Signals from Trunk Paraxial Mesoderm Induce Pronephros Formation in Chick Intermediate Mesoderm

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We used Pax-2 mRNA expression and Lim 1/2 antibody staining as markers for the conversion of chick intermediate mesoderm (IM) to pronephric tissue and Lmx-1 mRNA expression as a marker for mesonephros. Pronephric markers were strongly expressed caudal to the fifth somite by stage 9. To determine whether the pronephros was induced by adjacent tissues and, if so, to identify the inducing tissues and the timing of induction, we microsurgically dissected one side of chick embryos developing in culture and then incubated them for up to 3 days. The undisturbed contralateral side served as a control. Most embryos cut parallel to the rostrocaudal axis between the trunk paraxial mesoderm and IM before stage 8 developed a pronephros on the control side only. Embryos manipulated after stage 9 developed pronephric structures on both sides, but the caudal pronephric extension was attenuated on the cut side. These results suggest that a medial signal is required for pronephric development and show that the signal is propagated in a rostral to caudal sequence. In manipulated embryos cultured for 3 days in ovo, the mesonephros as well as the pronephros failed to develop on the experimental side. In contrast, embryos cut between the notochord and the trunk paraxial mesoderm formed pronephric structures on both sides, regardless of the stage at which the operation was performed, indicating that the signal arises from the paraxial mesoderm (PM) and not from axial mesoderm. This cut also served as a control for cuts between the PM and the IM and showed that signaling itself was blocked in the former experiments, not the migration of pronephric or mesonephric precursor cells from the primitive streak. Additional control experiments ruled out the need for signals from lateral plate mesoderm, ectoderm, or endoderm. To determine whether the trunk paraxial mesoderm caudal to the fifth somite maintains its inductive capacity in the absence of contact with more rostral tissue, embryos were transected. Those transected below the prospective level of the fifth somite expressed Pax-2 in both the rostral and the caudal isolates, whereas embryos transected rostral to this level expressed Pax-2 in the caudal isolate only. Thus, a rostral signal is not required to establish the normal pattern of Pax-2 expression and pronephros formation. To determine whether paraxial mesoderm is sufficient for pronephros induction, stage 7 or earlier chick lateral plate mesoderm was cocultured with caudal stage 8 or 9 quail somites in collagen gels. Pax-2 was expressed in chick tissues in 21 of 25 embryos. Isochronic transplantation of stage 4 or 5 quail node into caudal chick primitive streak resulted in the generation of ectopic somites. These somites induced ectopic pronephroi in lateral plate mesoderm, and the IM that received signals from both native and ectopic somites formed enlarged pronephroi with increased Pax-2 expression. We conclude that signals from a localized region of the trunk paraxial mesoderm are both required and sufficient for the induction of the pronephros from the chick IM. Studies to identify the molecular nature of the induction are in progress.

Key Words: Pax-2; Lim 1/2; Lmx-1; kidney development; pronephros; in situ hybridization; immunohistochemistry; paraxis; somites.

INTRODUCTION

Congenital anomalies of the genitourinary tract are detected in about 1 in 500 fetal ultrasounds (Colodny, 1987), and dysplasia, or abnormal kidney development, accounts for one-third of end-stage renal disease in children (USRDS, 1997). Embryonic kidney development has been described morphologically (Waddington, 1938; Abdel-Malek, 1950; Saxén and Sariola, 1987), and some of the genes involved in kidney development have now been identified (Eccles, 1998; Lipschutz, 1998).

Most studies of kidney induction have focused on the condensation of the metanephric mesenchyme in response
to the invading ureteric bud (Rothenpieler and Dressler, 1993; Dressler, 1995). Although this system is appropriate for the investigation of conversion of mesenchyme to epithelium, it is not particularly useful for studying how the kidney primordia are initially specified, because the nephrogenic mesenchyme is already determined by this developmental stage and lacks only the terminal condensation signals provided by the ureteric bud (Saxén and Sariola, 1987). The vertebrate kidney develops in three major steps via specific tissue interactions (Saxén and Sariola, 1987). The first kidney, the pronephros, is required for further development of the mesonephric and metanephric kidneys (Vise et al., 1997; Carroll et al., 1999a). The pronephros can be analyzed throughout the period of mesodermal patterning and it has the advantage of less complex anatomy, making it an ideal system in which to explore the early stages of intermediate mesodermal patterning (Vise et al., 1997).

