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Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms tumor

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ABSTRACT **The murine *Pax-2* gene contains a protein coding domain homologous to the *Drosophila* paired-box, first described in certain developmental control genes of the segmentation type. Polyclonal antibodies recognize two Pax-2 proteins that are encoded by differentially spliced mRNAs. The Pax-2 proteins can bind a DNA sequence known to interact with the paired domain of a *Drosophila* protein. By immunocytochemistry, expression of Pax-2 could be localized to the nuclei of condensing mesenchyme cells and their epithelial derivatives in the developing kidney. Expression is abruptly downregulated as the tubular epithelium differentiates. High levels of Pax-2 expression could also be detected in the epithelial cells of human Wilms tumors. These data suggest that Pax-2 is a transcription factor active during the mesenchyme-to-epithelium transition in early kidney development and in Wilms tumor.**

Although the mammalian kidney has long been an excellent model system for investigating epithelial–mesenchyme inductive interactions during development (1, 2), the genetic basis of the resulting morphological changes in both inducing and responding tissues remains unclear. The development of the mammalian kidney is initiated as the metanephric mesenchyme cells are induced by the ureteric bud to condense and form the metanephric blastema (for review, see ref. 3). In turn, the ureter is induced reciprocally to branch repeatedly and thus to form the collecting duct system. With the formation of early epithelial structures derived from the mesenchyme, changes in extracellular protein components are clearly evident. The adhesion molecule uvomorulin, or E-cadherin, is expressed 48 hr after induction (4), whereas the neural cell adhesion molecule is reduced (5). The basement membrane protein laminin undergoes transient expression of the A chain during epithelial cell polarization (6–8). Proteins that function in intercellular adhesive junction formation, such as desmosomal proteins 1 and 2 (9) and syndecan (10), are also expressed in the early epithelium. These changes in structural gene expression must be accompanied by activation or induction of specific transcription factors that coordinate these morphological changes in the developing kidney.

Recently, a family of murine *Pax* genes has been identified (11) based on a common protein coding domain, the paired-box, first described in the *Drosophila* segmentation genes paired and gooseberry (12). The *Pax* genes are expressed during embryogenesis in a tissue-restricted manner (13–18). The *Pax-1* gene is expressed in sclerotome and intervertebral disc cells and corresponds to the mouse developmental mutation undulated (*un*) (19), indicating that the Pax-1 protein functions during segmentation or migration of vertebral column structures. That the *Pax-2* gene product may function in early kidney epithelium formation has already been suggested by *in situ* hybridization analysis of Pax-2 mRNA in the developing mouse embryo (14). The *Pax-2* gene is also expressed in the developing neural tube in a pattern suggestive of a function during differentiation of specific neural cell types (15). Yet, very little is known about the Pax-2 protein.

In this report, polyclonal antibodies generated against a unique domain of Pax-2 were used to analyze Pax-2 proteins in developing mouse kidney and in human Wilms tumors. The expression of Pax-2 is localized to the nucleus of cells in condensing kidney mesenchyme and early epithelial structures derived from the mesenchyme. In Wilms tumor, Pax-2 expression is restricted to the epithelial component. In addition, it is demonstrated that Pax-2 protein can bind a specific sequence found in the *Drosophila* even-skipped gene promoter. These data suggest that Pax-2 is a transcription factor active in the early kidney development and in Wilms tumor.

MATERIALS AND METHODS

Construction, Purification, and Immunization of Pax-2 Fusion Protein. The Pax-2 cDNA sequences corresponding to amino acids 188–385 were isolated as a *Bam*HI–*Eco*RI fragment from the cDNA plasmid c31-A (14) and were cloned into the *Bam*HI–*Eco*RI sites of the vector pGex-3X encoding the 26-kDa glutathione *S*-transferase gene of *Schistosoma japonicum* (20). The resulting plasmid, pF-Pax-2, produces a fusion protein with a predicted molecular size of approximately 48 kDa. Expression and purification of the fusion protein were as described (20). Rabbits were immunized intramuscularly and intradermally with 250 μg of fusion protein in complete Freund's adjuvant. Rabbits were boosted in 3-week intervals with 150 μg of fusion protein in incomplete Freund's adjuvant. The IgG fractions were purified on protein A-Sepharose columns.

