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Characterization of *Pax-2* Regulatory Sequences That Direct Transgene Expression in the Wolffian Duct and Its Derivatives

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The *Pax* family of transcription factors plays important roles in vertebrate organogenesis. *Pax-2* is a critical factor in the development of the mammalian urogenital system. *Pax-2* is expressed in the epithelia of the ureter, the Müllerian duct, and the Wolffian duct and in the nephrogenic mesenchyme. Gene targeting in the mouse as well as natural mutations in mouse and man have demonstrated the requirement of *Pax-2* in the development of these structures. Little is known about the molecular mechanisms regulating *Pax-2* expression in the developing urogenital system. As a first step to reveal these mechanisms and to search for the elements and factors controlling *Pax-2* expression we have characterized regulatory sequences of the *Pax-2* gene in an *in vivo* reporter assay in the mouse. An 8.5-kb genomic region upstream of the *Pax-2* transcription start site directed reporter gene activity in the epithelium of the pronephric duct at 8.25 days postcoitum (dpc) and in the Wolffian duct starting from 9.0 dpc. Expression in the Wolffian duct and its derivatives, the ureter, the collecting duct system, the seminal vesicles, the vas deferens, and the epididymis, was maintained at least until 18.5 dpc. Hence, an element(s) in the 8.5-kb upstream region is sufficient to initiate and maintain *Pax-2* expression in the Wolffian duct and its derivatives. In order to more precisely map the Wolffian duct regulatory sequences, a deletion analysis of the 8.5-kb upstream region was performed in a transient *in vivo* reporter assay. A 0.4-kb subfragment was required for marker gene expression in the Wolffian duct. Misexpression of *fgf8* under the control of the 8.5-kb upstream region resulted in polycystic kidneys, demonstrating the general usefulness of *Pax-2* regulatory sequences in misexpression of foreign genes in the ureter and collecting duct system of the kidney in transgenic approaches in mice. © 2001 Academic Press

Key Words: **kidney; metanephros; urogenital system; promoter analysis; enhancer; Wolffian duct; Pax; Pax-2.**

INTRODUCTION

The formation of vertebrate organs is governed by complex inductive interactions ensuring correct tissue morphogenesis and cellular differentiation. Urogenital development is a particularly intriguing example of organogenesis since two distinct organ systems, the excretory system of the kidneys and the reproductive system with gonads and sex ducts, are functionally connected and derive from a common precursor, the intermediate mesoderm, which lies between the somitic and the lateral plate mesoderm. In the mouse, renal development is initiated at 8.0 days postcoitum (dpc) when pronephric tubules arise at the cervical level of the intermediate mesoderm. Pronephric tubules fuse to form the pronephric duct and together form the pronephros, a transient and nonfunctional organ. The extension of the pronephric duct, the Wolffian (or nephric) duct, grows caudally as a simple epithelial tube. It induces the adjacent nephrogenic cord to form nephrons. In mice, Wolffian duct and mesonephric tubules form the mesonephros, which in contrast to that in birds is a nonfunctional excretory organ. At 10.5 dpc, the ureteric bud emerges at the caudal end of the Wolffian duct, at the level of the hindlimb buds, and invades a posterior condensation of the intermediate mesoderm, the metanephric blastema. The ureteric bud undergoes complex branching morphogenesis

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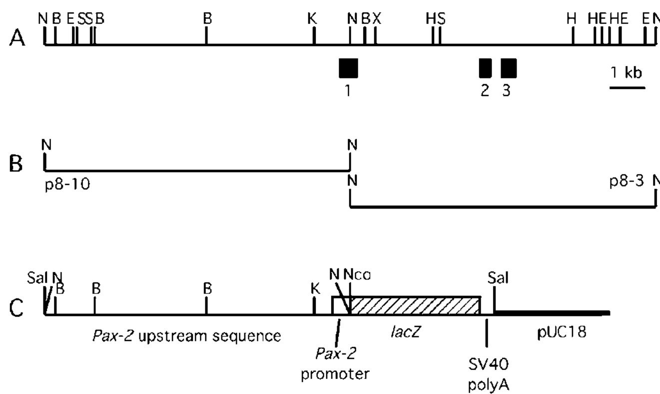

FIG. 1. Cloning and characterization of the *Pax-2* genomic region. (A) Restriction map of the partial *Pax-2* locus analyzed in this work. Exons 1 to 3 are indicated (filled boxes). The internal *Not*I site lies within the 5′ untranslated region of the *Pax-2* transcription unit. (B) Subclones p8-3 and p8-10 used for the further characterization of the *Pax-2* regulatory region. (C) Schematic representation of the pPax2.Not(8.5).lacZ vector used for transgenic analysis of the *Pax-2* regulatory region. The *lacZ* gene is in frame with the initiation codon of *Pax-2*. *Sal*I indicates sites used to release the fragment for pronuclear injection experiments. N, *Not*I; B, *Bam*HI; S, *Sac*I; K, *Kpn*I; X, *Xho*I; H, *Hin*dIII; E, *Eco*RI.

and will eventually form the collecting duct system of the mature kidney, the metanephros. Upon signals from the ureter, mesenchymal cells condense around the ureter tip, aggregate, and undergo a mesenchymal-to-epithelial transition to form a renal vesicle which upon further morphogenesis and differentiation will form the major part of the mature nephron, the functional excretory unit.

The Wolffian duct differentiates into the major portion of the male sex duct system, the epididymis, the vas deferens, and the seminal vesicles. In the female, the Wolffian duct degenerates starting from 14.5 dpc under hormonal influence. Instead, a second epithelial tube, the Müllerian duct, which is derived from the coelomic epithelium of the urogenital ridge, will differentiate into the female sex ducts, the oviduct, the uterus, and the upper part of the vagina. In the male, the Müllerian ducts are also established but degenerate quickly under the influence of anti-Müllerian hormone (for reviews on kidney development see Bard *et al.,* 1994; Lechner and Dressler, 1997; Saxén, 1987; Vainio and Muller, 1997).

The *Pax* genes constitute a family of developmental control genes characterized by a 128-amino-acid domain, the paired domain. The paired domain mediates specific DNA binding, suggesting that *Pax* genes encode transcription factors. Originally recognized in *Drosophila,* paired-domain-containing proteins have also been identified in vertebrates, including nine family members (*Pax-1* to *Pax-9*) in mammals. Sequence conservation and expression analysis define subfamilies of *Pax* genes with *Pax-1* and *Pax-9* forming a first, *Pax-2, Pax-5,* and *Pax-8* a second, *Pax-3* and *Pax-7* a third, and *Pax-4* and *Pax-6* a fourth subfamily (for reviews on *Pax* genes see Mansouri *et al.,* 1996; St-Onge *et al.,* 1995; Strachan and Read, 1994).

Pax genes show highly specific expression patterns during development. In particular, expression of members of the *Pax-2/5/8* family have been found in the developing urogenital system of all vertebrates studied (Dressler *et al.,* 1990; Heller and Brandli, 1999; Pfeffer *et al.,* 1998; for reviews see Rothenspieler, 1996; Torban and Goodyer, 1998). In the mouse, *Pax-8* is expressed in the renal vesicles, the comma- and S-shaped bodies of the metanephric kidney, and the developing Müllerian duct (Plachov *et al.,* 1990). Mice mutant for *Pax-8* do not exhibit a phenotype in the urogenital system but in thyroid gland development (Mansouri *et al.,* 1998), suggesting redundancies with other members of the gene family, like *Pax-2. Pax-2* is expressed in the ductal and mesenchymal components of the developing urogenital system, the Müllerian duct, the Wolffian duct, the ureter, and the condensing mesenchyme of the meso- and metanephric kidney (Dressler *et al.,* 1990; Fickenscher *et al.,* 1993; Oefelein *et al.,* 1996). Outside the kidney *Pax-2* is also expressed in the central nervous system, in interneurons of the spinal cord, and in the ear and eye (Nornes *et al.,* 1990).

