and posterior segments duct was often found in both. A portion of the duct primordia extending along the ventral side of the pronephric anlage would explain this observation without invoking transformation of tubule to duct (Fig. 5). The capacity of urodeles to form posterior explants containing duct alone at a much higher frequency than we find in *Xenopus* may be due to a larger duct field which extends farther caudally at this stage of development. The *Xenopus* duct anlage extends caudally a little later in development, and this would explain the appearance of posterior explants taken from stage 19–21 embryos that contain only duct tissue. We propose that a difference in timing rather than organization is responsible for the conflicting observations and that stage 20 *Xenopus* have a similar organization to mid/late-neurula urodele embryos.

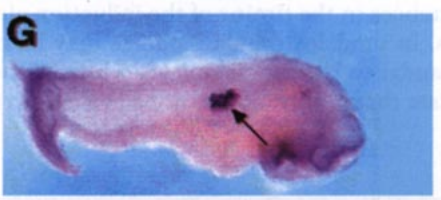
The removal of dorsal structures adjacent to the pronephric field has quite a different effect than removing anterior structures. This operation generated a similar frequency of duct only explants from stage 12.5 (13%) and 13–14 (11%) embryos, while later dorsal–ventral separations (stage 17–18 and 19–21) produced a lower (4%) but existent number of such explants. These data are consistent with the duct primordia lying completely ventral to the posterior portion of the tubule primordia in early neurulae and less ventral and more posterior in late neurulae and tailbud. It is possible

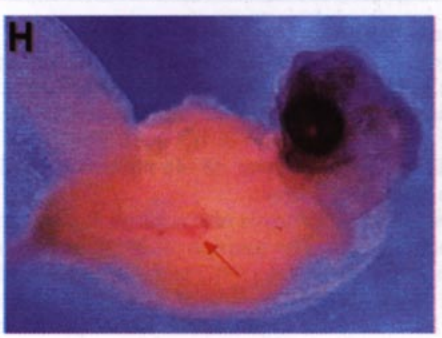
FIG. 3. Antibody 4A6 recognizes the pronephric duct. A, B, and D are lateral views of the right hand side of embryos, with dorsal facing up and anterior to the right. (A) Double staining of stage 39 embryo with antibody 3G8, developed in blue, and antibody 4A6, developed in red. The black arrow indicates staining of the nephrostomes by 3G8, and the green arrow 3G8 staining of the otic vesicle. (B) Stage 40 embryo stained with 4A6. Note the reactivity with the three nephrostomes above the clear area occupied by the pronephric tubules and the patchy nature of the stain in the duct. The arrow indicates one of the nephrostomes. (C) 4A6 staining of stage 43 embryo. Dorsal view. (D) Lateral view of stage 43–44 embryo stained with 4A6. Note the coiling of the anterior duct. Staining is solid and extends caudally to the cloaca (arrow).

FIG. 4. Dissection of embryos into anterior and posterior segments (A and B) or dorsal and ventral segments (C and D) through the plane of the pronephric field. The position of the pronephric region has not been accurately mapped in anuran neurulae. The figure is based on the urodele maps of Yamada (1937) and has been modified to account for the more anterior position of the pronephric field predicted by the *Xlim-1 in situ* data of Taira *et al.* (1994). Early neurulae are shown on the left (A and C) and mid-late neurulae on the right (B and D). Some variation in the angle and the precise point along the A–P and D–V axes was introduced to increase the probability of separating the tubule and duct precursors. Explants were developed to stage 41 and then fixed and double stained with 3G8 using a blue histochemical substrate and 4A6 using a red histochemical substrate. Posterior (E) or anterior (F) explants of a single embryo, with the posterior explant containing both tubule (black arrow) and duct (red arrow), and the anterior explant containing only tubule (black arrow). Dorsal (G) and ventral (H) explants of a single embryo with tubule only (black arrow) in the dorsal explant and duct only (red arrow) in the ventral explant.









