Kidney Development

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Pax genes encode a family of highly conserved DNA-binding transcription factors. These proteins play key roles in regulating a number of vertebrate and invertebrate developmental processes. Mutations in *Pax*-6 result in eye defects in flies, mice, and humans, and ectopic expression of this gene can trigger the development of ectopic compound eyes in flies. Likewise, mutation of other *Pax* genes in vertebrates results in the failure of specific differentiation programs—*Pax*-1 causes skeletal defects; *Pax*-2, kidney defects; *Pax*-3 or *Pax*-7, neural crest defects; *Pax*-4, pancreatic β-cell defects; *Pax*-5, B-cell defects; *Pax*-8, thyroid defects; and *Pax*-9, tooth defects. Although this class of genes is obviously required for the normal differentiation of a number of distinct organ systems, they have not previously been demonstrated to be capable of directing the embryonic development of organs in vertebrates. In this report, it is demonstrated that *Pax*-8 plays such a role in the establishment of the *Xenopus* embryonic kidney, the pronephros. However, in order to efficiently direct cells to form pronephric kidneys, *XPax*-8 requires cofactors, one of which may be the homeobox transcription factor *Xlim*-1. These two genes are initially expressed in overlapping domains in late gastrulae, and cells expressing both genes will go on to form the kidney. Ectopic expression of either gene alone has a moderate effect on pronephric patterning, while coexpression of *XPax*-8 plus *Xlim*-1 results in the development of embryonic kidneys of up to five times normal complexity and also leads to the development of ectopic pronephric tubules. This effect was synergistic rather than additive. *XPax*-2 can also synergize with *Xlim*-1, but the expression profile of this gene indicates that it normally functions later in pronephric development than does *XPax*-8. Together these data indicate that the interaction between *XPax*-8 and *Xlim*-1 is a key early step in the establishment of the pronephric primordium. © 1999 Academic Press

Key Words: pronephros; pronephric; *Xenopus;* nephric tubule; nephron; nephric duct.

INTRODUCTION

A succession of different kidney types is utilized during the development of vertebrates. The earliest embryonic kidney structures are simple and contain from 1 to 50 nephrons. During subsequent development, the simple kidney is replaced by distinct, complex, adult kidneys with up to one million nephrons (Burns, 1955; Saxén, 1987; Vize *et al.*, 1997). Similar genes are expressed in simple and complex kidneys and the molecular processes by which the different kidneys develop seem to be closely related (Carroll *et al.,* 1999a). The embryonic kidney of the frog, *Xenopus laevis,* is probably one of the most simple vertebrate kidneys, consisting of a single large nephron with an external glomus (for reviews see Fox, 1963; Vize *et al.,*1995, 1997). Given the ability to manipulate gene expression in the developing embryo via mRNA microinjection, *Xenopus* embryos represent an excellent system in which to study the molecular regulation of kidney development.

Pax genes encode a family of transcription factors that play essential roles in the development of a diverse array of organisms (Czerny *et al.*, 1997; Sun *et al.*, 1997). First identified in *Drosophila* segmentation mutants, Pax genes have since been identified in a number of metazoans. Ectopic expression of *Pax*-6 can lead to the formation of ectopic compound eyes in *Drosophila* (Halder *et al.*, 1995) and ectopic lenses in *Xenopus* (Altmann *et al.*, 1997), while ectopic expression of *Pax*-3 can lead to the expression of markers of presumptive muscle in some embryonic cell types (Maroto *et al.,* 1997). In vertebrates, nine Pax genes have been cloned and all are expressed during the develop-

ment of various embryonic tissues. All nine described vertebrate Pax genes have been mutated in mice, either by targeted ablation or through the analysis of naturally occurring lesions. In each case, mutation results in elimination or grossly abnormal development of specific tissues (e.g., Dahl *et al.*, 1997; Peters *et al.*, 1998). Molecular and morphologic analysis has shown that organogenesis in Pax mutants frequently fails during the earliest stages of development. However, despite the wealth of data on mutant phenotypes, the molecular basis of how Pax genes function in the establishment of vertebrate organ primordia remains poorly understood.

Pax genes can be grouped into four subfamilies based on sequence similarity and developmental expression patterns (Noll, 1993). One subfamily, group III, contains the orthologues of the Pax-2, -5, and -8 genes (Balczarek *et al.*, 1997). These three genes encode proteins with a high degree of sequence similarity and considerable overlap in their expression domains. Each gene has been mutated in mice by targeted ablation and each has a severe mutant phenotype. *Pax-2* mutants have defects in the development of the eyes, ears, and kidneys (Torres *et al.*, 1995, 1996), *Pax-5* mutants have defects in the development of B-lymphocytes (Urbanek *et al.*, 1994) and *Pax-8* mutants lack a thyroid gland (Mansouri *et al.*, 1998). Yet, in some cases, tissues expressing a particular Pax gene are not affected by mutation. For example, both *Pax-2* and *Pax-5* are expressed during mouse development at the mid-hindbrain boundary, yet functional ablation of either gene has only a mild effect on the development of this region. However, animals that lack both genes have a strong mutant phenotype, suggesting that *Pax-2* and *Pax-5* play functionally redundant roles in the development of the mid-hindbrain boundary (Schwarz *et al.*, 1997; but also see Favor *et al.*, 1996). A similar case most likely exists for *Pax-2* and *Pax-8* in the development of the metanephric kidney. Both genes are expressed in overlapping patterns in the induced metanephric mesenchyme, yet only the ablation of *Pax-2* affects mammalian kidney development (Torres *et al.*, 1995; Mansouri *et al.*, 1998). It is possible that if *Pax-2* is expressed earlier in the development of the metanephric kidney than *Pax-8*, *Pax-8* no longer plays a critical role in the development of this form of kidney.

In this study, the role of Pax genes in instructing cells to form the embryonic kidney was examined. The *Xenopus* orthologues of *Pax-2* and *Pax-8* were isolated and their expression patterns were determined. These data indicate that while both genes may play roles in pronephric development, *XPax-8* expression is initiated during the earliest stages of embryonic kidney specification, while *XPax-2* is only activated during later pronephric morphogenesis. The expression of ectopic Pax-8 mRNA was found to result in the development of large and ectopic pronephroi. This effect was greatly enhanced by coinjection with mRNA encoding another gene product expressed during the early stages of pronephric patterning, that of the LIM-type homeodomain protein, *Xlim*-1 (Taira *et al.,* 1994a). Coinjection of

these two mRNAs had a synergistic effect and resulted in the formation of greatly enlarged and ectopic kidney structures at a high frequency. As the expression domains of these two genes overlap in late gastrulae in the region that will later form the pronephros, these data indicate that synergism between these two proteins is very likely responsible for the establishment of the pronephric primordia during normal development. The closely related gene *XPax-2* is also able to synergize with *Xlim*-1 in a similar manner, suggesting that *Pax-2* and *Pax-8* are functionally redundant in pronephric development. However, *XPax-2* expression normally commences in tailbud stage embryos, 8 to 9 h after the pronephros is specified (Brennan *et al.,* 1998) and the *XPax-8* and *Xlim*-1 domains are established. Therefore, *Pax-2* is more likely to be involved in *Xenopus* pronephric morphogenesis than in pronephric patterning.

These results establish that *XPax-8* functions as a primary component of the initial response to the inductive signals patterning the pronephric mesoderm. They also indicate a mode of action that may be utilized by other Pax family members; interaction with LIM class homeoproteins.

MATERIALS AND METHODS

Cloning

cDNA from a stage 22 *Xenopus* embryo was amplified using PCR primers designed within a conserved region of the paired boxes of zebrafish *pax(zf-b),* murine *Pax-2* and murine *Pax-8* (primer A, TTGGAATCCA/CGGGTCAGCCATGGCTGTGT; primer B, TTGAATTCAAAGGCTGCTGAACTTTGGT). The major PCR product was isolated, subcloned, and sequenced. This product most closely resembled the paired box of murine Pax-8. In order to screen for full-length clones, the purified product was used to generate a random primed ^{32}P-labeled probe. *Xenopus* adult kidney, stage 28 head, stage 22 whole embryo, and stage 15 whole embryo libraries were screened. Over 60 clones were isolated and 42 were characterized by sequence analysis and restriction digest. Thirty-six represented clones of the *Xenopus* version of *Pax-2* while 6 represented versions of *Pax-8*. Sequence analysis showed that none of the *Pax-8* clones contained full-length versions of the gene. PCR primers were designed to the 5′ and 3′ untranslated region of two of the partial length clones, *XPax-8(42)* and *XPax-8(26),* respectively. A full-length version of Pax-8 was amplified from *Xenopus* stage 22 whole embryo cDNA and subcloned into the expression vector CS 2+ (XMMR). Large stretches of *XPax-2* and *XPax-8* were sequenced on both stands, and open reading frames defined by this analysis have been submitted to GenBank under Accession Numbers AF179300 and AF179301.