The critical role of *Pax-2* gene expression during development has been demonstrated by generating knockout mice. Mice homozygous mutant for null alleles of *Pax-2* have defects in ear, eye, and brain development (Torres *et al.,* 1996; Urbanek *et al.,* 1997). In addition, homozygous mutant newborns lack kidneys, ureters, and genital tracts. The Wolffian duct and Müllerian duct develop only partially and degenerate during embryogenesis. The ureter is not formed, and the nephrogenic cord is not able to undergo epithelial transformation to form mesonephric tubules, demonstrating a requirement both in the epithelial and in the mesenchymal compartments of the urogenital system (Dressler and Woolf, 1999; Torres *et al.,* 1995).

Spontaneous mutations of *PAX2* in humans have been reported (Eccles and Schimmenti, 1999; Sanyanusin *et al.,* 1995). Heterozygosity of the *PAX2* locus is associated with bilateral optic nerve colobomas, ear abnormalities, and renal deficiencies. A similar phenotypic spectrum has been seen in mice heterozygous for *Pax-2* (Favor *et al.,* 1996; Torres *et al.,* 1995). The severe dosage dependence of *Pax-2* function is reflected not only by haploinsufficiency in human and in mouse but also by a transgenic approach overexpressing *Pax-2* in mice (Dressler *et al.,* 1993). These mice are born with large multicystic kidneys, congenital nephritic syndrome, and end-stage renal failure.

These experiments have clearly shown the importance of regulating both the levels and the time and space of *Pax-2* expression during urogenital development. However, little is known about the *cis*-acting elements and the factors binding to them. In an earlier report we characterized an element responsible for *Pax-2* expression in the mid/hindbrain region of the developing brain (Rowitch *et al.,* 1999). Here we use a transgenic mouse reporter assay to identify and analyze a *Pax-2* upstream region that mediates

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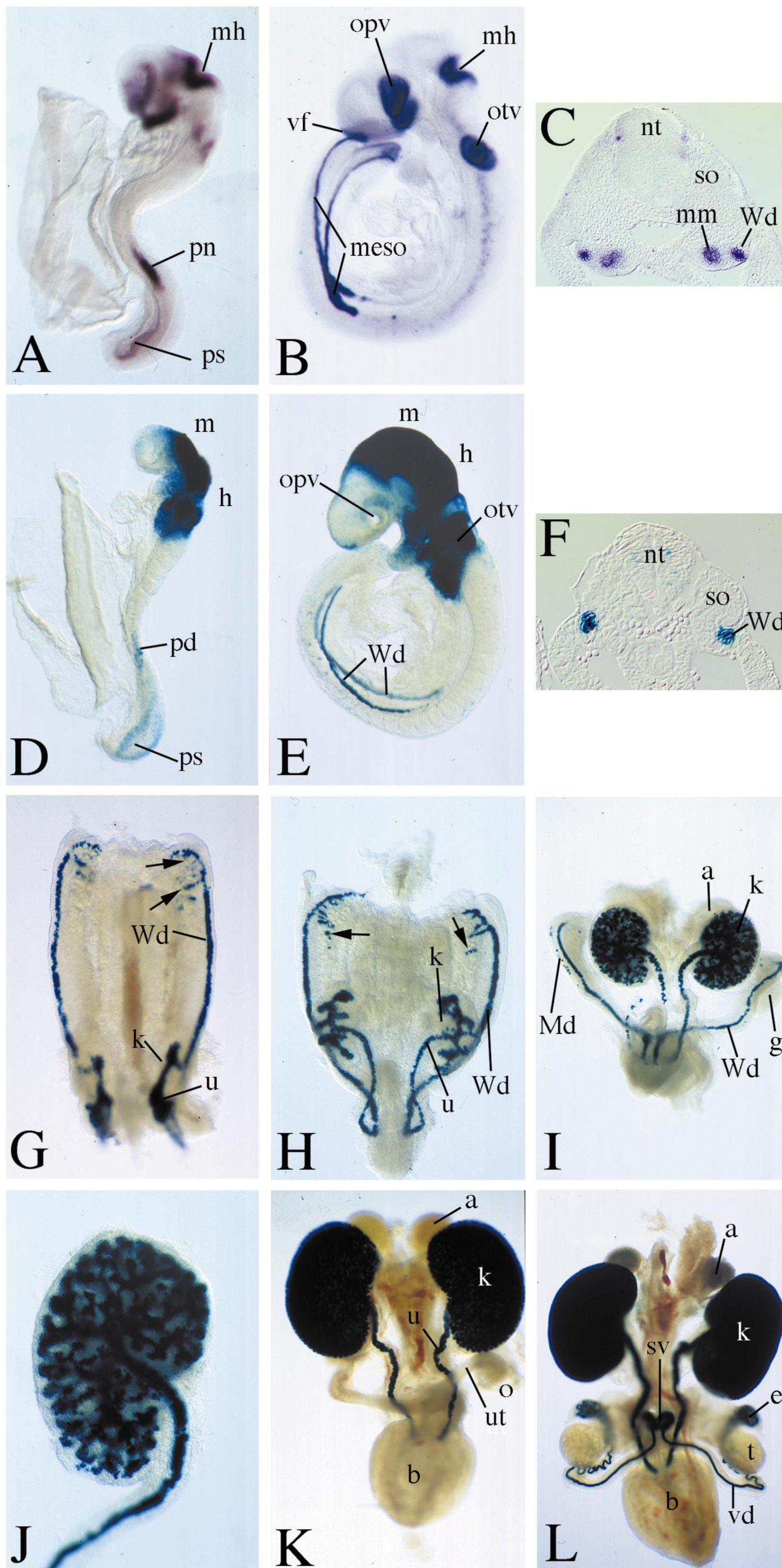

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expression in the developing urogenital system. While this region is sufficient both for initiation and for maintenance of marker gene expression in the Wolffian duct and its derivatives, it does not contain elements that govern *Pax-2* expression in the Müllerian duct or in the nephrogenic mesenchyme. The *Pax-2* regulatory region confers the ability to misexpress genes of interest in the Wolffian duct and its derivatives. Using a deletion analysis we have identified a short genomic fragment which is required to initiate Wolffian duct expression, opening the avenue for molecular identification of factors involved in regulating *Pax-2* expression in this tissue.

MATERIALS AND METHODS

Mouse Stocks

Embryos for *Pax-2* expression analysis were derived from matings of NMRI wild-type animals. For timed pregnancies plugs were checked on the morning after mating; noon was taken as 0.5 dpc.

DNA Constructs

The cloning of the *Pax-2* genomic region was performed as described recently (Rowitch *et al.,* 1999). In brief, a murine 129/Sv genomic library was screened using the *Pax-2* cDNA as a probe (Church and Gilbert, 1984; Dressler *et al.,* 1990). Two identical phages were isolated and the 17-kb genomic insert was subcloned into pBluescript as two 8.5-kb *Not*I fragments. Plasmid p8-10 harbors 8.5 kb of *Pax-2* upstream sequence, plasmid p8-3 contains exons 1–3 of the *Pax-2* locus. The 8.5-kb *Not*I fragment of plasmid 8-10 was inserted in front of the *Escherichia coli lacZ* gene to create the reporter construct pPax2.Not(8.5).lacZ.