Conversion of undifferentiated tissue to committed cell lineages requires the transcriptional activation of structural genes. The Pax genes are paired-box transcription factors expressed in distinct spatiotemporal patterns during embryogenesis (Gruss and Walther, 1992; Eccles, 1998). Pax-2 is important in the differentiation of the definitive or metanephric kidney, is expressed during early embryonic development in the pronephros, eyes, otic vesicles, and neural tube (Dressler et al., 1999; Lechner and Dressler, 1997). Pax-2 expression is tightly regulated during normal human development; overexpression has been observed in Wilms' tumors and renal cell carcinomas (Dressler, 1996). Haploinsufficiency of Pax-2 results in kidney hypoplasia, vesicouretal reflux, and optic nerve dysplasia in the autosomal dominant renal coloboma syndrome (Sanyanusin et al., 1995) and renal agenesis or hypoplasia in Senior–Loken syndrome (Warady et al., 1994). Haploinsufficiency of Pax-2 in three mouse mutants is associated with ear, eye, and brain anomalies as well as renal cystic changes and hypoplasia of varying degrees (Keller et al., 1994; Torres et al., 1995; Favor et al., 1996). In the studies described here, we used Pax-2 expression as a molecular marker for pronephros induction.

Like Pax-2, Lim 1 is expressed in both the tubules and the duct of the early pronephros and persists in the adult Xenopus (Carroll et al., 1999a,b), zebrafish (Carroll et al., 1999a), and murine (Barnes et al., 1994) kidney. Mice lacking functional Lim 1 do not have pro-, meso-, or metanephric (Shawlot and Behringer, 1995). Staining with anti-Lim 1/2 antibody served as a confirmatory marker for pronephros induction.

Another LIM homeobox gene, Lmx-1, is first expressed in the mesonephros (Fernandez et al., 1997). Mutations in Lmx-1B have been described in the nail–patella syndrome, which includes proteinuria and renal dysplasia (Chen et al., 1998; Dreyer et al., 1998).

All three kidney forms arise from embryonic intermediate mesoderm, which lies between the trunk paraxial mesoderm and lateral plate mesoderm in the early embryo.

Little is known about the regional specification of the intermediate mesoderm (Vise et al., 1997). Mesoderm cultured in isolation does not differentiate into specialized tissue, making self-determination unlikely (Slack, 1994). Rather, inducing signals presumably control when and where structures form. A midline signal is required for the upregulation of Pax-2 expression in the developing eye (Macleod et al., 1995). In Xenopus, somitic tissue will form pronephric tubules if explanted and grown away from the influence of the notochord (Yamada, 1940), and anterior somites have recently been identified as the inducing tissue in frog pronephric development (Seufert et al., 1999).

We hypothesized that signals from adjacent tissues are involved in embryonic kidney induction. Using whole-mount in situ hybridization and immunohistochemistry, we monitored Pax-2 and Lmx-1 expression and Lim 1/2 antibody staining during the conversion of intermediate mesoderm into pronephric tissue. We sought to identify the tissue interactions responsible for pronephros induction in the early chick kidney.

**MATERIALS AND METHODS**

**Embryos**

Fertile White Leghorn chicken or Japanese quail (Coturnix japonica) eggs were incubated at 38°C in forced-draft, humidified incubators until embryos had reached the appropriate stage (Hamburger and Hamilton, 1951).

**Microsurgery and Culture**

For most experiments, embryos were placed into Spratt culture (Spratt, 1947), subjected to microsurgery, and incubated overnight. Operations were done aseptically using the tips of cactus needles. Longitudinal cuts were made between the trunk paraxial mesoderm (PM) and the intermediate mesoderm (PM/IM), between the notochord and the paraxial mesoderm (N/PM), or between the intermediate and the lateral plate mesoderm (IM/LP), and the tissues were teased apart. Rostral/caudal (R/C) transsections were made at several somitic levels. Following manipulation, embryos were either fixed immediately for in situ hybridization or cultured in vitro for up to 24 h (Spratt, 1947).

For longer incubations, embryos were cultured in ovo (Criley, 1966). A window was made in the shell and the embryo was lightly stained with 0.1% neutral red. Longitudinal incisions were made using cactus needle tips between the PM and the IM, and the tissues were teased apart. Windows were sealed with a glass coverslip and paraffin, and eggs were incubated for up to 3 days at 38°C in forced-draft, humidified incubators, then fixed and subjected to in situ hybridization.

Putative inducing and responding tissues were isolated and recombined in collagen gels. Quail somites caudal to the fifth somite were excised, along with their overlying ectoderm and 63

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2 Abbreviations used: IM, intermediate mesoderm; LP, lateral plate mesoderm; N, notochord; PM, paraxial mesoderm; R/C, rostral caudal transsection.
underlying endoderm. One-half of the somite explants also contained notochord. Chick lateral mesoderm (i.e., prospective intermediate and lateral plate mesoderm) was isolated from HH4 or HH7 embryos. The endoderm was removed to expose the mesoderm, and the mesoderm was wrapped around the quail somites. These tissues were embedded in a collagen gel, overlaid with FIG. 1. Whole-mount in situ hybridization shows Pax-2 expression in IM destined to become pronephros (arrows). Arrowheads indicate Pax-2 expression in the paraxial mesoderm (fifth somitic level) of a HH stage 8+ embryo. The bottom shows Pax-2 expression in cross section. The Hamburger and Hamilton stage is indicated in the lower left corner of each photo. Abbreviations: N, notochord; NT, neural tube; S, somite; LP, lateral plate; MB/HB, midbrain/hindbrain level of the neural tube; PS, primitive streak; TB, tailbud. Bars: whole mount, 300 μm; section, 100 μm.