Immunoblotting (Western Blotting). Whole protein extracts were made by boiling cell pellets or embryonic tissues in 1× SDS loading buffer [50 mM Tris base/1% SDS/10% (vol/vol) glycerol/1% 2-mercaptoethanol/0.01 mg of bromophenol blue per ml]. Samples were loaded onto SDS/8% acrylamide gels, electrophoresed, and electroblotted onto nitrocellulose (21). Proteins were stained with dilutions of anti-Pax-2 IgG (1:250–1:1000) and an alkaline phosphatase-conjugated second antibody by using a Bio-Rad immunostaining kit according to the manufacturer's protocol.

S1 Nuclease Protection. The cDNA subclone containing the *Bam*HI–*Hin*dIII fragment of cPX (14) in the vector Bluescript KS(+) (Stratagene) was digested with *Bst*EII and dephosphorylated with calf intestinal alkaline phosphatase. After digestion with *Sac* I, a probe of 490 base pairs (bp) was isolated that contains the *Pax-2* sequences from nucleotides

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Abbreviation: E11–E18, embryonic days 11–18.

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887–1339 and an additional 38 bp of vector sequence. The double-stranded probe was labeled by using polynucleotide kinase and [γ-^{32}P]ATP. The S1 mapping procedure was as described (22).

Preparation of Nuclear Extracts and Gel Shifts. Kidneys were isolated from embryonic day 17 (E17) embryos, 3 to 4 litters at a time, and washed in phosphate-buffered saline (PBS). The nuclear extracts were prepared as described (23) and dialyzed in Z-buffer (25 mM Hepes, pH 7.8/12.5 mM $MgCl_2$/1 mM dithiothreitol/0.1% Nonidet P-40/20% glycerol) with 0.1 M KCl. For the electrophoretic mobility-shift assay, 5 μl of extract was mixed with 1 μg of poly(dI-dC) and 10,000 dpm of gel-purified ^{32}P-labeled oligonucleotide (0.2–0.5 ng) in a 10-μl volume. For the antibody control, DNA and extract were preincubated for 15 min and ammonium sulfate-precipitated antibodies were added to 100 μg/ml. After incubation at 30°C for 15 min, the DNA–protein complexes were run on 6% neutral acrylamide gels in 0.5× TBE (45 mM Tris borate/1 mM EDTA) running buffer.

To purify Pax-2 proteins, the IgG fractions of anti-Pax-2 antiserum were amino-coupled to agarose beads by using the ImmunoPure antibody immobilization kit (Pierce) according to the manufacturer's directions. The nuclear extract was run through the column three times. After being washed repeatedly with PBS, bound proteins were eluted with 0.1 M glycine (pH 3.0), neutralized with 1 M Tris (pH 8.0), and dialyzed in Z-buffer with 0.1 M KCl.

Immunocytochemistry. Embryos were dissected free of extraembryonic tissue and frozen on dry ice. Cryostat sections were cut at 8 μm, collected on gelatinized slides, and air-dried for 30–60 min. Immunostaining was as described by Harlow and Lane (24). Sections were fixed in acetone for 10 min at −20°C and then washed in PBS containing 0.05% Tween-20 (PBS/Tween) twice for 5 min. A dilution of rabbit anti-Pax-2 IgG to 10 μg/ml in a 1:500 dilution of rat anti-uvomorulin (Sigma) was prepared in 2% goat serum in PBS; 20 μl was applied to each section, which was incubated at room temperature for 1 hr in a humid chamber. Slides were washed twice in PBS/Tween. The second antibodies were diluted 1:32 for the tetramethylrhodamine B isothiocyanate (TRITC)-conjugated anti-rabbit antibody (Sigma) and 1:20 for the fluorescein isothiocyanate (FITC)-conjugated anti-rat antibody (Sigma) in 2% goat serum in PBS. After a 30-min incubation, slides were washed twice in PBS/Tween and covered with Gelvatol (24). Control sections were incubated with a preimmune rabbit IgG purified fraction and both second antibodies. No specific staining could be detected with the preimmune IgG in either embryonic kidney or Wilms tumor (data not shown).