Deletion constructs of the pPax2.Not(8.5).lacZ reporter plasmid were made by deleting the genomic region between the *Bam*HI and the *Kpn*I sites and inserting smaller genomic fragments of plasmid p8-10.

The expression vector pSG5-2.Pax2(8.5).fgf8 was constructed as follows: First, the eukaryotic expression vector pSG5 (Green *et al.,* 1988) was modified to pSG5-2 by linker insertion. An *Eco*RI–*Eco*RV–*Sma*I–*Pme*I–*Swa*I–*Bgl*II linker was inserted between the *Eco*RI and the *Bgl*II site for cDNA insertion, and the linker *Pvu*II–*Sal*I–*Not*I–*Xho*I–*Hpa*I–*Stu*I replaced a *Pvu*II–*Stu*I fragment harboring the SV40 enhancer/promoter in pSG5 to allow insertion of enhancer/promoter fragments. In the 8.5-kb *Pax-2* enhancer/promoter fragment of p8-10 the 3′-*Not*I site was modified to *Hpa*I and the *Not*I–*Hpa*I fragment directionally cloned into the respective sites of pSG5-2 to create the *Pax-2* misexpression vector pSG5-2.Pax2(8.5). The *fgf8* cDNA (Mahmood *et al.,* 1995) was inserted into the unique *Eco*RV site of pSG5-2.Pax2(8.5) after being released from pGEM9z.fgf8 as an *Eco*RI–*Xba*I fragment and a Klenow fill-in reaction.

The 0.7-kb *Nar*I–*Sph*I subfragment of p8-10.Not(8.5) found to contain a region for Wolffian duct expression was sequenced by cycle sequencing using an ABI Prism 310 genetic analyzer. A search for potential transcription factor binding sites was performed using the TRANSFAC program (http://transfac.gbf-braunschweig.de/TRANSFAC/index.html).

Pronuclear Injection and Genotyping of Mice

Transgenic mice were generated by pronuclear injection as described (Echelard *et al.,* 1994). For pSG5-2.Pax2(8.5).fgf8 and the pPax2.Not(8.5).lacZ constructs C57BL6CBA/J1 embryos were used, for Pax2.Not(8.5) deletion constructs NMRI embryos were preferred. Vector sequences were removed by *Sal*I digestion, and transgene fragments were separated by gel electrophoresis and purified with QIAquick (Qiagen). Fragments were diluted to 2 ng/μl for injection. For genotyping of embryos in transient reporter assays, yolk sac DNA was prepared (Echelard *et al.,* 1994) and analyzed by Southern blot analysis. A *lacZ* fragment was used as a probe in the case of the *lacZ* constructs. For the transient *fgf8*

FIG. 2. An 8.5-kb *Pax-2* upstream genomic fragment directs reporter gene expression in the Wolffian duct and its derivatives. (A–C) Whole-mount *in situ* hybridization analysis of *Pax-2* mRNA expression. (A) Onset of *Pax-2* expression in the pronephric region at 8.25 dpc. Other expression domains are the mid/hindbrain border, the forming optic and otic vesicles, and, weakly, the primitive streak region. (B) *Pax-2* expression marks the Wolffian duct and the adjacent mesonephric mesenchyme at 9.5 dpc. In addition to the expression domains at 8.25 dpc expression is now also detected in the forebrain and in spinal interneurons. (C) Section of a 9.5-dpc embryo posterior to the forelimbs clearly showing expression in the condensing mesenchyme and the Wolffian duct of the mesonephros. (D–L) Whole-mount β-galactosidase histochemistry from a permanent line transgenic for the pPax2.Not(8.5).lacZ reporter construct. (D) Onset of the marker gene expression in the pronephric region at 8.25 dpc. Strong β-galactosidase activity is observed in the midbrain and hindbrain, but is weak in the primitive streak region. (E) At 9.5 dpc β-galactosidase activity is detected in the Wolffian duct, in the midbrain and hindbrain, and in the branchial arches. (F) Section of a 9.5-dpc embryo posterior to the forelimbs showing restriction of *lacZ* expression to the Wolffian duct. (G) Isolated urogenital system of an 11.5-dpc embryo. β-Galactosidase activity is detected in the Wolffian duct and in the ureter, which has branched once at this time point. At the cranial end of the Wolffian duct dispersed cells are seen, indicating disintegration of the Wolffian duct (arrows). (H) 12.5-dpc urogenital system. Maintenance of expression in the domains of the 11.5-dpc urogenital system. The ureter has branched several times. The cells of the cranial Wolffian duct still disperse and may contribute to gonad and sex ducts (arrows). (I) 14.5-dpc urogenital system. Strong *lacZ* expression in the ureter tree and in the Wolffian duct. Discontinuous and weak staining in the Müllerian duct. (J) 16.5-dpc kidney. β-Galactosidase activity is seen in the epithelium of the collecting duct system, the ureter tips, and the ureter. (K) 18.5-dpc female urogenital system. β-Galactosidase activity is found in the ureter and in the collecting duct system, but not in the uterus. (L) In the male urogenital system the derivatives of the Wolffian duct, the epididymis, the vas deferens, and the seminal vesicles, are *lacZ* positive. a, adrenal; b, bladder; e, epididymis; g, gonad; h, hindbrain; k, metanephric kidney; m, midbrain; Md, Müllerian duct; mh, mid/hindbrain border; meso, mesonephros; mm, mesonephric mesenchyme; nt, neural tube; o, ovary; opv, optic vesicle; otv, otic vesicle; pd, pronephric duct; pn, pronephros; ps, primitive streak; so, somite; sv, seminal vesicles; t, testis; u, ureter; ut, uterus; vd, vas deferens; vf, ventral forebrain; Wd, Wolffian duct.

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misexpression assays, embryos were harvested at 15.5 and 17.5 dpc and genotyped using the *fgf8* cDNA as a probe. For permanent lines tail DNA was prepared, digested with *Eco*RI, and screened by Southern blot analysis employing a probe for *lacZ* sequences. The Pax2.Not(8.5).lacZ transgenic lines were kept on a mixed (NMRI/C57BL6) background.

Kidney Cultures

Cultures of 11.5-dpc metanephric kidney rudiments were done as described (Kispert *et al.,* 1996).

In Situ Hybridization Analysis, Histochemistry, and Documentation

The protocol used for whole-mount *in situ* hybridization is based on a published procedure (Knecht *et al.,* 1995; Parr *et al.,* 1993). *In situ* hybridization on sections followed the protocol by Lescher *et al.* (1998). The synthesis of the *Pax-2* and the *fgf8* antisense digoxigenin probes was carried out using a Digoxigenin RNA Labeling Kit (Roche Diagnostics). Riboprobes were used at approximately 1 μg/ml after DNase treatment and ethanol precipitation of the reaction. A probe to *Pax-2* was generated from the full-length cDNA (Dressler *et al.,* 1990) by linearization with *Xba*I and transcription with T3 RNA polymerase; for *fgf8,* the cDNA was linearized with *Hin*dIII and transcribed with T7 RNA polymerase. Whole-mount histochemistry for β-galactosidase was carried out as described (Echelard *et al.,* 1994).

After completion of the color reaction, the embryos, tissues, and sections were washed 3× in PBT (PBS with 0.1% Tween 20) and fixed in 4% paraformaldehyde/0.1% glutaraldehyde overnight at 4°C, washed in PBT, and transferred in 80% glycerol/PBT for storage and photography. Alternatively, samples were dehydrated, embedded in wax, and sectioned at 7 μm. Sections were dewaxed, rehydrated, and stained with hematoxylin and eosin or left unstained. Stained embryos and organs were photographed on Ektachrome Tungsten 64 color slide film (Kodak) using a Zeiss Stemi SV11 Apo photomicroscope. Sections were photographed under Nomarski optics on a Zeiss Axiophot. Slides were scanned and color composites were generated using Adobe PhotoShop v4.0 on a Power Macintosh.