In Situ Hybridization

Single-probe *in situ* hybridization was performed according to Harland (1991), and double-probe hybridizations were performed according to Knecht *et al.* (1996). Antisense digoxygenin (DIG) or fluorescein-labeled RNA probes were transcribed from DNA from the longest Pax-8 clone isolated, *XPax-8(26),* and from an *Xlim*-1 clone (Taira *et al.*, 1994b) and a 3.5-kb *XPax-2* clone, *XPax-2(10)*.

DNA was linearized using *Sac*II, *Sac*I, and *Xba*I for *XPax*-8, *Xlim*-1, and *XPax*-2, respectively. The linearized DNA was transcribed with T3 (*Pax*-8) or T7 (*Xlim*-1 and *Pax*-2) RNA polymerase to generate antisense transcripts. A developmental series of heterozygous albino embryos ranging in stage from mid-blastula to late tadpole stages were hybridized with the labeled probes. The color reaction was performed using NBT/BCIP (dark blue) in the case of single probes or BCIP (light blue) and magenta phos (magenta) for double hybridizations.

mRNA Injection

For mRNA production, the open reading frames of *XPax*-8 and *XPax*-2 were subcloned into CS2+ and pSP64TS (XMMR). *Xlim*-1, *Xlim*-1 3M, chordin, and β-galactosidase cDNAs had all previously been cloned into 64TS or its precursor, pSP64T, by others (Taira *et al.*, 1994b; Sasai *et al.*, 1994; Vize *et al.*, 1991). Sense, capped mRNAs were transcribed from linearized DNA of *Xlim*-1, *Xlim* 3M, *XPax*-2, *XPax*-8, chordin, and β-galactosidase plasmids using SP6 RNA polymerase and a cap analog (Krieg and Melton, 1984). RNA was purified on G-50 Sephadex columns and recovered material was quantitated by estimating the incorporation of ^{32}P-labeled UTP. For chordin, 100 pg of mRNA was injected as higher doses caused axial defects. For *Xlim*-1, *Xlim*-1 3M, *XPax*-8, and *XPax*-2, 500 pg of test mRNA plus 125 pg of β-galactosidase mRNA was injected for single injections and a total of 500 pg of test mRNA plus 125 pg of β-galactosidase mRNA for coinjections. β-Galactosidase mRNA control injections contained 500 pg. In order to evaluate if the test mRNAs were translated with equal efficiencies, mRNA samples were translated *in vitro* in the presence of [^{35}S]methionine and the labeled proteins were run on 10% polyacrylamide SDS gels and autoradiographed. *XPax*-8 and *Xlim*-1 were translated at equivalent levels, so coinjections contained 250 pg of each mRNA. *XPax*-2 was translated approximately fourfold less efficiently than *Xlim*-1 (not shown), so coinjections of these two mRNAs contained 100 pg of *Xlim*-1 and 400 pg of *XPax*-2. Embryos were injected into the C tier of 16- to 32-cell stage *Xenopus* embryos (Dale and Slack, 1987). Embryo staging was performed according to Nieuwkoop and Faber (1994).

Immunohistochemistry

Injected embryos were fixed at late tadpole stages for 10 min in MEMFA (Harland, 1991), assayed for β-galactosidase activity (Vize *et al.,* 1991), refixed for 20 min, and then probed with the pronephric tubule-specific antibody 3G8, duct-specific antibody 4A6 (Vize *et al.,* 1995), or the somite-specific antibody 12/101 (Kintner and Brockes, 1984). These antibodies are very useful for identifying components on the pronephros as 3G8 stains only the pronephric tubules (and the otic vesicle more weakly) and 4A6 stains only the pronephric duct. Other available markers expressed in the pronephros are also present in a variety of other tissues, making identification on the basis of whole-mount immunohistochemistry alone more difficult. As the epitopes for the different antibodies are expressed at different stages during development, embryos were fixed at either stage 36/37 (3G8) or stage 40 (4A6). In cases where embryos were double stained, a combination of either NBT/BCIP (dark blue) and BCIP (light blue) or NBT/BCIP and Histomark red (Kirkgaard and Perry Laboratories) was used. Embryos were postfixed in Bouins and either immediately analyzed or embedded in paraffin and sectioned.

Histology

Stained embryos were dehydrated then rehydrated in a methanol series. Embryos were then fixed overnight in Bouins fixative. Fixed embryos were dehydrated in ethanol and then washed in xylene twice for 10 min each. The xylene was replaced with 50% xylene/50% paraplast for 10 min, and embryos were subsequently washed in 100% paraplast at 60°C three times, once for 10 min, once for 30 min, and finally overnight. The following morning, the embryos were embedded and then sectioned at 10 μm.

RESULTS

Isolation of XPax-8

A combination of library screening and RT-PCR methods was used to isolate the full-length version of *Xenopus Pax*-8, *XPax*-8. The *Xenopus* Pax-8 protein is 465 amino acids long (Fig. 1) and has extensive homology to other vertebrate Pax genes of the 2/5/8 subfamily. It is 72, 59, and 52% identical to murine Pax-8, zebrafish Pax-8, and *Xenopus* Pax-2, respectively. Within the paired domain, the highly conserved DNA-binding region, it shows 98, 95, and 94% identity, respectively. Many Pax proteins contain a highly conserved octapeptide downstream of the paired domain that has been implicated in protein–protein interactions. The Pax-8 octapeptide, YSISGLLG, is identical to that of murine Pax-5 and differs by only one amino acid from the murine and zebrafish Pax-8 octapeptide, YSINGLLG (Fig. 1).

Several full-length cDNA clones containing *Xenopus Pax*-2 sequences were also isolated in this screen (Fig. 1). The clones correspond to several of the alternatively spliced versions of *XPax*-2 predicted by Heller and Brandli (1997). As the sequence and some aspects of the expression pattern of *XPax*-2 have been previously described, we will limit our discussion of this gene to its developmental function (see below).

Expression of Pax-8 and Xlim-1 in Normal Xenopus Development

Whole-mount *in situ* hybridization was used to determine the expression pattern of *XPax*-8 during early *Xenopus* development. These experiments reveal that *XPax*-8 expression commences at late gastrula stages in two distinct populations of cells, the otic vesicle and pronephric precursors. Expression is maintained in these two structures through late tailbud stages. In contrast to mouse and zebrafish, *XPax*-8 is not expressed at the mid-hindbrain boundary. Furthermore, *XPax*-8 is not expressed in the thyroid gland, a tissue that requires Pax-8 in mice (Mansouri *et al.*, 1998).