RESULTS

Two Pax-2 Proteins in Embryonic Kidney and Wilms Tumor. In the mouse embryonic kidney, two proteins were detected with the Pax-2 antibodies (Fig. 1*a*): a major band at 46 kDa, Pax-2b, and a minor band migrating slightly higher at 48 kDa, Pax-2a. The ratio of Pax-2a to Pax-2b appeared similar from E12 to E17. Very little Pax-2 was detected in the newborn, and none was detected in the adult kidney. Because previous *in situ* hybridization data suggested *Pax-2* expression in the induced mesenchyme and early epithelium (14), we examined human Wilms tumors for *Pax-2* expression, since these are tumors of embryonic origin with an epithelial component. Two of the three tumor samples, AB and LO, reacted strongly with the anti-Pax-2 antibodies (Fig. 1*b*). The tumor marked GM was almost completely necrotic, with very few distinguishable cells observed upon histological analysis (data not shown). The size and relative abundance of the two human Pax-2 proteins were similar to those of the embryonic mouse kidney, thus indicating strong evolutionary conservation of the *Pax-2* gene. Note that an embryonic stem cell line transformed with a plasmid containing the Pax-2a form of the cDNA expressed only the larger 48-kDa protein, although the level of expression was low. Thus, it is unlikely that the larger 48-kDa protein is a posttranslational modified form of the smaller 46-kDa Pax-2 protein. It also should be noted that a smaller 42-kDa species was observed upon prolonged staining, particularly in samples with high levels of Pax-2. The level of this 42-kDa species was variable, and presently it is not clear whether this represents a breakdown product of Pax-2, a cross-reacting protein, or an additional minor form of Pax-2.

To determine if the ratio of Pax-2a and Pax-2b mRNAs corresponds to the difference in protein levels, an S1 nuclease protection experiment was designed to determine the relative abundance of Pax-2a and Pax-2b mRNAs (Fig. 1*c*). Fig. 1*d* shows the results of an S1 nuclease mapping experiment using E17 and E18 kidney RNAs, E12 neural tube RNAs, E11 whole embryonic RNAs, and control RNAs. The protected probe that corresponds to the Pax-2b mRNA was predominant, being 5- to 8-fold more abundant than Pax-2a as determined by densitometer scanning. The relative ratio of