RESULTS

An 8.5-kb Pax-2 Genomic Upstream Fragment Directs Reporter Gene Activity in the Developing Wolffian Duct and Its Derivatives

In order to identify enhancer elements directing *Pax-2* expression in the developing urogenital system we partially cloned the genomic region of the *Pax-2* gene. In total, 17 kb of the *Pax-2* locus was cloned and characterized by restriction analysis and mapping of exonic sequences. The first three exons of the *Pax-2* gene were preceded by 8.5 kb of upstream sequences (Figs. 1A and 1B). These genomic sequences were contained within a single *Not*I fragment and were tested for elements driving expression in the urogenital system in an *in vivo* reporter assay. In the construct pPax2.Not(8.5).lacZ used for that purpose the ATG of the reporter gene *lacZ* replaced the endogenous start codon of *Pax-2* in exon 1 (Fig. 1C). Thus, the endogenous *Pax-2* promoter was utilized. The pPax2.Not(8.5).lacZ fragment stripped of vector sequences was used in pronuclear injections. A total of nine transgenic male-derived founder lines were identified. Seven of these demonstrated a consistent pattern of β-galactosidase activity in the embryo. Representative results for one of these permanent lines are shown and compared to endogenous *Pax-2* expression in the urogenital system (Fig. 2).

At 9.5 dpc *Pax-2* is expressed in the ventral forebrain, in the mid/hindbrain region, in the spinal interneurons, in the otic and optic vesicles, and in the Wolffian duct and the mesonephric mesenchyme (Fig. 2B). In contrast β-galactosidase activity was confined to the Wolffian duct, the mid/hindbrain region, the otic vesicle, and the branchial arches at the same stage (Fig. 2E). Hence, the 8.5-kb upstream region harbors elements driving marker gene expression only in a subset of the endogenous *Pax-2* expression domains. Aspects of the *Pax-2* upstream region driving expression in the brain have been reported on separately (Rowitch *et al.,* 1999); here we will focus on the urogenital aspect of *Pax-2* expression.

To analyze the onset and the continuation of the β-galactosidase activity in the developing urogenital system more carefully, we analyzed embryos derived from intercrosses of the heterozygous line 79 transgenic for pPax2.Not(8.5).lacZ between 7.0 and 18.5 dpc (Figs. 2D–2L). First expression in the urogenital system was detected at 8.25 dpc at the 8-somite stage, when a scattered staining was found extending lateral between somites ~4–6 and 8 (Fig. 2D). This staining probably reflects the pronephric duct. In an age-matched embryo analyzed for *Pax-2* mRNA expression, the expression domain comprises the entire pronephros (Fig. 2A). At 9.5 dpc the Wolffian duct was positive for β-galactosidase activity. At this stage this duct extends from the forelimb buds (approximately somite 10) posteriorly. The staining in the region of somites 6–10 was not detected any longer, compatible with the idea that the pronephros has disintegrated at this point. Sectioning of whole embryos stained for β-galactosidase activity or *Pax-2* expression clearly demonstrated the confinement of β-galactosidase activity to the Wolffian duct, whereas *Pax-2* mRNA is expressed in the Wolffian duct and the adjacent mesonephric mesenchyme (Figs. 2C and 2F). At 10.5 dpc the Wolffian duct reaches its maximal extension between fore- and hindlimb levels. The ureteric bud appears and invades the metanephric blastema. With branching morphogenesis of the ureter in the metanephric kidney, β-galactosidase activity outlined both the ureter and the collecting duct system (Figs. 2G–2L). This pattern of β-galactosidase activity in the metanephric kidney persisted at least until 18.5 dpc, the last time point analyzed. Interestingly, protuberances were detected at the anterior end of the Wolffian duct starting from 10.5 dpc and lasting until 12.5 dpc (Figs. 2G and 2H). At these stages *lacZ*-positive cells were occasionally seen in the gonads and sex ducts (data not shown). We cannot exclude that this staining reflects the presence of an independent enhancer element in the 8.5-kb upstream

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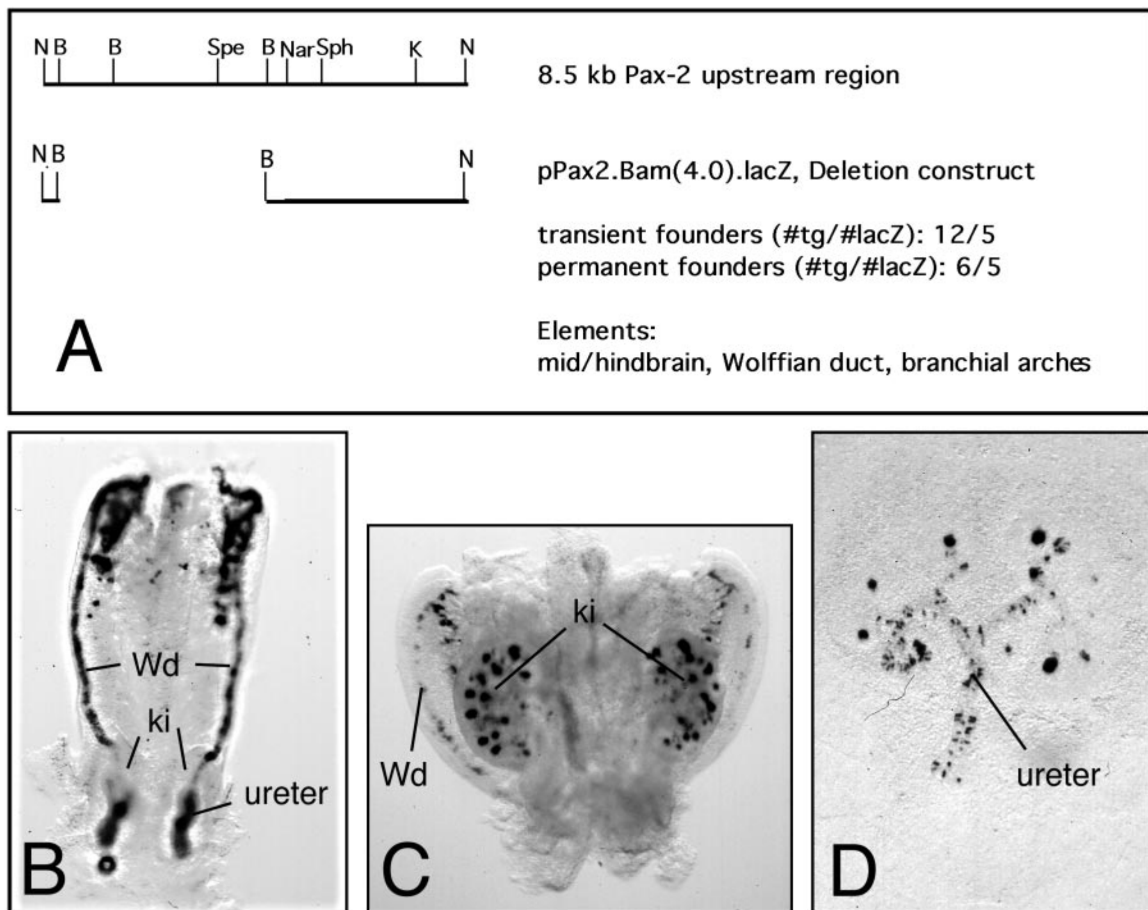