FIG. 2. Whole-mount in situ hybridization shows Pax-2 and Lmx-1 expression in mesonephros. The top row shows Pax-2 expression, and the lower row shows Lmx-1. At the right, cross sections show expression in mesonephric tubules (arrows), connecting segments (small arrowheads), and mesonephric ducts (large arrowheads). The Hamburger and Hamilton stage is indicated in the lower left corner of each photo. Abbreviations: N, notochord; NT, neural tube; A, aorta; V, postcardinal vein. Bars: whole mount, 300 μm; section, 100 μm.

FIG. 3. Microsurgical disruption of embryos developing for 24 h in Spratt culture. (Row 1) Longitudinal cuts between PM and IM prior to stage 8 prevented Pax-2 expression. Embryos cut at stage 9 already expressed Pax-2 at the time of manipulation; such expression persisted but did not extend caudally with further incubation. (Row 2) Tissue sections of an embryo cut at HH7 show that Pax-2 expression correlated with the morphologic pronephric anlage. The Hamburger and Hamilton stage at the time of manipulation is indicated in each photo. Abbreviations: N, notochord; NT, neural tube; S, somite; LP, lateral plate. Bars: whole mount, 300 μm; section, 100 μm.
FIG. 4. Lim 1/2 antibody staining shows that pronephros formation requires a medial signal. A longitudinal cut between PM and IM at HH7 prevented pronephros formation. Left: A whole-mount embryo fixed after incubation for 24 h following manipulation. The pronephros (arrows) was stained with Lim 1/2 antibody. Right: Tissue sections show that Lim 1/2 antibody staining correlated with the morphologic pronephric structure in the control, but not the cut side.

FIG. 5. Time course of pronephros formation following manipulation. Pax-2 expression (arrows) was used as a marker for pronephros formation in whole mount and serial sections at time = 4 or 8 h after IM was separated from PM. By t = 8 h, the pronephros was detectable morphologically, as well as by Pax-2 expression, on the control, but not the cut, side.

FIG. 6. Pronephros formation is required for mesonephros development. An embryo was incised longitudinally between PM and IM and cultured for 3 days in ovo. Lmx-1 was expressed in limbs (L) and mesonephric tubules (arrows), but not in mesonephric ducts (arrowhead). Abbreviations: N, notochord; NT, neural tube; H, head; A, aorta. Bars: whole mount, 300 μm; section, 100 μm.
Dulbecco's minimal essential medium supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (Artlinger and Bronner-Fraser, 1993), and cultured for up to 28 h. Cultured chick explants that included presumptive chick PM served as positive controls; negative control cultures comprised only chick lateral mesoderm (absent the endoderm and any prospective PM).

In some experiments Hamburger and Hamilton stage 4 or 5 (HH4, HH5) (Hamburger and Hamilton, 1951) quail node (the rostral 125 μm of the primitive streak) was transplanted isochronically and heterotopically to the chick caudal primitive streak (250–500 or 500–750 μm caudal to the chick node). Chimeric embryos were cultured in vitro for up to 28 h (NEW, 1955) prior to fixation and analysis.

**In Situ Hybridization**

When experiments were terminated, embryos were fixed in 4% buffered paraformaldehyde, dehydrated in graded methanol, and frozen at −20°C. Rehydrated embryos were subjected to whole-mount in situ hybridization with digoxigenin-labeled antisense RNA probes for Pax-2 or Lmx-1 (Nieto et al., 1996). Domingos Henrique provided pDNA containing a 1-kb fragment of chick Pax-2 cDNA obtained by PCR and inserted into the EcoRV site of pKS by TA cloning. It was linearized with EcoRI and transcribed with T3 RNA polymerase in the presence of UTPγS. Sheep anti-digoxigenin antibody conjugated to alkaline phosphatase revealed the probe in pro- and mesonephros (Figs. 1–3, 5, and 7–10). Randy Johnson kindly provided a probe to Lmx-1 that labeled mesonephros and limbs (Figs. 2 and 6). The antisense strand was cut from PBSK using T7 and EcoRI. A probe for paraxial (Fig 10) was obtained from D. Sosic and E. Olson (Garcia-Martinez et al., 1997). Histologic examination of cross sections was performed by sectioning paraffin-embedded embryos previously subjected to whole-mount in situ hybridization.