The timing of *XPax*-8 transcriptional activation in the pronephric primordia overlaps with the time at which explants of pronephric mesoderm can be removed from embryos and differentiate into pronephric tubules when cultured in isolation (Brennan *et al.,* 1998). The observed
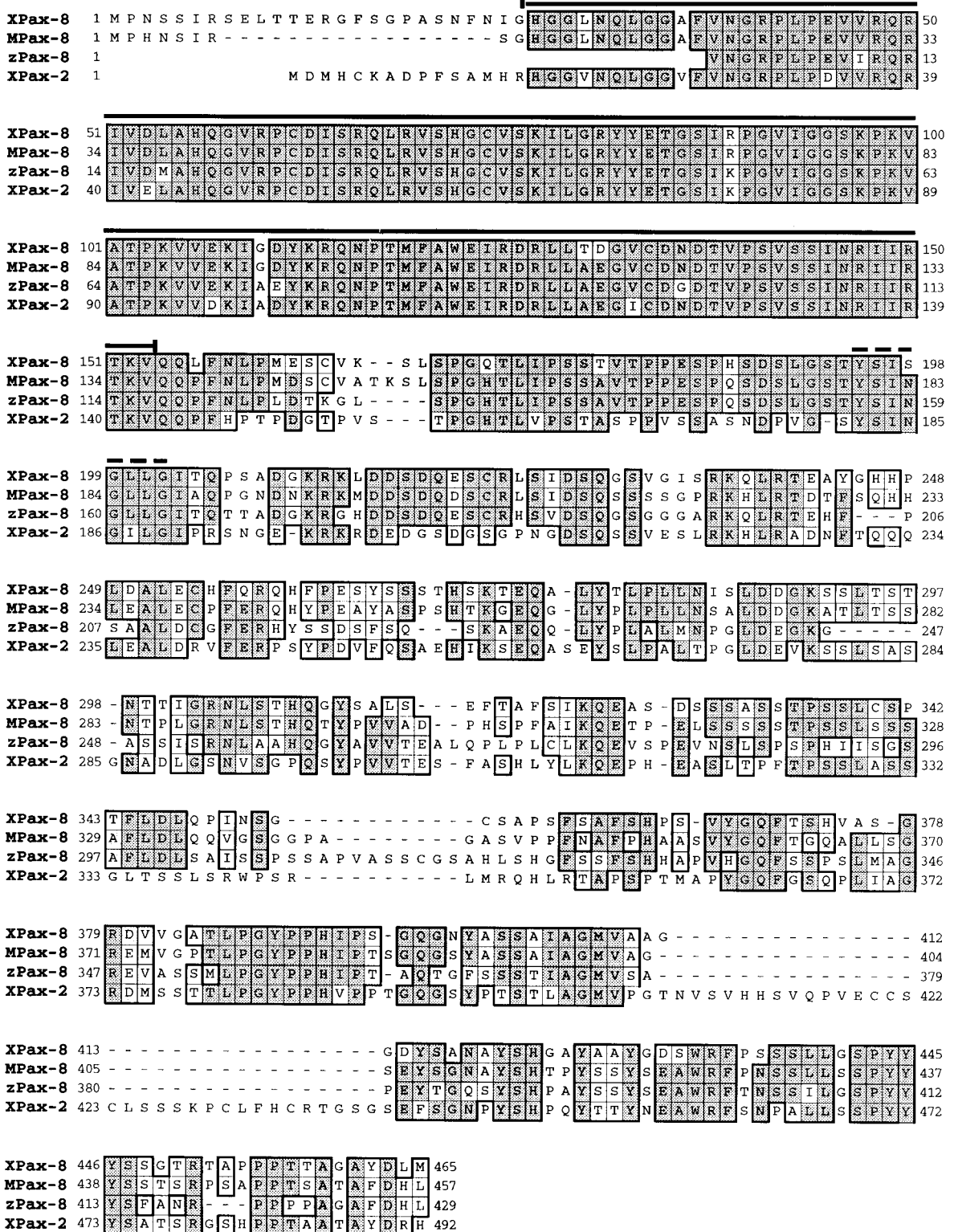
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XPax-8    1 M P N S S I R S E L T T E R G F S G P A S N F N I G H G G L N Q L G G A F V N G R P L P E V V R Q R  50
MPax-8    1 M P H N S I R - - - - - - - - - - - - - - - - - S G H G G L N Q L G G A F V N G R P L P E V V R Q R  33
zPax-8    1                                                         V N G R P L P E V I R Q R  13
XPax-2    1           M D M H C K A D P F S A M H R H G G V N Q L G G V F V N G R P L P D V V R Q R  39

XPax-8   51 I V D L A H Q G V R P C D I S R Q L R V S H G C V S K I L G R Y Y E T G S I R P G V I G G S K P K V 100
MPax-8   34 I V D L A H Q G V R P C D I S R Q L R V S H G C V S K I L G R Y Y E T G S I R P G V I G G S K P K V  83
zPax-8   14 I V D M A H Q G V R P C D I S R Q L R V S H G C V S K I L G R Y Y E T G S I K P G V I G G S K P K V  63
XPax-2   40 I V E L A H Q G V R P C D I S R Q L R V S H G C V S K I L G R Y Y E T G S I K P G V I G G S K P K V  89

XPax-8  101 A T P K V V E K I G D Y K R Q N P T M F A W E I R D R L L T D G V C D N D T V P S V S S I N R I I R 150
MPax-8   84 A T P K V V E K I G D Y K R Q N P T M F A W E I R D R L L A E G V C D N D T V P S V S S I N R I I R 133
zPax-8   64 A T P K V V E K I A E Y K R Q N P T M F A W E I R D R L L A E G V C D G D T V P S V S S I N R I I R 113
XPax-2   90 A T P K V V D K I A D Y K R Q N P T M F A W E I R D R L L A E G I C D N D T V P S V S S I N R I I R 139

XPax-8  151 T K V Q Q L F N L P M E S C V K - - S L S P G Q T L I P S S T V T P P E S P H S D S L G S T Y S I S 198
MPax-8  134 T K V Q Q F N L P M D S C V A T K S L S P G H T L I P S S A V T P P E S P Q S D S L G S T Y S I N 183
zPax-8  114 T K V Q Q F N L P L D T K G L - - - - S P G H T L I P S S A V T P P E S P Q S D S L G S T Y S I N 159
XPax-2  140 T K V Q Q F H P T P D G T P V S - - - T P G H T L V P S T A S P P V S S A S N D P V G - S Y S I N 185

XPax-8  199 G L L G I T Q P S A D G K R K L D D S D Q E S C R L S I D S Q G S V G I S R K Q L R T E A Y G H H P 248
MPax-8  184 G L L G I A Q P G N D N K R K M D D S D Q D S C R L S I D S Q S S S G P R K H L R T D T F S Q H H 233
zPax-8  160 G L L G I T Q T T A D G K R G H D D S D Q E S C R H S V D S Q G S G G G A R K Q L R T E H F - - - P 206
XPax-2  186 G I L G I P R S N G E - K R K R D E D G S D G S G P N G D S Q S S V E S L R K H L R A D N F T Q Q Q 234

XPax-8  249 L D A L E C H F Q R Q H F P E S Y S S T H S K T E Q A - L Y T L P L L N I S L D D G K S S L T S T 297
MPax-8  234 L E A L E C P F E R Q H Y P E A Y A S P S H T K G E Q G - L Y P L P L L N S A L D D G K A T L T S S 282
zPax-8  207 S A A L D C G F E R H Y S S D S F S Q - - S K A E Q Q - L Y P L A L M N P G L D E G K G - - - - - 247
XPax-2  235 L E A L D R V F E R P S Y P D V F Q S A E H I K S E Q A S E Y S L P A L T P G L D E V K S S L S A S 284

XPax-8  298 - N T T I G R N L S T H Q G Y S A L S - - - E F T A F S I K Q E A S - D S S S A S S T P S S L C S P 342
MPax-8  283 - N T P L G R N L S T H Q T Y P V V A D - - P H S P F A I K Q E T P - E L S S S S S T P S S L S S S 328
zPax-8  248 - A S S I S R N L A A H Q G Y A V V T E A L Q P L P L C L K Q E V S P E V N S L S P S P H I I S G S 296
XPax-2  285 G N A D L G S N V S G P Q S Y P V V T E S - F A S H L Y L K Q E P H - E A S L T P F T P S S L A S S 332

XPax-8  343 T F L D L Q P I N S G - - - - - - - - - - - - C S A P S F S A F S H P S - V Y G Q F T S H V A S - G 378
MPax-8  329 A F L D L Q Q V G S G G P A - - - - - - - G A S V P P F N A F P H A A S V Y G Q F T G Q A L L S G 370
zPax-8  297 A F L D L S A I S S P S S A P V A S S C G S A H L S H G F S S F S H H A P V H G Q F S S P S L M A G 346
XPax-2  333 G L T S S L S R W P S R - - - - - - - - - L M R Q H L R T A P S P T M A P Y G Q F G S Q P L I A G 372

XPax-8  379 R D V V G A T L P G Y P P H I P S - G Q G N Y A S S A I A G M V A A G - - - - - - - - - - - - - - - 412
MPax-8  371 R E M V G P T L P G Y P P H I P T S G Q G S Y A S S A I A G M V A G - - - - - - - - - - - - - - - 404
zPax-8  347 R E V A S S M L P G Y P P H I P T - A Q T G F S S S T I A G M V S A - - - - - - - - - - - - - - - 379
XPax-2  373 R D M S S T T L P G Y P P H V P P T G Q G S Y P T S T L A G M V P G T N V S V H H S V Q P V E C C S 422

XPax-8  413 - - - - - - - - - - - - - - - - - G D Y S A N A Y S H G A Y A A Y G D S W R F P S S S L L G S P Y Y 445
MPax-8  405 - - - - - - - - - - - - - - - - - S E Y S G N A Y S H T P Y S S Y S E A W R F P N S S L L S S P Y Y 437
zPax-8  380 - - - - - - - - - - - - - - - - P E Y T G Q S V S H P A Y S S Y S E A W R F T N S S I L G S P Y Y 412
XPax-2  423 C L S S S K P C L F H C R T G S G S E F S G N P Y S H P Q Y T T Y N E A W R F S N P A L L S S P Y Y 472

XPax-8  446 Y S S G T R T A P P P T T A G A Y D L M 465
MPax-8  438 Y S S T S R P S A P P T S A T A F D H L 457
zPax-8  413 Y S F A N R - - - P P P P A G A F D H L 429
XPax-2  473 Y S A T S R G S H P P T A A T A Y D R H 492
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FIG. 1. Sequence of *Xenopus* Pax-8. The predicted protein sequence of *Xenopus* (X) Pax-8 is compared to that of its vertebrate relatives, murine (M) and zebrafish (z) Pax-8 and *Xenopus* Pax-2. A solid bar indicates the Paired box, and a dashed line, the octapeptide. The DNA sequence of the *XPax*-8 and *XPax*-2 can be accessed in GenBank using Accession Numbers CAF179300 and AF179301.