FIG. 3. Deletion analysis of the *Pax-2* Wolffian duct enhancer element using a permanent line for the 4-kb *Pax-2* upstream regulatory region. (A) The deletion construct pPax2.Bam(4.0).lacZ is shown in relation to the 8.5-kb upstream region which was tested in the construct pPax2.Not(8.5).lacZ. In a transient assay, 5 of 12 transgenic embryos showed *lacZ* staining including the Wolffian duct. Six permanent lines were derived, of which 5 showed *lacZ* expression. Of the 3 lines tested all showed staining in the Wolffian duct and its derivatives, the mid/hindbrain region and the branchial arches. (B–D) Urogenital systems and kidney cultures derived from embryos from a permanent line transgenic for pPax2.Bam(4.0).lacZ, stained for β-galactosidase activity as a whole mount. (B) 11.5-dpc urogenital system, (C)12.5-dpc urogenital system, (D) 2-day kidney culture. *lacZ*-positive cells are scattered in the Wolffian duct and the ureter. ki, kidney; Wd, Wolffian duct.

region. However, continuity of staining with the Wolffian duct was observed at all times. At 14.5 dpc weak staining of some cells was also seen in the Müllerian duct (Fig. 2I). Since the Wolffian duct is established in both sexes and degenerates only in the female after 14.5 dpc, Wolffian duct staining after this time was found only in the male, in the epithelium of the epididymis, in the vas deferens, and in the seminal vesicles (Figs. 2K and 2L). In addition to the Wolffian duct and its derivatives, *Pax-2* is expressed in the nephrogenic mesenchyme of the pronephros, mesonephros, and metanephros and in the Müllerian duct and its derivatives in the female as far as the urogenital system is concerned. Since these expression domains fail to express *lacZ* in the pPax2.Not(8.5).lacZ transgenics, this upstream region contains only the regulatory elements for Wolffian duct expression.

A 0.4-kb Genomic Fragment Is Required to Activate Reporter Gene Activity in the Developing Wolffian Duct

To more precisely localize the elements driving *Pax-2* expression in the Wolffian duct, we tested 4 kb of genomic region upstream of the *Pax-2* initiation codon in the construct pPax2.Bam(4.0).lacZ (Fig. 3A). Of six male-founder-derived lines, five expressed *lacZ,* and all three established lines that were tested showed a consistent pattern of β-galactosidase activity in the Wolffian duct and its derivatives. Expression in the Wolffian duct between 9.0 and 11.0 days was similar to that described for the 8.5-kb upstream region (data not shown). At later stages, expression in the Wolffian duct and its derivative, the ureter, was much weaker and more punctuate compared to the 8.5-kb con-

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struct, indicating that sequences between −4 and −8 kb are required to enhance and/or maintain expression in the Wolffian duct and its derivatives (Figs. 3B–3D). Thus, the region between −4 kb and the start codon still harbors elements required for the initiation of reporter activity in the Wolffian duct as well as the elements for mid/hindbrain and branchial arch expression (Rowitch *et al.,* 1999).

In the next set of experiments, we attempted to restrict the genomic region required for the initiation of *Pax-2* expression in the Wolffian duct further by deleting more sequences in our reporter constructs and looking at the consequences of *lacZ* expression in a transient reporter assay, analyzing embryos between 9.5 and 10.5 dpc (Fig. 4A). In all our constructs, we tested the *Pax-2* upstream region in the context of the *Pax-2* promoter. In the construct pPax2.Nar-Not(3.7), 3.7 kb of upstream region was sufficient to drive *lacZ* expression in the Wolffian duct (Fig. 4B), but 3.0 kb in the construct pPax2.Sph-Not(3.0).lacZ failed to do so although expression was observed in other regions of the embryo. Consequently, the intervening 0.7-kb *Nar*I–*Sph*I fragment was injected in conjunction with the *Pax-2* promoter. Indeed, the 0.7-kb fragment contains a region for *Pax-2* expression in the Wolffian duct as shown by our transient reporter assays (Fig. 4C). The 0.7-kb *Nar*I–*Sph*I fragment was roughly divided in two and the subfragments were again tested in the reporter assay. A 0.4-kb *Nar*I–*Apa*I fragment was required to drive reporter gene expression in the developing Wolffian duct (Fig. 4D), but the adjacent 0.3-kb *Apa*I–*Sph*I fragment failed to give Wolffian duct expression. Interestingly, our deletion analysis also restricted the genomic regions for the mid/hindbrain element as well as for the branchial arch element. While the 0.5-kb *Pvu*II–*Xho*II fragment conferred expression of the marker gene in the mid/hindbrain region, the 0.4-kb *Nar*I–*Apa*I fragment as well as the 0.3-kb *Apa*I–*Sph*I fragment failed to do so. Thus, it is possible that the mid/hindbrain element resides around the *Apa*I site or that the element is bipartite in nature. The region for expression in the branchial arches could not be separated from the Wolffian duct enhancer (Fig. 4E).

This analysis opens the possibility of directly searching for transcription factors mediating *Pax-2* expression in the Wolffian duct using biochemical methods. As a first step toward this, we have sequenced the 0.7-kb *Nar*I–*Sph*I fragment and have searched the 0.4-kb minimal region for potential transcription factor binding sites using the TRANSFAC program. Candidate binding sites include an E box for binding of a basic HLH protein, a homeobox transcription factor binding site, a Smad binding site, and a zinc finger protein binding site (Fig. 5 and data not shown).

Pax-2 Regulatory Sequences Are Capable of Misexpression of Foreign Genes in the Developing Ureter

In order to test whether the 8.5-kb *Pax-2* upstream region is sufficient to misexpress foreign genes in the Wolffian duct and its derivatives, in particular the ureter, we sought to create a versatile misexpression vector based on the *Pax-2* 8.5-kb upstream region. In brief, we exchanged the SV40 enhancer/promoter in the eukaryotic expression vector pSG5 with the 8.5-kb *Pax-2* enhancer/promoter and introduced a multicloning site for easy insertion of foreign genes. The resulting vector was designated pSG5-2.Pax2(8.5).

To test the capability of this construct in the misexpression of foreign genes, we decided to use an *fgf8* cDNA, which was formerly shown to direct patterning defects and overproliferation when misexpressed under the control of the *Wnt-1* enhancer in the mid/hindbrain region of embryos (Lee *et al.,* 1997).

The resulting construct pSG5-2.Pax2(8.5).fgf8 was used in pronuclear injections (Fig. 6A). Due to the deleterious consequences of *fgf8* misexpression, no attempts were made to establish permanent lines. Instead, embryos were isolated and kidneys analyzed at 15.5 and 17.5 dpc in a transient assay. For 15.5 dpc, 23 embryos were harvested, 4 of which were transgenic. Two embryos with a high copy number of the transgene showed obvious kidney and CNS phenotypes, 2 embryos with a low-copy transgene insertion had no gross morphological defects and were not studied in more detail. For 17.5 dpc, 33 embryos were collected. All 4 transgenic embryos showed an obvious alteration in kidney morphology (Fig. 6B).