**Immunohistochemistry**

Whole embryos were fixed in 4% buffered paraformaldehyde and then subjected to immunohistochemistry (Darnell and Schoenwolf, 1995). An epitope in early embryonic quail cells (Figs. 9 and 10) was detected using a monoclonal antibody (QCPN; B. Carlson, University of Michigan, Ann Arbor). For Lim 1/2 detection (Figs. 4 and 10), embryos were blocked with normal goat serum, then stained with a 1:2 dilution of mouse anti-Lim 1/2. The secondary antibody was goat anti-mouse IgG conjugated to peroxidase (Jackson No. 115-035-003), used at a dilution of 1:200. Hybridoma cells secreting anti-Lim 1/2 or anti-quail were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD), and the Department of Biology, University of Iowa (Iowa City), under Contract 1-HD-6-2915 from the National Institute of Child Health and Human Development.

**RESULTS**

**Pax-2, but Not Lmx-1, Is Expressed in Pronephros, whereas Both Are Expressed in Mesonephros**

Previous studies have shown that Pax-2 is expressed in the developing kidney, brain, eye, and ear (Dressler et al., 1990). Figure 1 shows that Pax-2 is a molecular marker for pronephric tissue. In some embryos, Pax-2 was weakly expressed in the intermediate mesoderm at HH7 (Hamburger and Hamilton, 1951) (not shown); its level of expression increased with maturation. By HH9, pronephric Pax-2 expression was limited to intermediate mesoderm caudal to the fifth somite (Fig. 1). In the earlier embryo (HH7 and HH8, Fig. 1), Pax-2 was also transiently expressed in trunk paraxial mesoderm at the level of the fifth somite. Pax-2 expression persisted in the mesonephros, which was also labeled with Lmx-1 (Fig. 2).

In contrast to Pax-2, Lmx-1 (Riddle et al., 1995; Fernandez et al., 1997) was not expressed in the pronephros; rather, its expression was first seen in the mesonephros and limb buds after HH15. Thus, the pronephros was molecularly characterized by expression of Pax-2, but not Lmx-1 (Fig. 1). In contrast, the mesonephros expressed both markers, but their expression patterns differed. Tissue sections of labeled embryos showed Pax-2 expression in the nephric duct, its connecting segment, and the mesonephric tubules, whereas Lmx-1 was expressed only in the tubules (Fig. 2).

**Trunk Paraxial Mesoderm Is Required for Pronephros Formation from Intermediate Mesoderm**

To determine whether the intermediate mesoderm self-differentiates or whether inductive or suppressive signals from adjacent tissues regulate the conversion of intermediate mesoderm to pronephric tissue, we microsurgically dissected embryos to separate putative inducing tissues from intermediate mesoderm. Embryos at several developmental stages were incised along the rostrocaudal axis and the tissues were teased apart. The contralateral uncult side served as the control. Dissected embryos were cultured overnight (Spratt, 1947), fixed, and subjected to in situ hybridization (Nieto et al., 1996).

When embryos were cut between trunk PM and IM prior to HH8, the majority of embryos formed a pronephros from
intermediate mesoderm on the control side only (Fig. 3, Table 1). Pronephros formation was ascertained based on Pax-2 expression and characteristic morphology and location in tissue sections. Embryos cut at HH9 or later already had bilateral Pax-2 expression at the time of manipulation (not shown) that persisted after culture, but the caudal extent of Pax-2 expression was attenuated on the cut side (Fig. 3, row 1). Embryos manipulated at HH8 gave intermediate results; Pax-2 expression on the cut side was undetectable in some embryos, whereas others had attenuated Pax-2 expression in truncated pronephros.

Some embryos were stained with an alternate marker for pronephros formation. PM was separated from IM at HH7 and the embryo was cultured overnight as described above (Spratt, 1947). Figure 4 shows a representative embryo stained with a mouse antibody to Lim 1/2. In 100% of 13 manipulated embryos, Lim 1/2 antibody staining and pronephros development were seen on the control side only. These results are in complete agreement with those obtained with Pax-2 labeling and morphologic analysis of pronephric structure, providing additional support for our conclusion that pronephros formation requires a medial signal.

Quantitative results are summarized in Table 1. In 43 of 45 (96%) embryos manipulated prior to HH8, separation of trunk paraxial mesoderm from intermediate mesoderm prevented pronephros development on the experimental side. Two (4%) embryos had truncated Pax-2 expression on the experimental side, and none had normal expression. In embryos manipulated at HH8, 5 of 12 (42%) embryos had no pronephros formation on the experimental side; a truncated pronephros was seen in 7 (58%). The effect of surgical manipulation on pronephros development decreased with advancing developmental stage, such that 50, 14, and 0% of embryos manipulated at HH9, 9, and GH9+, respectively, had no Pax-2 expression or histologically evident pronephros on the experimental side. However, the pronephros was caudally truncated on the experimental side, even at later stages in which some development was evident at the time of microsurgery.