expression of *XPax*-8 in the presumptive pronephric region differs in a number of ways from that of *Xlim*-1, the previously described early pronephric marker (Taira *et al.,* 1994a). In order to compare and contrast the distinct expression patterns of these two genes a parallel series of single and double staining was performed with *XPax*-8 and *Xlim*-1 mRNA probes. The results of this comparison are shown in Fig. 2. By late gastrula, stage 12.5, expression of both *XPax*-8 and *Xlim*-1 can be detected in pronephric precursors (images available on the WWW, see XMMR). At stage 14, *XPax*-8 expression in the embryonic kidney is a large round patch ventral to the presumptive anterior somites. In contrast to *Xlim*-1, the *XPax*-8 expression domain does not extend into the ventral lateral plate mesoderm (Fig. 2). Although these two genes seem to be activated within the intermediate mesoderm at the same time, *XPax*-8 is only expressed in a dorsal subset of cells that express *Xlim*-1, implying that the transcription of the two genes is controlled independently. Over the next few hours, the *XPax*-8 expression pattern refines to a teardrop shape that corresponds to the future pronephric primordium (Fig. 2; Yamada, 1937; Pasteels, 1942). Over the same time period, expression of *Xlim*-1 in mesoderm ventral to the future pronephros weakens, and the *Xlim*-1 pattern gradually refines to correspond very closely to that of *XPax*-8. By stage 23, the pronephric expression patterns of the two genes are indistinguishable (Fig. 2).

Ectopic XPax-8 or Xlim-1 Expression Leads to the Development of Enlarged Pronephroi

Given the temporal and spatial expression patterns of *XPax*-8 and *Xlim*-1 in the region fated to form the pronephros, it is possible that either or both of these genes are involved in the commitment of cells to a pronephric fate. In order to test for a functional role in kidney development, 500 pg of mRNA encoding either *XPax*-8 or *Xlim*-1 was injected into different regions of the marginal zone of 16- to 32-cell-stage *Xenopus* embryos. A quantity of 125 pg of mRNA encoding β-galactosidase was coinjected as a lineage tracer to follow the fate of the injected cell. Cells injected included the C-2, C-3, and C-4 blastomeres of 32-cell-stage embryos (nomenclature of Dale and Slack, 1987), which correspond to cells fated to form anterior somites and heart (C-2), more posterior somites, pronephroi and lateral plate (C-3), and lateral plate, ventral mesoderm, and posterior somites (C-4). Injected embryos were raised to stage 36 and then fixed and processed to visualize pronephric tubules using the monoclonal antibody 3G8 and the distribution of the lineage tracer using a histochemical substrate for β-galactosidase. Only embryos that were phenotypically normal were scored for effects on pronephric development.

Normal stage 35–37 *Xenopus* pronephric tubules always have three dorsal branches linked to a more ventral common tubule (Wallingford *et al.,* 1998). Ectopic expression of either *XPax*-8 or *Xlim*-1 alone resulted in the development of pronephric tubules that were often larger in size than
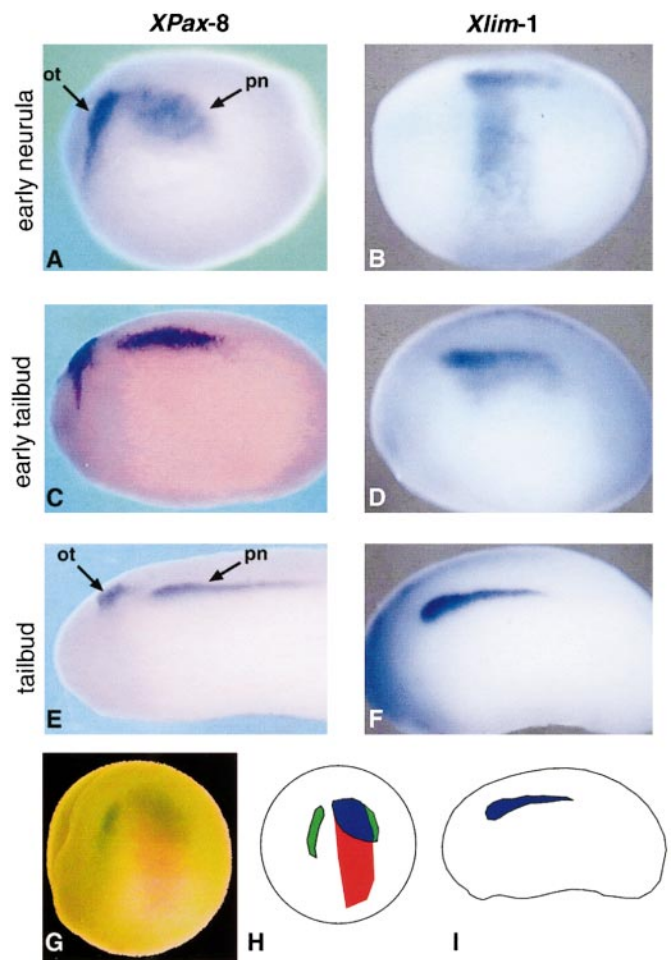
FIG. 2. *XPax*-8 and *Xlim*-1 expression in the presumptive embryonic kidney. Expression of *Pax*-8 as revealed by whole-mount *in situ* hybridization of *Xenopus* embryos of various developmental stages (samples on the left). *Xlim*-1 (Taira *et al.,* 1994a) expression at similar stages is illustrated on the right. The two distinct patterns of pronephric expression converge until they are indistinguishable by stage 25. A, stage 14, *XPax*-8. B, stage 14, *Xlim*-1. C, stage 20, *XPax*-8. D, stage 20, *Xlim*-1. E, stage 24, *XPax*-8. F, stage 23, *Xlim*-1. G, double staining of stage 16 embryo with *XPax*-8 in dark brown and *Xlim*-1 in pink/red. Computer-generated false coloring was used to enhance the difference between the two immunohistochemical substrates. H, graphical interpretation of data shown in G. Green represents *XPax*-8 only expression domains in the presumptive otic vesicle and possibly in the posterior pronephric region. Red represents the *Xlim*-1 only domain in the ventral mesoderm belt. Blue represents the area of overlap. I, graphical representation of data in E and F; both *XPax*-8 and *Xlim*-1 are expressed in overlapping domains throughout the presumptive pronephros (blue). ot, otic vesicle; pn, pronephros. Anterior is to the left, and dorsal up, in all samples.

control pronephroi (Table 1). Pronephroi were classified as enlarged if they contained more than three dorsal branches. The increase in the number of branches was not at the
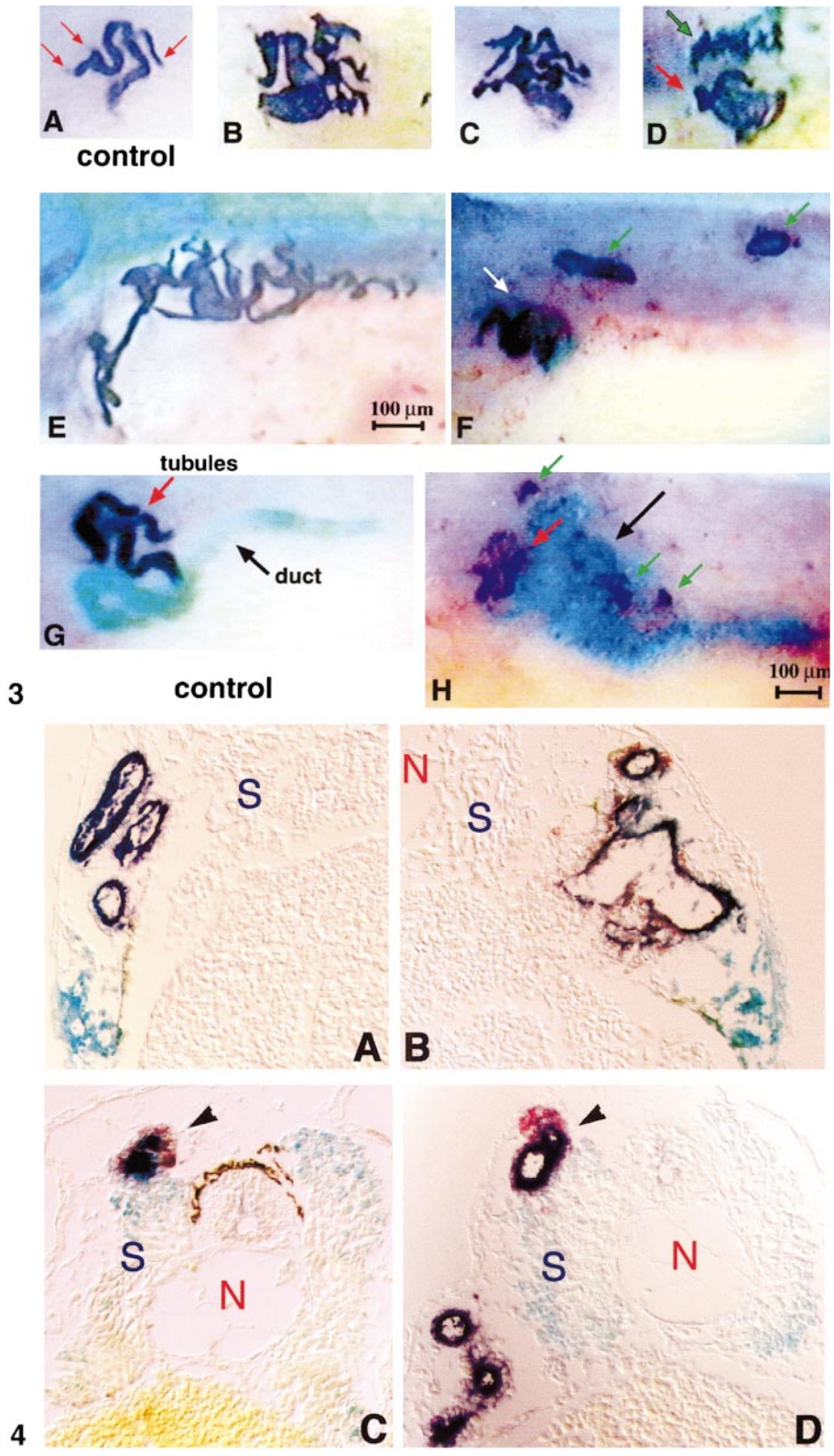
control