Kidneys transgenic for the construct pSG5-2.Pax2(8.5).fgf8 appeared enlarged compared to nontransgenic controls (Fig. 6B). Histological analysis revealed the presence of large cysts in the medulla region of the kidney (Fig. 6C). *In situ* hybridization analysis for *fgf8* expression indicated strong but spotty expression in the transgenic kidneys but not in the wild-type controls. *fgf8* expression was confined to the ureteric epithelium. *fgf8* expression was largely absent from the epithelium surrounding the cysts, suggesting that the activity of the *Pax-2* upstream region is greatly reduced upon differentiation into collecting duct epithelium (Figs. 6D and 6E). Analysis of *Pax2, c-ret, Wnt-11,* and *Wnt-7b* expression did not detect differences between wild-type and transgenic kidneys (data not shown), arguing that *fgf8* misexpression did not lead to patterning defects but caused overproliferation of the ureteric epithelium, leading to enlarged cyst-like structures in the collecting duct system. All embryos exhibiting a kidney phenotype also showed gross abnormalities in the CNS (data not shown). Overproliferation of the mid/hindbrain region resulted in exencephalic structures similar to the ones described for *fgf8* misexpression under the *Wnt-1* enhancer (Lee *et al.,* 1997). Hence, phenotypes of *fgf8* misexpression were restricted to tissues in which the 8.5-kb *Pax-2* upstream region drives expression. This demonstrates the potential of the expression vector pSG5-2.Pax2(8.5) in manipulation of gene expression in kidney development *in vivo.*

DISCUSSION

The Wolffian duct plays a pivotal role in the development of the urogenital system. Starting as an extension of the

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A

N B B Spe B Nar Sph K N

8.5 kb Pax-2 upstream region

Deletion construct	#tg	#lacZ	#Wd	#mh	#ba
pPax2.Nar-Not(3.7).lacZ	17	12	12	12	12
pPax2.Sph-Not(3.0).lacZ	11	3	0	0	0
pPax2.Nar-Sph(0.7).lacZ	8	5	3	3	3
pPax2.Apa2(0.3).lacZ	4	4	0	0	0
pPax2.Apa1(0.4).lacZ	7	6	6	0	6
pPax2.PvuII-XhoII(0.5).lacZ	3	3	2	2	2

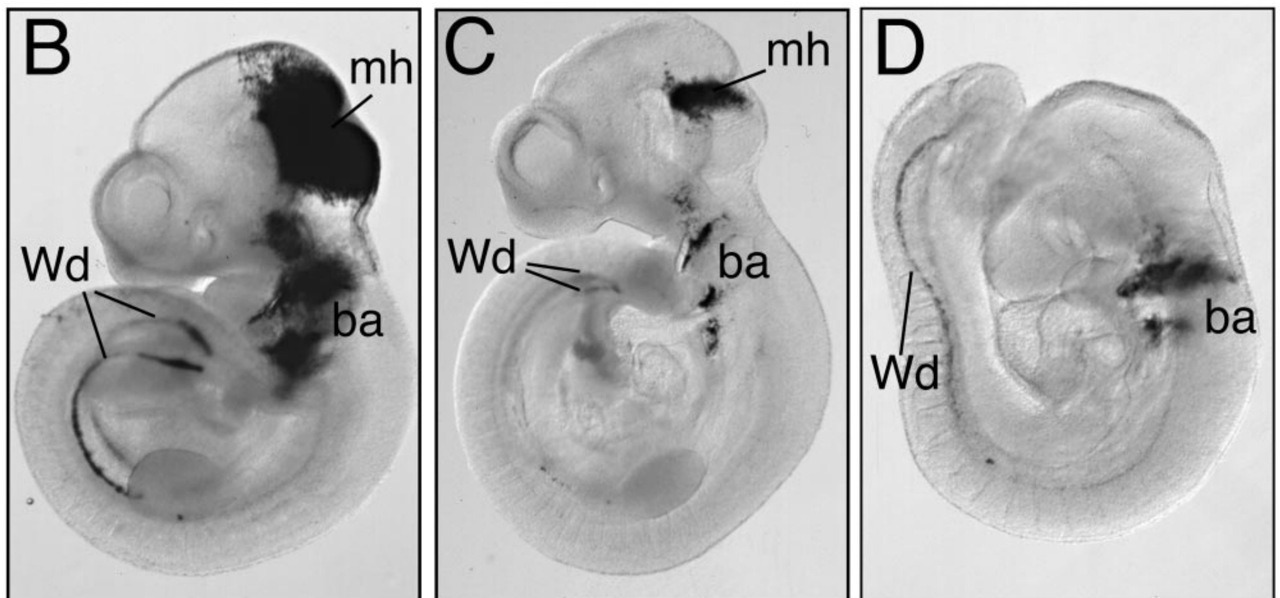

FIG. 4. Deletion analysis of the *Pax-2* Wolffian duct enhancer element in a transient *in vivo* reporter assay. (A) All deletion constructs are shown in relation to the 8.5-kb upstream region, which was tested in the construct pPax2.Not(8.5).lacZ. Subfragments of the 8.5-kb *Pax-2* upstream region were tested in a transient *in vivo* reporter assay for expression in the Wolffian duct in 9.5- or 10.5-dpc embryos. On the left the subfragments contained within the deletion constructs are indicated. #tg indicates the number of transgenic embryos obtained in total, #lacZ the total number of *lacZ*-positive embryos, #Wd the number of embryos with β-galactosidase activity in the Wolffian duct, #mh the number of embryos with β-galactosidase activity in the mid/hindbrain region, and #ba the number of embryos with β-galactosidase activity in the branchial arches. (B) 10.5-dpc embryo transgenic for the deletion construct pPax2.Nar-Not(3.8).lacZ. (C) 10.5-dpc embryo transgenic for the deletion construct pPax2.Nar-Sph(0.8).lacZ. Wolffian duct, branchial arches, and mid/hindbrain region are *lacZ* positive. (D) 9.5-dpc embryo transgenic for the deletion construct pPax2.Apa1(0.4).lacZ. Wolffian duct and branchial arches are the only *lacZ*-positive domains. All embryos were stained as whole mounts for β-galactosidase activity. ba, branchial arches; mh, mid/hindbrain region; Wd, Wolffian duct.

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```
  NarI                                                                 XhoII
GGCGCCAAGC GAGCATGGCA GAGGTGGCAG CAGAGAAGGC GAAGGAACTG AGATCCACAA GGAAGATTTA    70
CCGCGGTTCG CTCGTACCGT CTCCACCGTC GTCTCTTCCG CTTCCTTGAC TCTAGGTGTT CCTTCTAAAT


TTGGGCAGAT CAGATGCACA GAGGCGACTA ATGAAGCAAA TCCCGAGATG GGTATCAGAG CAACTCCCCA    140
AACCCGTCTA GTCTACGTGT CTCCGCTGAT TACTTCGTTT AGGGCTCTAC CCATAGTCTC GTTGAGGGGT


AAAGTTTATT TGCCTTTAAA TTTCCGCAGG GAGGCGGGCT CCTTGTTTGA AGTGTAAATG CCCCTAGGTT    210
TTTCAAATAA ACGGAAATTT AAAGGCGTCC CTCCGCCCGA GGAACAAACT TCACATTTAC GGGGATCCAA


GTGGGGGGGT GTTGAAGGGC TGCTTTGAAA ACACTAGAGA GAAAAGGTTC ATTTAGAGTC AGATGAGGGA    280
CACCCCCCCA CAACTTCCCG ACGAAACTTT TGTGATCTCT CTTTTCCAAG TAAATCTCAG TCTACTCCCT


AAAGCAACCA AGCCTGACAG GTCGAGCCCT GGCTGTGTTT GGGGTTGGGT TGTTGTCTTT CTTTTTCTTT    350
TTTCGTTGGT TCGGACTGTC CAGCTCGGGA CCGACACAAA CCCCAACCCA ACAACAGAAA GAAAAAGAAA

                                                                    ApaI
CCTCCCTTTC CTCTTCTTTC TTCTTTTCTT TTTCTACTCT TTCCCCTTCT GTTAGGGCCC ATAGAAGAAG    420
GGAGGGAAAG GAGAAGAAAG AAGAAAAGAA AAAGATGAGA AAGGGGAAGA CAATCCCGGG TATCTTCTTC