Because some embryos already expressed Pax-2 at the time of manipulation, we examined the time course of pronephros development in embryos in which the PM was separated from the IM at HH7. Some embryos had weak Pax-2 expression in IM immediately after manipulation, but most were negative, and pronephros formation was not yet morphologically evident (not shown). After incubation for 4 h, weak Pax-2 expression was detected on the unmanipulated control side, but had been lost from the cut side, and there was no morphologic evidence for pronephros formation on the cut side (Fig. 5, row 1). Embryos fixed at 8 h had increased Pax-2 expression in the pronephric anlage of the control side only (Fig. 5, row 2), and by 24 h of culture, Pax-2 was strongly expressed in the pronephros developing in the control, but not the cut, side of the manipulated embryo (Fig. 3, rows 1 and 2). These studies confirmed that proximity to the PM was required for both Pax-2 expression and morphologic development of the pronephros in embryos manipulated at HH7.

Pronephros Induction Is Required for Mesonephros Development

Pronephros formation was required for further kidney development. We separated PM from IM in HH7 embryos and cultured the embryos in ovo for up to 3 days, until they had reached a developmental stage consistent with mesonephros formation (GH11.5). In 5 of 5 embryos, mesonephros developed only on the unmanipulated side, as evidenced by both Lmx-1 expression and morphologic criteria (Fig. 6).

Neither Axial nor Lateral Signals Are Required for Pronephros Induction, and Failure to Form Pronephros after Microsurgery Is Not Due to Blocking the Migration of Prospective IM Cells from the Primitive Streak

To determine whether the inducing signal arose directly from the PM or more medially (e.g., from the notochord or
the floor plate of the neural tube, embryos were dissected parallel to the rostrocaudal axis between the notochord and the trunk PM. Even embryos dissected at HH7 formed normal pronephroi on both the cut and the uncut sides after overnight culture (Fig. 7, row 1). Importantly, these cuts also showed that microsurgery did not block the migration of prospective IM cells from the primitive streak. That is, if the more lateral cuts blocked migration, so should the more medial cuts; these results show clearly that the latter do not. Rather, the pronephros failed to develop following separation of IM from the inducing signals produced by the PM. Separation of IM from LP did not impair or enhance pronephros formation in the IM on the experimental side (Fig. 7, row 2), showing that a more lateral signal from the lateral plate mesoderm, in contrast to the medial signal from the PM, is not required for pronephros induction. Quantitative results are summarized in Table 2.

Neither Endoderm, Ectoderm, nor Rostral Tissues Are Required for Pronephros Induction

To ascertain whether the inducing signal arises in the PM or in its underlying endoderm or overlying ectoderm, each of these tissues was removed prior to culture. To avoid the possibility of regrowth of ectoderm (Obara-Ishihara et al., 1999), the lateral portion of some embryos was also removed prior to Spratt culture (Spratt, 1947). Examination of serial sections demonstrated that regrowth of endoderm and ectoderm did not occur (Fig. 8, rows 1 and 2). Removal of endoderm or ectoderm neither impaired nor enhanced pronephros formation (Fig. 8, rows 1 and 2). Results of embryos dissected between HH6 and HH8 are summarized in Table 2.

To establish the rostral boundaries of the pronephric field, embryos were transected at various somitic levels. In these embryos, Pax-2 expression in the midbrain/hindbrain region of the neural tube served as a positive control. Embryos transected above the level of the fifth somite had bilateral Pax-2 expression only in the caudal isolate (Fig. 8, row 3, and Table 3), whereas embryos transected below the level of the sixth somite at HH8 or later expressed Pax-2 in both the rostral and the caudal isolates (Fig. 8, row 3, and Table 3). Five of ten embryos transected between the fifth and the sixth somites expressed Pax-2 in both the rostral and the caudal isolates. These studies showed that rostral tissues such as head mesoderm are unlikely to be the source of the inducing signal and indicated that there was no rostrocaudal shift over time in the character of either inducing or responding tissue; tissue that had the characteristics found below the fifth somitic level at the time of microsurgery retained those characteristics later and despite microsurgical separation from more rostral tissues.