tubules

duct

3   control

4

TABLE 1

Frequency of Enlarged or Ectopic Pronephric Tubules
in mRNA-Injected Embryos

	% Enlarged tubules	% Ectopic tubules	% No effect (or smaller)	N
XPax-8	7	23	70	61
Xlim-1	15	19	66	106
Xlim-1 3M	10	14	76	184
XPax-8 + Xlim-1	19	47	17	85
XPax-8 + Xlim-1 3M	27	37	36	83
Chordin	0.6	6	93	177
β-Galactosidase	0	0	100	66

Note. The injected mRNA(s) are listed on the left. 500 pg of
mRNA (total) was injected in each case except chordin, where 100
pg was injected.

expense of the size of the pronephric tubules, as the area
occupied by the pronephric tubules also increased (dis-
cussed below). Injected embryos also developed small, ec-
topic 3G8-positive structures within somites or intermedi-
ate mesoderm (Table 1). The fact that either *Xlim*-1 or
XPax-8 can lead to enlarged and ectopic pronephroi sug-
gested that these two genes may both play significant roles
in pronephric development. However, many cells that re-
ceived the injected mRNA, including some in the vicinity
of the normal pronephros, failed to develop into pronephric
tissue and the large pronephric phenotype was only ob-

served in 7 to 15% of samples. Two possibilities could
explain the lack of penetrance of the individual mRNAs.
First, greater levels of the protein activity may be required
to achieve pronephric patterning, or second, additional
cofactors may be required for function. As the amount of
protein produced from injected mRNAs is probably already
much higher than normal *in vivo* levels, it seems more
likely that the lack of cofactors is the limiting factor. Based
on their expression patterns, it is possible that cooperation
between *XPax*-8 and *Xlim*-1 is required to achieve proneph-
ric specification. In order to test this possibility coinjec-
tions of *XPax*-8 plus *Xlim*-1 were performed.

XPax-8 and Xlim-1 Synergize to Pattern the Pronephric Mesoderm

A total of 250 pg of mRNA encoding each protein was
coinjected into *Xenopus* embryos. In order to distinguish
synergistic versus additive effects, the total amount of
mRNA coinjected was the same as in single injections (500
pg), that is, one-half of the amount of each mRNA used in
the above experiments. Reducing the amount of *XPax*-8 or
Xlim-1 injected normally reduces the frequency with which
enlarged pronephroi are observed (not shown). Injected
embryos were raised to stage 36 and then processed to
detect β-galactosidase activity histochemically and pro-
nephric tubules by immunohistochemistry using 3G8. Tad-
poles derived from embryos injected with *XPax*-8 plus
Xlim-1 mRNA were found to have a much higher frequency
of grossly enlarged and ectopic pronephroi than do embryos
injected with 500 pg of either mRNA alone (Fig. 3, Table 1).

FIG. 3. Ectopic expression of *Pax*-8 plus *Xlim*-1 leads to the development of abnormally large pronephroi and ectopic pronephroi. All
samples were stained with antibody 3G8. A–E reproduced at same magnification, as are G and H (100-μm scale bars are in E and H). Anterior
is to the left, dorsal is up. (A) Normal stage 36 pronephric tubules. Red arrows indicate the three normal dorsal branches. Faintly stained
nephrostomes can be seen extending from the two left branches. (B–E, G) Enlarged pronephroi in *XPax*-8 plus *Xlim*-1- (1:1 ratio) injected
embryos. In B the tubules to the left are slightly distended, probably due to osmotic pressure, and this thickness probably represents
distortion rather than enlargement. However, the right side of this same pronephros contains many additional tubule branches that are all
of normal thickness. In C–E, all tubules are of normal width. In D the pronephros (red arrow) is of only slightly greater than normal size,
but it is adjacent to an ectopic pronephros (green arrow) which is almost as large. In F, two additional ectopic pronephroi (green arrows) are
obvious, dorsal and posterior to the normal position of the organ (white arrow). (G) Control stage 39 embryo stained for pronephric tubules
using 3G8 and a dark blue substrate and pronephric duct using 4A6 and a light blue substrate. Note that G and H are at a later stage of
development than A–F, and the scale is also slightly different. (H) *XPax*-8 plus *Xlim*-1-injected embryo, stage 39, stained as in G. Note the
enlarged region of nonmigratory duct staining (light blue) in the vicinity of the pronephric tubules and also the presence of small ectopic
pronephric tubules (dark blue stain, green arrows). Anterior is to the left, and dorsal is up in all panels.

FIG. 4. Histological analysis of enlarged and ectopic pronephroi. Dorsal is up in all samples. (A) Control. Transverse section through a
normal pronephros, pronephric tubules stained with 3G8 in dark blue and pronephric duct stained with 4A6 in light blue. (B) Enlarged
pronephros. The pronephros on the injected side of the same embryo as shown in A. This sample developed from an embryo injected with
XPax-8, *Xlim*-1, and β-galactosidase mRNA. The lineage tracer was developed with a red substrate and can be observed in the epithelia of
the enlarged pronephric tubules. Once again, tubules are stained dark blue, and duct stained light blue. The red tracer stain can be clearly
visualized in tubules due to the restriction of the 3G8 epitope to the apical surface of tubule epithelia. (C) Ectopic pronephric tubule. The
ectopic tubules (arrowhead) are stained with 3G8 and a dark blue substrate and contain the β-galactosidase lineage tracer (red). Somites were
stained with 12/101 and a light blue substrate. Note the reduction in size of the somites on the left side below the ectopic tubules. (D)
Ectopic pronephric tubule. A sample processed in the same manner as that shown in C. Both normal (β-galactosidase negative) and ectopic
(β-galactosidase positive, arrowhead) pronephric tubules are visible on the left side. Once again, the somites are smaller on the side with
the ectopic pronephric tubules. S, somite; N, notochord.

Control injections with 500 pg of β-galactosidase mRNA never resulted in such enlarged pronephroi (Table 1), nor has the ectopic expression of dozens of other mRNAs tested in our laboratory (not shown).

The enlarged pronephric tubules observed in coinjected embryos have up to five times the number of branches observed in control pronephroi and are up to 3.6-fold larger in area than normal tubules (see below), a phenotype never observed in singly injected embryos. In some cases, the entire intermediate mesoderm seems to be converted into pronephric material (Fig. 3). Coinjected embryos developed pronephroi with up to 16 branches rather than the normal 3 (Fig. 3). When the size of the pronephric tubules was quantified using NIH Image software, the tubules in Fig. 3E were found to occupy 3.6 times the area occupied by control tubules, and those shown in Fig. 3B occupy 2.2 times the area of the control. This is probably an underestimation of the increase in size of the organs, as these measurements were made on two-dimensional as opposed to three-dimensional images. The difference in size is significant (χ^2 test, $P > 0.05$), and control experiments indicate that there was no statistical difference between the injected and uninjected side of β-galactosidase-injected embryos.