AAAAAGAACA GGAAAGGGGG AGTCAGAAGG AGAGGCCATC GAGAAGGGAC ATCTGCAAAT CAGCCCTTCC    490
TTTTTCTTGT CCTTTCCCCC TCAGTCTTCC TCTCCGGTAG CTCTTCCCTG TAGACGTTTA GTCGGGAAGG


TTCTAAAGTC CAAAGAGAGG GCTGTGCAGA GGCTAACGGG CTTCCAAAGC AGCAGGGTAG ATCGCCTTTT    560
AAGATTTCAG GTTTCTCTCC CGACACGTCT CCGATTGCCC GAAGGTTTCG TCGTCCCATC TAGCGGAAAA

                      PvuII
GATTATCTAG ACCCAGAAAC CAGCTGGAAA ACTAACAGGG AAACTGAAGG TGTGTGTGTG TGTGTGTGTG    630
CTAATAGATC TGGGTCTTTG GTCGACCTTT TGATTGTCCC TTTGACTTCC ACACACACAC ACACACACAC


TGTGTGTGTG TGTGTGTGTG TGTGTGTATG TGTGTGTGTG TGCGCGCGCG CGTCCCAGGA GTCCTGGGAA    700
ACACACACAC ACACACACAC ACACACATAC ACACACACAC ACGCGCGCGC GCAGGGTCCT CAGGACCCTT