<table>
<thead>
<tr>
<th>Hamburger and Hamilton stage</th>
<th>Cut between N and PM</th>
<th>Cut between IM and LP</th>
<th>Ectoderm removed</th>
<th>Endoderm removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>2/2 (100%)</td>
<td>—</td>
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<tr>
<td>7</td>
<td>12/12 (100%)</td>
<td>7/7 (100%)</td>
<td>12/12 (100%)</td>
<td>11/11 (100%)</td>
</tr>
<tr>
<td>8</td>
<td>10/10 (100%)</td>
<td>1/1 (100%)</td>
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</table>

Note. Data show the number of expressing embryos/total number of cases, with the percentage expressing Pax-2 on the manipulated side in parentheses.
Paraxial Mesoderm Is Sufficient for Pronephros Induction

Collectively, the experiments described above show that signals from paraxial mesoderm are required for pronephros induction from intermediate mesoderm. To determine whether paraxial mesoderm is also sufficient for pronephros induction, we cocultured the putative inducing and responding tissues in collagen gels (Artinger and Bronner-Fraser, 1993). Caudal somites isolated from HH8 to HH9 quail embryos were recombined with lateral mesoderm (containing prospective IM and LP) isolated from HH4 or HH7 chick embryos, embedded in a collagen gel, and cultured in vitro for up to 28 h. Pax-2 expression served as a marker of pronephros induction. Fixed tissues were also stained with anti-quail antibody to determine the origin of the pronephric tissue (Darnell and Schoenwolf, 1995). A representative specimen (Fig. 9) shows that the inducing PM was quail derived, whereas the responding (i.e., Pax-2-expressing) tissue was of chick origin. Of 25 tissue recombinants, 21 expressed Pax-2 in chick tissue, indicating that the quail somites had induced pronephric anlage in the chick mesoderm. This rate is comparable to that observed in positive controls in which caudal chick PM was left in apposition to IM and cultured (i.e., in isolation from the endoderm and the rostral and more medial tissues) in collagen gel (Table 4). It compares favorably with rates reported by Seufert and co-workers for experiments in which they recombined anterior somites with two stage 9 Xenopus animal caps. In their studies, 7 of 20 samples developed pronephric tubules (Seufert et al., 1999). In contrast, chick IM and LP cultured without PM (negative controls) did not express Pax-2. Quantitative results are shown in Table 4.

<table>
<thead>
<tr>
<th>Hamburger and Hamilton stage</th>
<th>1/2</th>
<th>2/3</th>
<th>3/4</th>
<th>4/5</th>
<th>5/6*</th>
<th>6/7*</th>
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<tr>
<td>C</td>
<td>R</td>
<td>C</td>
<td>R</td>
<td>C</td>
<td>R</td>
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<td>1/1</td>
<td>0/1</td>
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Note. The designation “1/2” indicates transection between the first and the second pairs of somites. Asterisks indicate that the transection was made at the level at which somites were expected to appear, in embryos that had not yet developed that number of somites. For example, a HH8+ embryo has only five somites, but some of the cuts were made more caudally, at the level at which the demarcation between the sixth and the seventh somite was expected to appear. Abbreviations: C, Pax-2 was expressed in the IM in the caudal isolate; R, Pax-2 was expressed in the IM in the rostral isolate.

Transformation of Tissues in Collagen Gels

Tissues cocultured | Pax-2 expressed | No Pax-2 expression
<table>
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<tr>
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<tr>
<td>Negative control:</td>
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<tr>
<td>HH7 chick LP</td>
<td>0/8 (0%)</td>
<td>8/8 (100%)</td>
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<tr>
<td>Positive controls:</td>
<td></td>
<td></td>
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<tr>
<td>HH7 chick PM + LP</td>
<td>9/11 (82%)</td>
<td>2/11 (18%)</td>
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<tr>
<td>HH7 chick N + PM + LP</td>
<td>7/7 (100%)</td>
<td>0/7 (0%)</td>
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<tr>
<td>Experimental:</td>
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<tr>
<td>HH7 chick LP + HH9 quail PM</td>
<td>17/21 (81%)</td>
<td>4/21 (19%)</td>
</tr>
<tr>
<td>HH7 chick LP + HH8 quail PM</td>
<td>2/2 (100%)</td>
<td>0/2 (0%)</td>
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<tr>
<td>HH4 chick LP + HH8 quail PM</td>
<td>2/2 (100%)</td>
<td>0/2 (0%)</td>
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Note. Chick LP includes chick intermediate and lateral mesoderm and associated ectoderm. Quail PM includes overlying ectoderm and underlying endoderm. The Hamburger and Hamilton stage at the time of isolation is noted. At stage 8, the fourth somite and more caudal prospective PM was cultured with chick tissues. At stage 9, only PM caudal to the fifth somite was used. Data show the number of expressing embryos/total number of cases, with the percentage expressing Pax-2 in parentheses.
The Generation of Ectopic Somites Leads to the Development of Ectopic and Enlarged Pronephroi