The pronephric tubules are linked to the pronephric duct, which develops from the ventroposterior portion of the pronephric anlage (Vize *et al.*, 1995). Both *XPax-8* and *Xlim-1* are expressed in this region in early neurulae (Fig. 2). In order to test if additional pronephric duct tissue was also formed in response to *XPax-8* plus *Xlim-1*, coinjected embryos were raised to stage 39/40 when the duct acquires the antigen detected by the antibody 4A6. Embryos were double stained using both 3G8 and 4A6 (see Materials and Methods). As Figure 3 illustrates, a number of *XPax-8* plus *Xlim-1*-injected embryos indeed contain additional pronephric duct tissue. Ectopic duct was also sometimes observed in the absence of any ectopic tubules. Forty-two percent of *XPax-8* plus *Xlim-1*-injected embryos were found to contain additional duct tissue. Forty-three percent of embryos with additional duct tissue have no additional 3G8-positive pronephric tubules. If the duct phenotype is added to the ectopic tubule phenotype, the overall frequency of ectopic pronephric tissue in coinjected embryos is therefore raised to 85%. Other than the effect on pronephric tubules and ducts the only other responses to coinjection of *XPax-8* plus *Xlim-1* were mild defects in the surrounding somites. Such defects can be visualized when injected embryos are stained for both pronephric and somitic structures. The somites adjacent to enlarged pronephroi sometimes fail to stain with antibody 12/101 and are sometimes smaller than control somites. The importance of this observation will be discussed below.

The larger than normal pronephroi are not caused by tubule distention because of a blockage of pronephric function. In normal frog pronephroi, the connecting tubules are linked to the coelom via ciliated nephrostomes (Vize *et al.*, 1997). When pronephric tubules are not functional or when they are blocked, osmotic pressure causes tubule distention

giving them a misleadingly enlarged appearance (Howland, 1921). The giant pronephroi observed in injected embryos resemble normal pronephric tubules despite their increased size. Immunostaining (Fig. 3) and histological sections (Fig. 4) of embryos coinjected with *XPax-8* and *Xlim-1* showed that the tubules are of normal thickness indicating that these giant pronephroi are not caused by osmotic distention. The enlarged pronephroi are quite clearly due to the development of a larger organ.

Coinjection of 250 pg of each mRNA produced a higher frequency of enlarged kidneys than did 500 pg of either mRNA alone (Table 1), indicating synergy between *Xlim-1* and *XPax-8*. Once again, ectopic pronephroi were also observed and at a much higher frequency than in single injection experiments, but such ectopic tubules were only observed within the somites and intermediate mesoderm (Fig. 3 and data not shown). Histological examination of stained samples indicated that the ectopic 3G8-positive structures are epithelial and indeed appear to be pronephric tubules (Fig. 4). The 3G8 staining pattern of pronephric tubules is distinct from that of otic vesicle in that pronephric staining is localized to the lumen of the tubule (see Vize *et al.*, 1995). The ectopic structures formed in injected embryos have a staining pattern that is characteristic of pronephric tubules and not of otic vesicle (Fig. 4). In addition, independent markers of pronephric tissue, *XPax-2* and *Xwnt-4* (McGrew *et al.*, 1992; Carroll *et al.*, 1999b), were also observed to be ectopically activated in response to injected *XPax-8* plus *Xlim-1* (not shown), providing further evidence that these structures are indeed ectopic pronephric tubules. Many of these ectopic pronephric tubules were completely surrounded by somitic tissue and were separated from the normal position of the pronephros by four somites or more, indicating that they represent completely distinct structures that have formed in response to the ectopic mRNAs present in presumptive somitic cells (Fig. 4). Ectopic pronephroi always contain the coinjected lineage tracer, indicating that they form in response to the injected mRNAs rather than due to indirect induction (see below).

In order to determine if XPax-8 contributes to the development of enlarged pronephroi by enhancing the activity of Xlim-1 in a manner analogous to LIM-binding proteins such as LDB-1 (Agulnick *et al.*, 1996), the activity of a mutant activated form of *Xlim-1*, *Xlim-1* 3M, (Taira *et al.*, 1994b) was tested in this assay. This form of *Xlim-1* contains key amino acid substitutions in the negative regulatory LIM domain. *Xlim-1* 3M mRNA was injected into embryos and the kidney phenotype was scored in a manner similar to that described above. The frequency of the pronephric phenotype in embryos injected with *Xlim-1* 3M was slightly lower than that observed with wild-type *Xlim-1* (Table 1). As the activated form of Xlim-1 has no greater activity than the native form, XPax-8 does not appear to function by enhancement of Xlim-1 activity.

Xlim-1 3M was also coinjected with *XPax-8* in order to determine whether the activated form could further increase the synergism with *XPax-8* or overcome the restric-
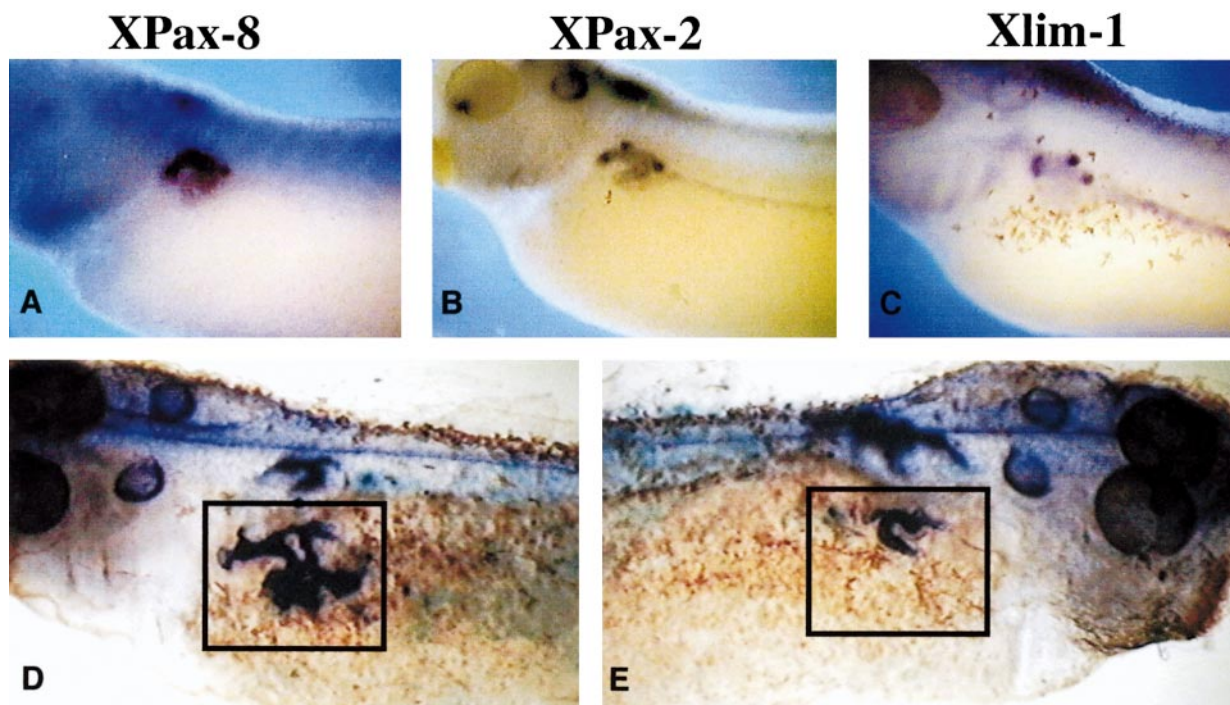
FIG. 5. *XPax-2* may supersede *XPax-8* function later in pronephric development. *In situ* hybridization of stage 36 embryos with antisense mRNA probes for *XPax-8* (A), *XPax-2* (B), and *Xlim-1* (C). Note the even levels of expression throughout the tubules for *XPax-8* compared to the restriction of high levels of expression to the dorsal tips of the tubules for *XPax-2* and *Xlim-1*. Also note that *XPax-2* and *Xlim-1* are expressed in the nephric duct while *XPax-8* is not. Embryos coinjected with *XPax-2* and *Xlim-1* mRNA show similar phenotypes to those of embryos injected with *XPax-8* and *Xlim-1*. D shows the injected side of a *XPax-2* plus *Xlim-1* coinjected embryo stained with the 3G8 antibody. Note the enlarged pronephros (boxed) compared to the uninjected side shown in E, indicating that *XPax-2* and *XPax-8* are functionally equivalent in this assay.

tion of tissues capable of forming ectopic pronephroi. The effect of 3M plus *XPax-8* coinjections was indistinguishable from those of wild-type *Xlim-1* and *XPax-8* (Table 1), indicating that the level of *Xlim-1* activity is not responsible for the tissue restriction of the phenotype.