   SphI
GGCATGC
CCGTACG
```

FIG. 5. Sequence of the 0.7-kb *Nar*I–*Sph*I fragment containing the enhancer element for Wolffian duct expression of *Pax-2.* Restriction sites used to generate further subfragments are indicated.

pronephric duct in the region of the future forelimb buds, the Wolffian duct grows caudally along the entire trunk region to the hindlimb levels and induces the formation of mesonephric tubules in the adjacent nephrogenic cord. Similarly the ureter, a budding product of the Wolffian duct, induces the metanephric mesenchyme to condense and to aggregate as a first step in forming metanephric tubules. Finally, in the male the Wolffian duct differentiates into the male sex duct components including the epididymis, the vas deferens, and the seminal vesicles, whereas in the female the Wolffian duct degenerates after 14.5 dpc.

An Enhancer Element in the Pax-2 Upstream Region Specific for the Wolffian Duct and Its Derivatives during All Stages of Development

Our analysis of the *Pax-2* locus has identified an 8.5-kb upstream region which is sufficient to mediate marker gene

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expression in the Wolffian duct during all developmental stages. We first detected *lacZ* expression in the pronephric region at 8.25 dpc. *lacZ* expression in the region between somites 4 and 10 is very transient, compatible with the idea that the pronephros degenerates quickly. Between 8.5 and 10.5 dpc the Wolffian duct extends caudally. During these stages we did not notice *lacZ*-expressing cells outside the epithelial Wolffian duct, arguing that expression in the nephrogenic mesenchyme is independently regulated. This is likely since *Pax-2* expression in the mesenchyme is induced by signals from the Wolffian duct (Dressler *et al.,* 1990; Obara-Ishihara *et al.,* 1999; Rothenspieler and Dressler, 1993). It has been argued that cells from the meso- and metanephric tubules may not entirely derive from nephrogenic mesenchyme but may also be contributed by the epithelium of the Wolffian duct and its derivative the ureter (Arend *et al.,* 1999; Qiao *et al.,* 1996). Due to the longevity of its gene product, β-galactosidase activity can be used as a short time lineage tracer. Thus, if cells leave the epithelial sheet of the Wolffian duct to contribute to the nephrogenic mesenchyme we should observe *lacZ*-positive cells in the mesenchyme adjacent to the epithelium. However, at our level of resolution we failed to detect β-galactosidase-positive cells outside the Wolffian duct epithelium.

In contrast, we did observe disintegration of the anterior end of the Wolffian duct starting from 10.5–11 dpc on. We noticed individual as well as groups of *lacZ*-expressing cells in the region of the caudal end of the urogenital system. These cells may be taken up in adjacent organ rudiments such as sex ducts but potentially also the gonads. A more detailed analysis is required to determine the contribution of the disintegrating Wolffian duct to the neighboring structures. However, it has been reported that cells from the cranial mesonephric tubules disintegrate and contribute to the male sex ducts (Sainio *et al.,* 1997) and that mesonephric cell migration induces testis cord formation in the mammalian gonad (Tilmann and Capel, 1999). Our data support the notion that possibly in both sexes cells from the anterior end of the Wolffian duct may become mesenchymal and be respecified in different cell lineages within the sex organs.

At 14.5 dpc we consistently detected dispersed groups of cells along the epithelium of the Müllerian ducts. It is unclear whether this *lacZ* expression domain reflects a weak independent Müllerian duct enhancer present in the *Pax-2* upstream region. Alternatively, the Wolffian duct enhancer may be transiently activated in the epithelium of the Müllerian duct. Third, cells from the anterior part of the Wolffian duct may have populated the Müllerian duct epithelium. Our data do not allow us to discriminate between these possibilities.

lacZ staining in the epithelia of the collecting duct system of the kidney, the ureter, the epididymis, the vas deferens, and the seminal vesicles persisted in the male until at least 18.5 dpc, the last stage analyzed. Since *Pax-2* expression continues into postnatal stages (Oefelein *et al.,* 1996) it is likely that the Wolffian duct enhancer operates for some time longer.

The *lacZ* staining in the kidney was found throughout the entire collecting duct system although endogenous *Pax-2* expression is confined to the ureter in the periphery of the kidney (Dressler *et al.,* 1990). Further work will be required to determine whether the persistence of β-galactosidase activity in the more mature collecting duct epithelium reflects the longevity of the protein or points to the presence of an element outside the 8.5-kb upstream region normally restricting *Pax-2* expression to the undifferentiated ureteric epithelium.

Initiation and Maintenance of Pax-2 Expression in the Wolffian Duct Epithelium Is Regulated by Independent Elements

Pax-2 is expressed in distinct cell types within the developing urogenital system: the developing and differentiated epithelia of Wolffian duct, ureter, and collecting duct system; the epithelium of the Müllerian duct; and the nephrogenic mesenchyme (Dressler and Douglas, 1992; Dressler *et al.,* 1990). Our analysis of the 8.5-kb upstream region (as well as 8.5-kb downstream region, data not shown) discovered the presence of only one region controlling expression in the Wolffian duct and its derivatives. Thus, other aspects of *Pax-2* urogenital expression are regulated independently.

Our deletion analysis identified a 0.4-kb region required for mediating initiation of marker gene expression in the Wolffian duct of 9.5- to 10.5-dpc embryos. Our analysis of lines transgenic for the 4-kb upstream region showed that *lacZ* expression in the Wolffian duct and the ureter from 11.5 dpc on is discontinuous, weak, and spotty. This points to the presence of an element within the −8 and −4 kb *Bam*HI fragment which is required for enhancement and/or maintenance of *Pax-2* expression in these structures. This result is in agreement with the finding that mouse embryos homozygous mutant for *Pax-2* develop a short Wolffian duct (Torres *et al.,* 1995). This indicates that *Pax-2* expression is not required for Wolffian duct establishment but for its maintenance and elongation.

We have recently reported that the same 8.5-kb element described here harbors an enhancer for mid/hindbrain expression of *Pax-2* (Rowitch *et al.,* 1999). Our deletion analysis could separate the elements for Wolffian duct expression from that for mid/hindbrain expression of *Pax-2,* suggesting that these expression domains are not regulated by a common factor. In contrast, we could not separate the element for branchial arch expression from the Wolffian duct element at our level of resolution. It is conceivable that this reflects regulation by a factor coexpressed in both structures. However, it is noticeable that *Pax-2* is not expressed in the branchial arch region. Thus, it is likely that the branchial arch element located in the 0.4-kb *Apa–Nar* fragment is normally silenced by factors binding to elements outside the 8.5-kb upstream region in the *Pax-2* genomic locus.

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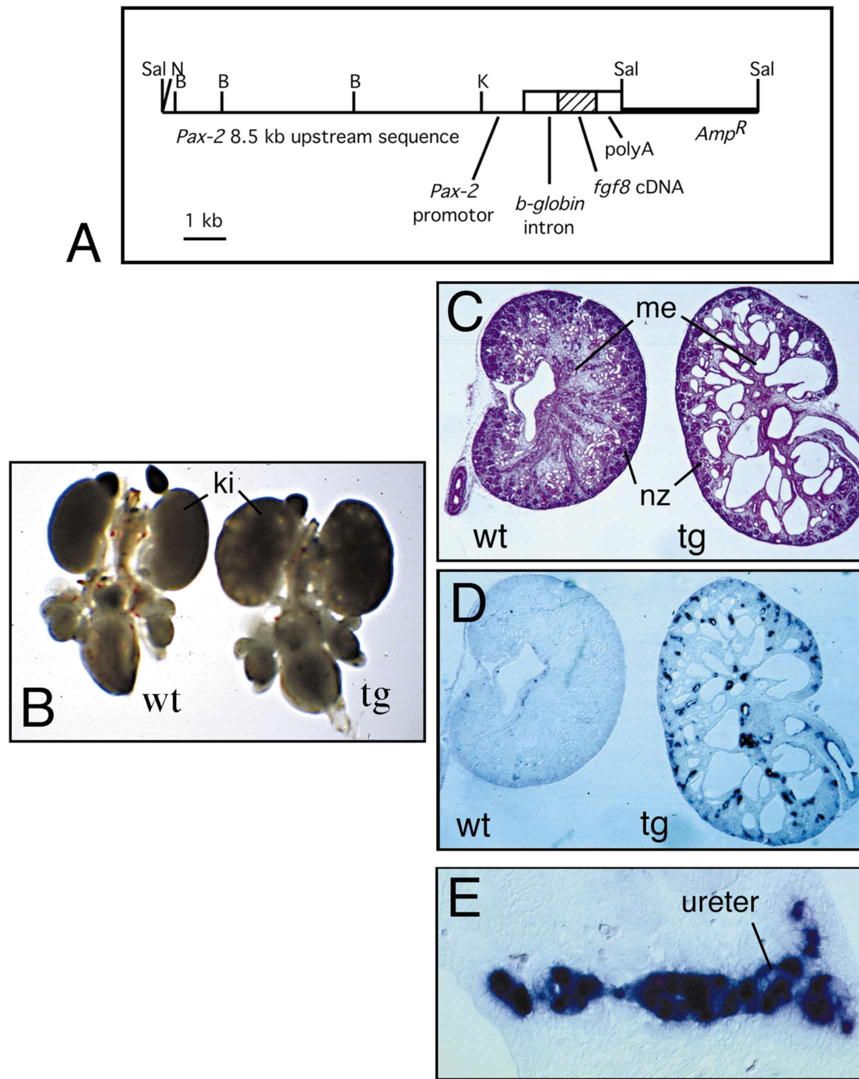

FIG. 6. Misexpression of *fgf8* under the control of the 8.5-kb *Pax-2* regulatory region results in polycystic kidneys. (A) Structure of the misexpression vector pSG5-2.Pax2(8.5).fgf8 comprising the 8.5-kb *Pax-2* enhancer/promoter, a β-globin intron, and a poly(A) signal. *fgf8* was cloned into an *Eco*RV site as described. (B) Whole-mount preparations of 17.5-dpc urogenital systems of a transgenic embryo and a nontransgenic littermate. Note the enlargement and the appearance of bright spots in the transgenic kidney. (C) Histological analysis of metanephric kidneys of a 17.5-dpc embryo transgenic for pSG5-2.Pax2(8.5).fgf8 and a nontransgenic littermate. The transgenic kidney is characterized by large cysts in the medulla of the kidney. The outer nephrogenic zone appears normal. (D) *In situ* hybridization analysis for *fgf8* expression in metanephric kidneys of a 17.5-dpc embryo transgenic for pSG5-2.Pax2(8.5).fgf8 and a nontransgenic littermate. Strong *fgf8* expression is found in the ureteric epithelium of the transgenic kidney. (E) Enlargement of the peripheral region of the transgenic kidney shown in D. *fgf8* expression is strong but spotty in the ureteric epithelium. ki, metanephric kidney; me, medulla; nz, nephrogenic zone; wt, wild type; tg, transgenic.

Factors Controlling Pax-2 Expression in the Wolffian Duct

As a first step toward identifying the factor(s) regulating initiation of *Pax-2* expression in the Wolffian duct we have sequenced the minimal region for marker gene activation, the 0.4 *Nar*I–*Apa*I fragment. Analysis of this sequence with the TRANSFAC program has identified consensus binding sites for a couple of transcription factors, including homeobox, Smad, and bHLH transcription factors. However, further deletion experiments will be required to define a smaller fragment suitable for a direct biochemical analysis of potential transcription factor binding sites. Recently embryological experiments in the chick demonstrated the importance of surrounding tissues for *Pax-2* expression in the pronephros and the Wolffian duct (Mauch *et al.,* 2000; Obara-Ishihara *et al.,* 1999) Mauch *et al.* (2000) showed that

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signals from the paraxial mesoderm induce pronephros formation (and *Pax-2* expression) in the intermediate mesoderm. Removal of ectoderm, endoderm, midline mesoderm, and lateral plate mesoderm does not affect pronephros induction. In contrast, Obara-Ishihara (1999) described that removal of surface ectoderm led to loss of *Pax-2* expression and a halt in Wolffian duct elongation. BMP-soaked beads placed in the intermediate mesoderm restored *Pax-2* expression and Wolffian duct elongation, pointing to the importance of BMP/activin/Smad signaling for *Pax-2* expression. Possibly, this experimental discrepancy can be resolved by assuming a temporal order of disparate signals from the surrounding tissues to the intermediate mesoderm. It is conceivable that pronephros and *Pax-2* induction in the intermediate mesoderm is triggered by signals from cervical somites, whereas Wolffian duct elongation and *Pax-2* maintenance in the mesonephric region is mediated by surface ectoderm- or lateral plate mesoderm-derived BMP-4 signal. In future work, we will use our Pax2(8.5).lacZ transgenic line to address the importance of various signaling pathways for *Pax-2* expression.

The Pax-2 Wolffian Duct Element Is Useful in Transgenic Misexpression Experiments

The general usefulness of the *Pax-2* upstream region to drive ectopic gene expression was tested using *fgf8* as a foreign cDNA. Recently, misexpression of *fgf8* in the developing brain led to patterning defects as well as overproliferation of the affected tissues (Lee *et al.,* 1997). Similarly, we observed overproliferation in the CNS, leading to exencephalic structures, and in the collecting duct epithelium, resulting in polycystic kidneys, a phenotype common to several congenital diseases in human (Consortium, 1994; Mochizuki *et al.,* 1996). Hence, misexpression of *fgf8* under the *Pax-2* upstream region affected only tissues for which the *Pax-2* upstream region harbors enhancer elements. We have started to misexpress other genes like *Wnt-11* in the collecting duct system to better understand their normal role in kidney development.

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