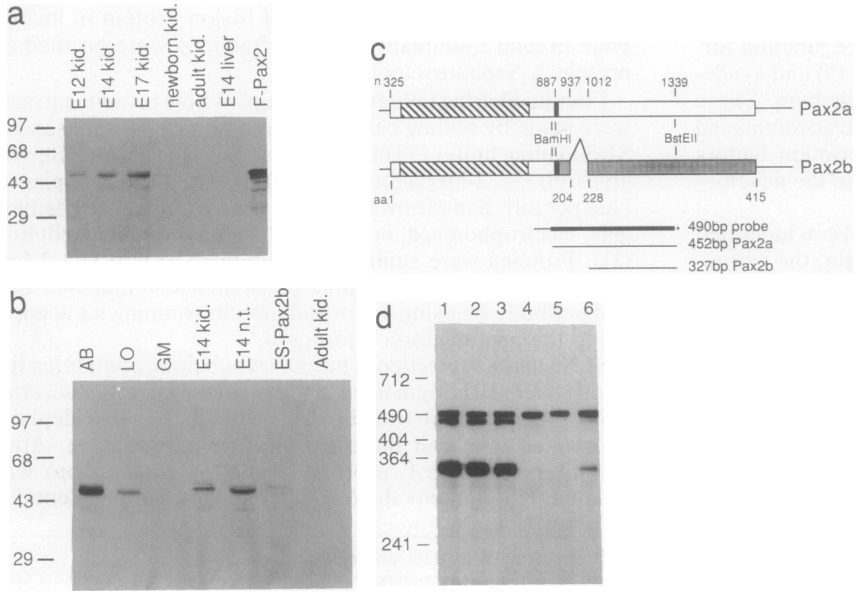

FIG. 1. (*a*) Western blot of Pax-2 proteins in embryonic mouse kidney (kid.). Tissue samples were obtained at the times indicated. F-Pax-2 is 2.5 ng of the purified bacterial fusion protein. (*b*) Western blot of Wilms tumor samples AB, LO, and GM and of embryonic kidney (kid.) and neural tube (n.t.) probed with Pax-2 antibodies. ES-Pax is a stably transformed cell line expressing Pax-2a (G.R.D. and M. L. Tremblay, unpublished data). (*c*) Schematic of the two potential Pax-2 mRNAs differing by alternative splicing at nucleotides 937–1012 (11). The hatched box represents the paired domain, the solid box is the paired octapeptide, and the shaded region of Pax-2b is the domain used in the fusion protein. The probe 490bp was designed to distinguish between Pax-2a and Pax-2b forms of the mRNAs. (*d*) Results of S1 nuclease protection experiment with total kidney RNA at E17 (lane 1) and E18 (lane 2), neural tube RNA at E12 (lane 3), no RNA (lane 4), yeast RNA (lane 5), and total RNA at E11 (lane 6).

Pax-2a to Pax-2b mRNA corresponds very well with the relative abundance of the two proteins detected with the antibodies. These data suggest that the 48-kDa and the 46-kDa Pax-2 proteins are derived from two alternatively spliced Pax-2a and Pax-2b mRNAs.

Pax-2 Proteins Bind DNA. Recent results with the *Drosophila* paired protein indicate binding to the *e4* and *e5* sequences of the 5′ region of the even-skipped gene promoter via the paired domain and the paired-type homeobox, each protecting two different but overlapping sequences in a DNase I footprinting assay (25). The *Drosophila* paired domain is capable of binding the *e5* sequence independently (25); and the mouse Pax-1 protein can bind derivatives of the *e5* sequence with good affinity (26).

To determine if the mouse Pax-2 protein can bind DNA, the 15-bp subsequence of the *e5* region that is specifically protected by the paired domain was used in an electrophoresis mobility shift assay. Initial experiments used a 45-bp trimer of the *e5* subsequence (Fig. 2*a*); this oligonucleotide is called *e5.3*. With an embryonic kidney nuclear extract, several shifted species were detected. By preincubating the nuclear extract with the *e5.3* oligonucleotide and then adding Pax-2 antibodies, a major band shift was no longer observed, and a very high molecular weight species was evident that barely ran into the gel (Fig. 2*a*). The additional faster migrating, shifted species were unaffected by the Pax-2 antibody addition. From the nuclear extract, the Pax-2 protein was purified by an immunoaffinity column and tested for binding. This purified protein fraction could shift *e5.3* and comigrated with a major band seen with the nuclear extract (Fig. 2*a*). The faster migrating species were not observed when using the purified Pax-2 fraction. All of the observed shifted species could be blocked by competition with an excess of unlabeled *e5.3* DNA, indicating specificity. With a ×50 molar excess, there was an 80% reduction of the Pax-2 specific shift as determined by densitometer scanning, and a ×250 molar excess almost blocked out all the labeled *e5.3*.

Two additional DNAs were tested for binding: the *e5.1* oligonucleotide contained only one copy of the paired domain

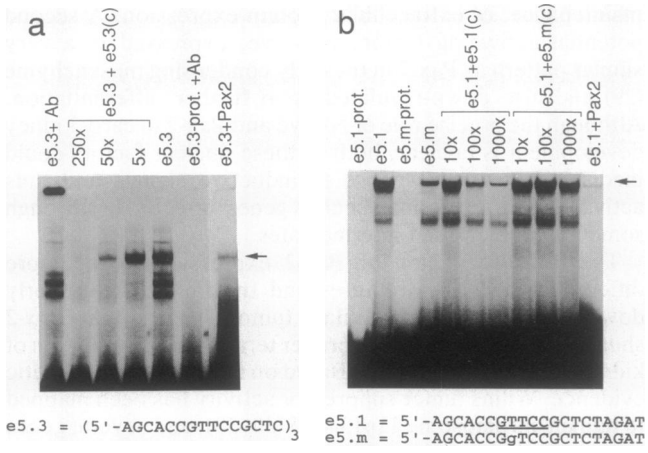

FIG. 2. Binding of Pax-2 to the *Drosophila e5* subsequence. Shifts are generated with embryonic kidney nuclear extracts, unless otherwise indicated, as −protein (Z-buffer alone) or +Pax-2 (immunoaffinity-purified Pax-2). The unlabeled competitor DNA, indicated as "(c)," was added in increasing molar excess amounts. Ab indicates the addition of 100 μg of Pax-2 antibodies per ml after preincubation of extract and DNA. The arrow indicates the migration position of the Pax-2–DNA complex. (*a*) Specific binding of *Pax-2* to *e5.3* DNA. The sequence of *e5.3* is shown. (*b*) Binding of Pax-2 to *e5.1* and *e5.m* DNAs. The *e5.1*–Pax-2 shift was subject to competition with unlabeled *e5.1* DNA and *e5.m* DNA. The sequences of the oligonucleotides are shown, and the core paired-domain binding sequence, as defined by Chalepakis (26), is underlined.