Additional Pronephric Tissue Is Not Due to Secondary Effects Caused by Axis Induction

Injection of *Xlim-1* or *XPax-8* mRNA into the ventral blastomeres of four-cell *Xenopus* embryos results in the formation of ectopic axial tissue in a small percentage of cases (not shown). The *Xlim-1* 3M mutant active form is a powerful dorsalizer and results in a very high frequency of well-developed secondary axes when expressed at this stage (Taira *et al.*, 1994b). As *XPax-8* appears to be synergizing with *Xlim-1* it was possible that in addition to enhancing the ability to pattern the pronephric mesoderm the coexpression of these two genes was also enhancing the dorsalizing activity of *Xlim-1*. Because the somites are thought to play a role in the induction of the pronephros (D. Seufert, E. Jones, and P. D. Vize, manuscript in preparation; H. Brennan and E. Jones, personal communication), it was important to test if ectopic pronephroi were induced by increased amounts of paraxial tissue or if they formed in direct response to the ectopic mRNAs. This issue was investigated in two ways. The first was to examine the organization of axial mesoderm in embryos in which *XPax-8* and *Xlim-1* coexpression resulted in the formation of large or ectopic pronephroi. The second approach was to induce the formation of secondary axes by other means and to determine the capability of these secondary axes to enhance pronephric development.

Hundreds of samples with enlarged or ectopic pronephric tubules were examined by double staining with antibody 3G8 and with the somitic muscle antibody 12/101. Although some embryos had less somitic tissue overlying enlarged pronephroi, very few contained additional somitic tissue that would be indicative of the development of an ectopic dorsal axis. Indeed, in all of the embryos exhibiting enlarged pronephroi shown in Figs. 3 and 5 and scored in Table 1, no axial duplications were observed. This indicates that the formation of giant or ectopic pronephroi is not dependent upon ectopic dorsal axis formation. Secondary axes were induced by *XPax-8* plus *Xlim-1* injection into

blastomere C4, but such embryos were not scored for effects on pronephric development.

When axial duplications were induced by microinjection of chordin mRNA or injection of 5 nl of 1.5 M lithium chloride (not shown), ectopic pronephroi were only observed in 20% of the secondary axes. Moreover, such ectopic pronephroi were only observed either when the duplication was very complete or when the induced axis was immediately adjacent to the pronephros present in the primary axis, in which case the normal pronephric field was probably responding to inductive signals from the ectopic dorsal axis (data not shown). More commonly, the pronephric tubules in embryos injected with dorsalizing agents are suppressed, presumably due to the respecification of the presumptive pronephric mesoderm to a more dorsal fate by the dorsalizing factors.

Neither *XPax*-8 nor *Xlim*-1 displayed any ventralizing activity in these experiments, and ventralization does not result in enlargement of the pronephroi in *Xenopus* (D. Seufert, J. Deguire, E. Jones, and P. D. Vize, submitted for publication), indicating that an indirect ventralizing effect is also unlikely to be responsible for the enlarged pronephric phenotype.

Increase in Pronephric Size Is Associated with a Decrease in Somite Size

In order to determine if enlarged pronephroi formed at the expense of other embryonic tissues, coinjected embryos were double stained with pronephric tubule-specific (3G8) and somitic (12/101) antibodies and then sectioned. The area of the somites in section was measured using NIH Image software and compared to the uninjected side. Although some somitic tissue that contains *XPax*-8 and *Xlim*-1 mRNA (as visualized by β-galactosidase expression) is recognized by the muscle differentiation marker, 12/101, the tissue is frequently disorganized and, in a majority of cases, significantly reduced in area ($P < 0.05$). Examples of these effects on somitic development are shown in Fig. 4. On average, somites adjacent to enlarged or ectopic pronephroi were 19.5% smaller than control somites in sections taken through the ectopic tubules. The reduction of somitic tissue area (19.5%) was approximately equal to the area occupied by ectopic pronephroi (20.8% of somite area), providing strong evidence that *XPax*-8 plus *Xlim*-1 expression has respecified presumptive somitic cells to a pronephric fate. β-Galactosidase-injected somites were not statistically different in size from uninjected controls (96% identical).

XPax-2 May Supersede XPax-8 Function in Later Stages of Pronephric Morphogenesis

Transcription of the *Xenopus* orthologue of Pax-2, *XPax*-2, commences at stage 21, several hours after *XPax*-8 begins to be transcribed in the pronephric anlage (Heller and Brandli, 1997; Carroll *et al.*, 1999a,b; this report). The onset of expression of *XPax*-2 coincides with the initiation of cell shape changes that signal the onset of pronephric anlage differentiation (Hausen and Reibesell, 1991; Vize *et al.*, 1997). As previously mentioned, the expression pattern of *Xlim*-1 refines to correspond to that of *XPax*-8 during neurulation (Fig. 2). However, at later stages of kidney development, the expression patterns of these two genes diverge and this divergence occurs following the onset of *XPax*-2 expression (Fig. 5). As *XPax*-2 expression becomes refined to the three tips of the dorsal connecting tubules, so does *Xlim*-1, while *XPax*-8 expression remains even throughout the pronephric tubules (Fig. 5). *Xlim*-1 and *XPax*-2 are also expressed in the pronephric duct at this stage, while *XPax*-8 is not. This series of observations suggests that the expression pattern of *Xlim*-1 initially refines to become coincident with *XPax*-8 and later possibly undergoes a second revision to become coincident with *XPax*-2. This expression data indicates that XPax-2 may substitute for XPax-8 as the cofactor of Xlim-1 during the later stages of kidney development. In order to investigate this possibility, *XPax*-2 and *Xlim*-1 were coinjected into the marginal zone of 16- to 32-cell-stage embryos that were then raised to tadpole stages and processed to visualize pronephric tubules.

Coexpression of *XPax*-2 and *Xlim*-1 was found to result in the formation of ectopic pronephric tubules in 42% of injected embryos ($n = 55$) and in the formation of enlarged pronephroi in 20% of samples (Fig. 5). The phenotype and the number of ectopic and enlarged pronephroi generated in this fashion were not distinguishable from those generated with *XPax*-8 and *Xlim*-1. These data demonstrate that *XPax*-2 and *XPax*-8 are functionally redundant in ectopic expression studies and that either can interact with *Xlim*-1 to pattern pronephric mesoderm. However, the *in vivo* expression data argue that *XPax*-8 plays this role during pronephric patterning in late gastrulae/early neurulae and *XPax*-2 during pronephric morphogenesis in tailbud stages.

DISCUSSION

The murine Lim-1 gene is essential for kidney development (Shawlot and Behringer, 1995), as are the murine (Torres *et al.*, 1995) and zebrafish Pax-2 genes (Pfeffer *et al.*, 1998). In mutant mice *Pax*-8 is not essential for normal kidney development (Mansouri *et al.*, 1998). In this report we demonstrate that Pax/lim-1 interactions are involved in the development of the pronephric kidney of *Xenopus.* As Pax-2 and Pax-8 are functionally redundant in ectopic expression experiments, the timing and place of expression of these two genes is likely to dictate which gene is important in the development of different kidneys.

Expression of Xenopus Pax Genes in the Pronephric Mesoderm

Expression of *XPax*-8 can first be detected by *in situ* hybridization at late gastrula stages in the rudiments of the

otic placode and the pronephros. Expression in the pronephric precursors commences at approximately the same time, stage 12.5, as *Xlim*-1, but the expression patterns of the two genes are distinct. *XPax*-8 mesodermal expression is limited to the region fated to form the pronephros (Yamada, 1937; Pasteels, 1942), while that of *Xlim*-1 overlaps this region but extends much further ventrally (Taira *et al.*, 1994a; Fig. 2). During subsequent development, the *Xlim*-1 pattern becomes more restricted and by early tailbud stages is coincident with that of *XPax*-8. By mid-tailbud stages, the pronephric expression of *XPax*-8 and *Xlim*-1 is no longer coincident. At this point *Xlim*-1 expression is coincident with another Pax family member, *XPax*-2. The overlapping expression of Pax and LIM genes within the pronephric region suggested that they may be cooperating in the establishment and maintenance of the pronephric mesoderm.

Synergistic Interactions between Pax-8 and lim-1 Pattern Pronephric Mesoderm

Coinjection of *XPax*-8 and *Xlim*-1 mRNA in the lateral marginal zone results in the development of ectopic and greatly enlarged pronephric tubules and ducts. Each gene can achieve this result independently but coexpression is at least twice as effective. Enlarged pronephroi formed in response to ectopic *XPax*-8 plus *Xlim*-1 can be up to 3.6 times larger in area than the normal pronephros. The size of the enlarged pronephroi is considerably larger in response to coexpression than is observed in response to either gene alone. Likewise, ectopic pronephroi can form in response to either gene within the somites and intermediate mesoderm, but the frequency with which such ectopic tissues are observed is much higher in response to coexpression. The increased frequency of both phenotypes is not an additive response, as the total amount of mRNA in single and coinjections is the same. Rather, it implies that these genes act synergistically to increase the amount of pronephric tissue.