Figure 10A depicts a fate map of the avian embryo. Hensen’s node gives rise to the notochord, and cells ingress from the more caudal primitive streak to give rise to PM, IM, and LP mesoderm in a rostrocaudal fashion (Schoenwolf et al., 1992). When Hensen’s node from HH4 or HH5 quail embryos was isochronically transplanted into the caudal primitive streak of chick embryos (Fig. 10B), an ectopic notochord self-differentiated, and the node induced ectopic somites (Streit and Stern, 1999; Schoenwolf, unpublished data). Pronephric induction by paraxial mesoderm (somites) varied, depending upon both having adequate responding tissue and the intensity of the inducing signal. In 10 of 13 embryos, the native pronephros was elongated on the side with ectopic somites, but in 3, a supernumerary pronephros was induced (Figs. 10C and 10D). In the embryo shown in Fig. 10C, the pronephros was labeled with Pax-2, and quail tissues were identified by immunohistochemistry. To confirm pronephric induction using another marker, and to better identify the somites, the pronephros in the embryo shown in Fig. 10D was labeled with antibody to Lim 1/2, and the somites were identified by paraxis expression. As shown in Fig. 10C, the notochord and part of one somite are quail derived; the remaining tissues arose from the chick embryo. In both embryos, on the control side (lacking ectopic notochord or somites), the pronephros developed normally in the intermediate mesoderm. On the experimental side, large ectopic somites formed between the native intermediate mesoderm and the lateral plate mesoderm. An additional pronephros developed lateral to the ectopic somite. In addition, between the native and the ectopic somites, competent intermediate mesoderm expressed pronephric markers. Moreover, presumably because it received signals from both the native and the large ectopic somite, the pronephros derived from this intermediate mesoderm was greatly enlarged (Figs. 10C and 10D). This indicates that the inducing signal produced by the paraxial mesoderm acts in a dose-dependent manner.

DISCUSSION

The Pax genes are paired-box transcription factors expressed in different spatiotemporal patterns during embryogenesis (Walther et al., 1991). Pax-2, important in the development of the metanephric kidney, is expressed during early embryonic development in a highly localized pattern in the pronephroi, eyes, otic vesicles, and midbrain/hindbrain level of the neural tube (Dressler et al., 1990; Torres et al., 1995). In our hands, weak Pax-2 expression was detected in the intermediate mesoderm by HH7, and Pax-2 was strongly expressed caudal to the fifth somite by HH9. Pax-2 expression correlated with morphologic evidence for pronephric development in tissue sections and was consistent with classical reports describing development of the pronephros in the intermediate mesoderm caudal to the level of the fifth somite (Abdel-Malek, 1950; Hamilton, 1952). Pax-2 expression persisted in the mesonephros, which was also labeled by Lmx-1 (Fernandez et al., 1997). Therefore, we used Pax-2 as our primary marker of conversion of pluripotent intermediate mesoderm to committed pronephric tissue. Antibody staining for Lim 1/2 was used as a confirmatory pronephric marker, whereas Lmx-1 served as a marker for the mesonephros.

The pronephros, mesonephros, and metanephros all arise from intermediate mesoderm, and similar gene expression patterns in all three kidney forms argue for conservation of developmental pathways and mechanisms of induction (Vise et al., 1997). These similarities are useful; by studying induction and patterning in the pronephros, we also learn about the development of the subsequent kidneys.

The differences between pronephric determination and development of later phases can also be exploited to understand better the molecular mechanisms responsible for kidney induction. Signals emanating from the nephric duct are required for meso- and metanephric kidney induction, whereas the pronephros arises directly from the intermediate mesoderm and gives rise to the aforementioned nephric duct (Vise et al., 1997). If pronephros development involves the same signaling molecules used at later kidney stages, then these molecules must be produced by other embryonic structures (Yates and Pate, 1989; Vise et al., 1997).

The experiments described here ruled out the possibility of self-determination. Lateral embryonic isolates containing intermediate and lateral plate mesoderm failed to develop pronephroi in the absence of a medial signal. The pronephros was absent or truncated when the intermediate mesoderm was separated from the adjacent medial tissues, demonstrating that the inducing signal was produced by the immediately adjacent PM or perhaps by the notochord/neural tube. Normal pronephros formation in the IM left in apposition to the PM but separated from the notochord and floor plate of the neural tube ruled out an axial signal. Seufert and co-workers similarly found that notochord was not required for pronephros induction in Xenopus embryos (Seufert et al., 1999).