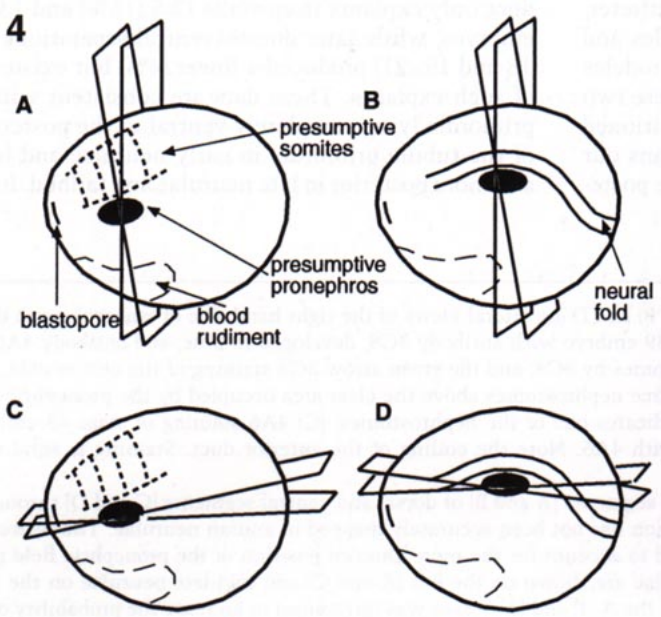


TABLE 1
Differentiation of Tubule and Duct in Embryos Divided into Anterior–Posterior or Dorsal–Ventral Explants

	Anterior	Posterior	Dorsal	Ventral	Stage
Tubule only			5	0	12.5
Tubule and duct			3	4	
Duct only	N.D.		0	2	
Neither			7	9	
Total			15	15	
Tubule only	17	2	26	0	13 to 14
Tubule and duct	2	76	4	25	
Duct only	0	0	0	5	
Neither	60	1	16	16	
Total	79	79	46	46	
Tubule only	12	3			14 to 15
Tubule and duct	4	44			
Duct only	0	0	N.D.		
Neither	32	1			
Total	48	48			
Tubule only	12	0	28	6	17 to 18
Tubule and duct	5	21	38	41	
Duct only	0	0	0	3	
Neither	5	1	14	30	
Total	22	22	80	80	
Tubule only	50	9	13	4	19 to 21
Tubule and duct	12	70	62	41	
Duct only	0	5	0	3	
Neither	24	2	9	36	
Total	86	86	84	84	

Note. Antibody 3G8 was used to score tubule and antibody 4A6 to score duct. Both the left and right hand side of each explant was scored independently for the presence of pronephric structures. The number of explants in each experiment is shown in Total. Note that in anterior–posterior separations no posterior explants with duct alone are found until stage 19 to 21, while duct alone ventral explants are found at all stages tested. N.D., not determined.

that the cells specified to form the pronephric duct migrate between these two positions, or different cells may be specified as tubule and duct at different times. The frequency of dorsal explants containing tubule alone drops from 57% at stage 13–14 and 35% at stage 17–18 to 15% at stage 19–21, consistent with the appearance of duct primordia posterior to the tubules. A model for the changing relationship of tubule and duct primordia is presented in Fig. 5.

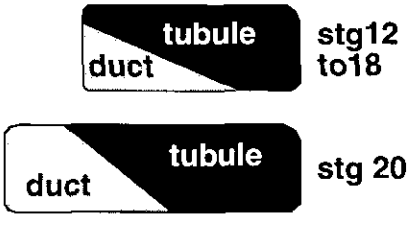


FIG. 5. Proposed relationship of tubule and duct primordia in *Xenopus*. Anterior is to the right and dorsal is up. In early neurula the duct primordia lies mostly ventral to that of the tubules and can be separated in D–V but not A–P dissections. In mid-neurula stages the duct primordia has shifted more posterior and cannot be easily separated by either plane of dissection. In late neurula/early tailbud the duct primordia lies posterior to the tubules and can be separated only by A–P dissection.

The data obtained indicate that specification status of tubule or duct is dynamic during the early neurula stages. That additional cells are being specified as tubule during this period is evidenced by the increase in the number of anterior explants containing tubule alone in stage 19–21 samples, where 50/86 (58%) have tubule alone, compared to stage 13–14 (22%) and stage 14–15 (25%, see Table 1). The evidence for additional cells being specified as duct during neurula stages is also provided by the anterior–posterior dissections, which show that duct can only be separated from tubule with this type of dissection after stage 19. Either specified cells are moving within the intermediate mesoderm prior to segregation of the pronephric primordia, or respecification is occurring. The latter possibility seems more likely. Although the capacity to induce ectopic pronephric structures has been demonstrated to exist well into

tailbud stages (Holtfreter, 1933), the data presented here indicate that this signal is also influencing development of the normal pronephros throughout the neurula stages.

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