Developmental Basis of the Large and Ectopic Pronephric Phenotypes

The three most likely explanations for how ectopic *XPax*-8 plus *Xlim*-1 expression results in the development of additional pronephric tissue are that: (1) synergy between these proteins results in an increase in the amount of the pronephric inductive tissues/signals and indirectly leads to the formation of additional pronephric tissue; (2) these proteins enhance proliferation rates specifically in pronephric tissues; and (3) these proteins synergize to instruct nonpronephric cells to adopt a pronephric fate. The first possibility, that the phenotype is the indirect effect of increasing the amount of pronephric inducing tissue or pronephric inducing signal, seems unlikely in light of the relatively normal phenotype of embryos with enlarged pronephroi, plus the demonstration that additional axial or paraxial tissue in secondary axes was not responsible for the additional pronephric tubules. In fact the amount of adjacent somitic tissue is reduced, rather than increased, in the region adjacent to enlarged pronephroi. The second possibility, enhanced proliferation, while it cannot be ruled out, seems very unlikely in light of the manner in which amphibian embryos develop. This is a closed developmental system fueled by maternal yolk with no external source of nutrients. Additional cell division would result in a greater number of smaller cells, not in a larger embryo or larger organs. An effect on proliferation would also not explain how ectopic pronephroi can form separated from the main organ by many somites. This leaves the third possibility, that synergism between XPax-8 and Xlim-1 leads nonpronephric cells to adopt a pronephric fate. These additional cells may be derived from the intermediate mesoderm or from the somitic mesoderm, which would explain the reduction in somite mass observed in the vicinity of enlarged pronephroi (Fig. 4). Although additional experiments tracing the fate of injected blastomeres will be required to analyze this phenomenon in detail, the most likely basis of the pronephric phenotype is that ectopic *XPax*-8 and *Xlim*-1 respecifies nonpronephric cells to form pronephric tubules or pronephric duct.

The Basis of XPax-8/Xlim-1 Synergism

The molecular nature of the interaction between XPax-8 and Xlim-1 is presently unknown. One possibility is that Pax-8 and lim-1 proteins physically interact to form a multimer with novel properties. Because ectopic expression of an activated form of *Xlim*-1, *Xlim*-1 3M, is less effective at inducing pronephric structures than wild type, the enhancement observed in coinjections is not simply due to XPax-8 activating the Xlim-1 protein, a mechanism that has been proposed for the LIM domain interacting factor, LDB-1 (Agulnick *et al.*, 1996). Instead, these results suggest that if there is a physical interaction between lim-1 and Pax-8, it may alter the specificity or functionality of one or both proteins. Pax and lim proteins have both previously been shown to interact with other proteins. The Pax-5 protein recruits members of the *ets* family of transcription factors to B-cell-specific promoters (Fitzsimmons *et al.*, 1996) and Pax-3 interacts with the retinoblastoma gene product to modulate transcription (Wiggan *et al.*, 1998). Several factors have been shown to interact with lim proteins (reviewed by Curtiss and Heilig, 1998). One example is the LIM domain containing protein *mec*-3 that is essential for touch receptor neuron development in *Caenorhabditis elegans. Mec*-3 requires the POU-homeodomain protein *Unc*-86 in order to activate transcription from target genes, including the *mec*-3 promoter itself (Xue *et al.*, 1993; Lichtsteiner and Tjian, 1995). If a similar scenario exists between *XPax*-8 and *Xlim*-1, both proteins may be essential for the maintenance of *Xlim*-1 expression. This would explain the restriction of the broad early *Xlim*-1 expression domain to one matching that of its proposed cofactor. In vertebrates, Lim-3 has been

demonstrated to synergize with the POU-homeodomain protein Pit-1 and the homeodomain protein Otx-2 to regulate target genes (Bach *et al.*, 1995, 1997). It is possible that similar interactions exist between lim-1 and the Pax proteins. However, preliminary experiments have not been able to detect a physical interaction between these proteins *in vitro,* and an alternative mode of action, such as each protein regulating a distinct but necessary parallel pathway, cannot be ruled out.

Additional Factors Involved in Pronephric Patterning

Although *XPax-8* and *Xlim-1* cooperate to bias differentiation along a specific developmental pathway, at least one additional cofactor may be required to achieve pronephric determination. This is evidenced by the fact that ectopic pronephroi are only formed in a subset of embryonic tissues: the somites and the intermediate mesoderm. Furthermore, not all cells within these tissues that receive ectopic *XPax-8* plus *Xlim-1* form pronephric structures, indicating that other intrinsic factors or some form of regulation must also be involved. Given the restriction in distribution of ectopic pronephroi, these additional factors may be localized. Such factors may be secreted proteins, such as members of the FGF, BMP, and Wnt families, which have been demonstrated to play key roles in metanephric development (for review, see Lechner and Dressler, 1997; Bard *et al.*, 1994) and are expressed in dynamic patterns in the pronephros and surrounding tissues (Carroll *et al.*, 1999b). Alternatively, they may be cell-autonomous factors such as *eyes absent* and *sine oculis,* transcription factors that have been demonstrated to interact with Pax-6 during eye specification (Chen *et al.,* 1997; Pignoni *et al.,* 1997). The amenability of *Xenopus* embryos to mRNA injection makes them an excellent system in which to investigate the activity of these proteins in pronephric patterning.

Functional Redundancy of Pax-2 and -8

Pax-8 has not previously been shown to play a key role in kidney development. While *Pax-8* is expressed in zebrafish embryonic kidneys (Pfeffer *et al.,* 1998) and mouse embryonic and adult kidneys (Asano and Gruss, 1992; Plachov *et al.*, 1990), *Pax-8* mutants generated by targeted disruption appear to have normal adult metanephric kidneys (Mansouri *et al.*, 1998). It is likely that *Pax-8* mutation has no effect on the adult kidney because of compensation by *Pax-2*, which is expressed at higher levels and in more cell types than *Pax-8* in the development of the metanephros (Plachov *et al.*, 1990). Analysis of *Pax-8* expression patterns in zebrafish (Pfeffer *et al.*, 1998) and *Xenopus* (this report) suggests that this gene does play an early, essential role in the development of the embryonic kidneys, the pronephroi, during the developmental window that precedes *Pax-2* activation. As pronephroi are probably not functional in the mouse (Saxén, 1987), the lack of a *Pax-8* mutant phenotype

is not surprising. However, in fish and frogs, pronephroi are essential (Howland, 1921; Fales, 1935), so ablation of *Pax-8* in these taxa should have a strong mutant kidney phenotype. Mutation of *Pax-2* also has strong effects on the development of the pronephroi in zebrafish (Brand *et al.*, 1996). In such mutants, the earliest stages of pronephric development, including activation of *Pax-8*, occur normally (Pfeffer *et al.*, 1998). The failure in development probably occurs at later stages when *Pax-2* has superseded *Pax-8* function in the pronephros. Once the *Xlim-1* expression pattern refines to match that of *XPax-2*, the two Pax genes are expressed in nonoverlapping domains, and therefore neither one would be able to compensate for the other.

Interestingly, although *Pax-8* is essential for normal thyroid development in the mouse (Mansouri *et al.*, 1998), *XPax-8* is not expressed in the frog thyroid, while *XPax-2* is (our unpublished observations; see the XMMR). This indicates that Pax-2 may be able to fulfill a necessary function of the Pax-8 gene product in *Xenopus* thyroid development, supporting the notion of redundancy. The fact that the phenotype of *XPax-2* plus *Xlim-1* overexpression is indistinguishable from that of *Pax-8* plus *Xlim-1* overexpression provides functional data in support of molecular redundancy of Pax family members.

Pax-8 as a Regulator of Pronephric Development

As the expression of either *XPax-8* or *Xlim-1* alone can result in the development of ectopic pronephroi, they could each be called "master regulators" of pronephric development. However, in reality both genes as well as additional factors are probably required in order to specify the pronephros. A similar scenario most likely exists in *Drosophila* eye development. Although ectopic expression of *eyeless/Pax-6* (Halder *et al.,* 1995), *dac* (Shen and Mardon, 1997), or *eya* (Bonini *et al.,* 1998) can form ectopic eyes, it is only in a limited number of cell types and in a subset of cells in which the ectopic gene expression occurs. Furthermore, coexpression of *dac* and *eya,* both capable of directing ectopic eye development independently, is greatly synergistic (Chen *et al.,* 1997). This suggests that the fly eye is specified by multiple genetic interactions rather than a linear molecular hierarchy. We propose a similar scenario for the development of the pronephros. We have demonstrated that *Xlim-1* and *XPax-8* synergize to specify the pronephric precursors. However, not all mesodermal cells that receive ectopic *XPax-8* plus *Xlim-1* form pronephroi, indicating that at least one additional factor is required to achieve pronephric specification. Future studies investigating the nature of these additional signals should allow the full spectrum of gene products required for the induction and patterning of this simple organ to be elucidated.

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