We considered the possibility that the potential responding tissue had been ablated by surgical manipulation. The width of the IM labeled by either Pax-2 in situ hybridization or Lim 1/2 antibody staining was 40–50 μm, whereas the tip of the cactus needle used for separation of IM from PM was significantly less than 10 μm in diameter, making extirpation of the entire width of responding tissue impossible. Each cut constituted an incision, not an ablation. Moreover, medial-cutting errors would result in apposition of the IM to the LP mesoderm, whereas lateral-cutting errors would result in apposition of IM to PM, with each serving as a useful control. Because cuts were made from about the first somitic level caudally through the tail end of the embryo, one might reason that ingestion of prospective IM from the primitive streak (García-Martínez et al., 1993) was disrupted by cuts between PM and IM. However, ingestion of pronephric precursor cells likely occurred prior to HH7, and if not, its occurrence would also be
prevented by cuts made between notochord/neural tube and PM, which did not affect pronephros development. Moreover, quail-chick chimeras have shown that adjacent mesoderm cells can migrate to new positions and form new intermediate mesoderm (Garcia-Martinez and Schoenwolf, 1992), making ablation of all potential responding tissue unlikely.

Over 95% of the embryos cut between the PM and the IM before HH8 formed pronephros on the control side only, whereas embryos manipulated after HH9 had pronephros development on both sides, but the length of the pronephros on the cut side was truncated rostrocaudally compared to the control side. The persistence of the pronephros at the rostral end of the cut side in HH9 embryos showed that the inducing signal was only transiently required. However, the caudal extent of pronephros development was truncated on the experimental side, even at later stages in which Pax-2 expression was evident at the time of microsurgery, indicating that the inducing signal was produced by PM mesoderm in a rostral to caudal fashion.

Separation of intermediate mesoderm from LP mesoderm neither impaired nor enhanced pronephros development, indicating that no lateral signal was required for IM induction. We reasoned that the lateral tissues could emit inhibitory signals that restrict or otherwise determine the competence of the IM to respond to the medial-inducing signal, much as noggin inhibits BMP signaling in the medial somite (Marcelle et al., 1997). However, based on the failure of the IM/LP isolates to show expansion of Pax-2 expression, this possibility can be ruled out.

Removal of endoderm did not impair pronephros development, showing that a ventral signal was not required for pronephros induction. Neither did we find evidence that the ectoderm produced a dorsal signal required for pronephros induction. Neither did we find evidence that ventral signal was required for pronephros induction. However, another explanation for the discrepancy between our results and those of Obara-Ishihara and co-workers exists. BMP-4 has been demonstrated to be important in somitogenesis (Pourquié et al., 1996), and the effect of BMP-4 on pronephros induction in the IM may be, therefore, indirectly mediated by trunk paraxial mesoderm.

Rostrocaudal transection experiments showed both that Pax-2 expression occurred only caudal to the level of the prospective fifth somite and that a rostral signal was not required for induction of Pax-2 expression in the IM. This pattern was observed regardless of the developmental stage at the time of microsurgery, indicating that the characteristics of the inducing and responding tissues around level of the prospective fifth somite did not change with time or manipulation.

Recombination experiments showed that quail somites are sufficient for the induction of Pax-2 expression (pronephros formation) in chick intermediate and lateral mesoderm. Our findings are consistent with those of Seufert et al., who reported that UV-ventralized Xenopus embryos lacked somites and subsequently failed to develop pronephroi. These investigators further reported that pronephric tubules, but not ducts, could be induced in embryonic isolates when lateral mesoderm was heterochronically cultured within an ectodermal sandwich that also contained anterior somitic tissue (Seufert et al., 1999). The embryonic kidney of Xenopus is among the simplest of vertebrate kidneys, consisting of a single large nephron with an external glomus (Vise et al., 1997). It is the functional embryonic kidney in fish and amphibia, whereas the mesonephros is the definitive kidney (Brändli, 1999). In contrast, avian kidney development more closely approximates that of mammals, in that both proceed from pronephros through mesonephros and have a metanephros as the definitive kidney. Our findings in the avian pronephros confirm the frog studies, and the evolutionary conservation of signaling stresses the importance of the somite in the patterning of the early embryonic kidney.

When quail node was transplanted into chick caudal primitive streak, ectopic somites were generated. Intermediate mesoderm that received signals from two rows of somites produced an enlarged pronephros. One might argue that an absence of suppressing signals from lateral plate mesoderm explains the enhanced pronephric development, but microsurgical separation of IM from LP did not enhance Pax-2 expression in the pronephros. Rather, these experiments provide the first evidence that the inducing signal produced by the paraxial mesoderm acts in a dose-dependent manner.

In summary, we have shown that rostral tissue, axial or lateral plate mesoderm, endoderm, and ectoderm neither induce nor inhibit pronephric induction. In contrast, interaction with trunk paraxial mesoderm is both required and sufficient for pronephros induction in the chick intermediate mesoderm. Studies are in progress to define the molecular nature of the inducing signals.
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