binding sequence, and the *e5.m* oligonucleotide had a specific point mutation in the core sequence that was known to decrease the binding efficiency (26). With whole nuclear extracts, Pax-2 bound at least a 5- to 10-fold greater amount of *e5.1* compared with *e5.m* (Fig. 2*b*). An excess of unlabeled *e5.1* could competitively block out the Pax-2 specific shift bound to the labeled *e5.1* DNA, although competition was not complete, even after a 1000-fold molar excess (Fig. 2*b*). However, the mutant *e5.m* DNA could not compete for binding to *e5.1* DNA even with a ×1000 molar excess (Fig. 2*b*). Thus, the T → G point mutation in the core of the binding site dramatically reduced the efficiency of Pax-2 specific binding to the *e5* sequence. Furthermore, the trimer DNA *e5.3* bound significantly better than the *e5.1* DNA, since a ×250 molar excess competitively blocked most of the specific shifted species. The purified Pax-2 fraction showed a single shifted species that comigrated with the major band seen with whole embryonic kidney nuclear extracts. Although these data indicate specific binding of Pax-2 to the *e5* subsequence, the binding was not of high affinity as evidenced by the inability to completely compete with unlabeled *e5.1*. It is probable that differences in the paired domains among the various mouse Pax proteins as well as the *Drosophila* proteins would result in differential recognition of the *e5* sequence and may indicate optimum binding to unique derivatives of the *e5* sequence for each protein.

Localization of Pax-2 in Embryonic Kidney and Wilms Tumor. To confirm the expression of protein in the developing kidney, to better resolve the morphological features of expressing cells and tissues, and to determine the intracellular localization, a detailed immunocytochemical analysis was begun. In the developing excretory system, Pax-2 proteins could be detected at E10 in the Wolffian duct and in pronephric and mesonephric tubules (data not shown). By E12, the ureter has branched four to eight times, and the kidney begins to assume its characteristic shape. Pax-2 protein is readily detected in the kidney, and protein levels are highest in the nuclei of condensing mesenchyme cells and in the nuclei of ductal epithelium furthest from the ureter (Fig. 3*a*). Fig. 3*b* shows a high magnification of the tip of a growing collecting duct where uvomorulin, an epithelial-specific adhesion molecule (27), is stained with fluorescein and Pax-2 is stained with rhodamine. Note the high level of protein in the nuclei of cells condensing at the tip of the growing duct. Nuclei of the ductal epithelium also contain Pax-2 protein, although at a somewhat lower level. At E14, Pax-2 protein is still seen in most of the early epithelial structures and is particularly evident in the nephrogenic region along the kidney perimeter (Fig. 3*c*). Upon closer examination (Fig. 3*d*), expression of Pax-2 is strong in the comma-shaped body but has begun to decrease in the proximal loop of the S-shaped body. A dramatic decrease in Pax-2 protein levels is evident in the more developed areas of an E17 kidney (Fig. 3 *e* and *f*). At this stage, the mesenchyme-derived tubular epithelium and the ureter-derived collecting duct system are extensive. Mature glomeruli are easily distinguished morphologically (Fig. 3*f*). Pax-2 protein is still expressed in the mesenchyme of the nephrogenic zone and in the end buds of the ductal epithelium. However, little protein was detected in the tubular epithelium, stained with anti-uvomorulin and in the mature podocytes of the glomeruli located more medially (Fig. 3*f*). Some of the major collecting ducts still contain detectable amounts of Pax-2 proteins (Fig. 3*e*).

Immunocytochemical analysis was extended to the Wilms tumor samples that had been examined by Western blotting. Wilms tumor typically exhibits a triphasic structure consisting of blastemal, epithelial, and stromal cell types (28), all of which can be at various stages of differentiation. Fig. 4 shows histological sections of two different tumors and adjacent

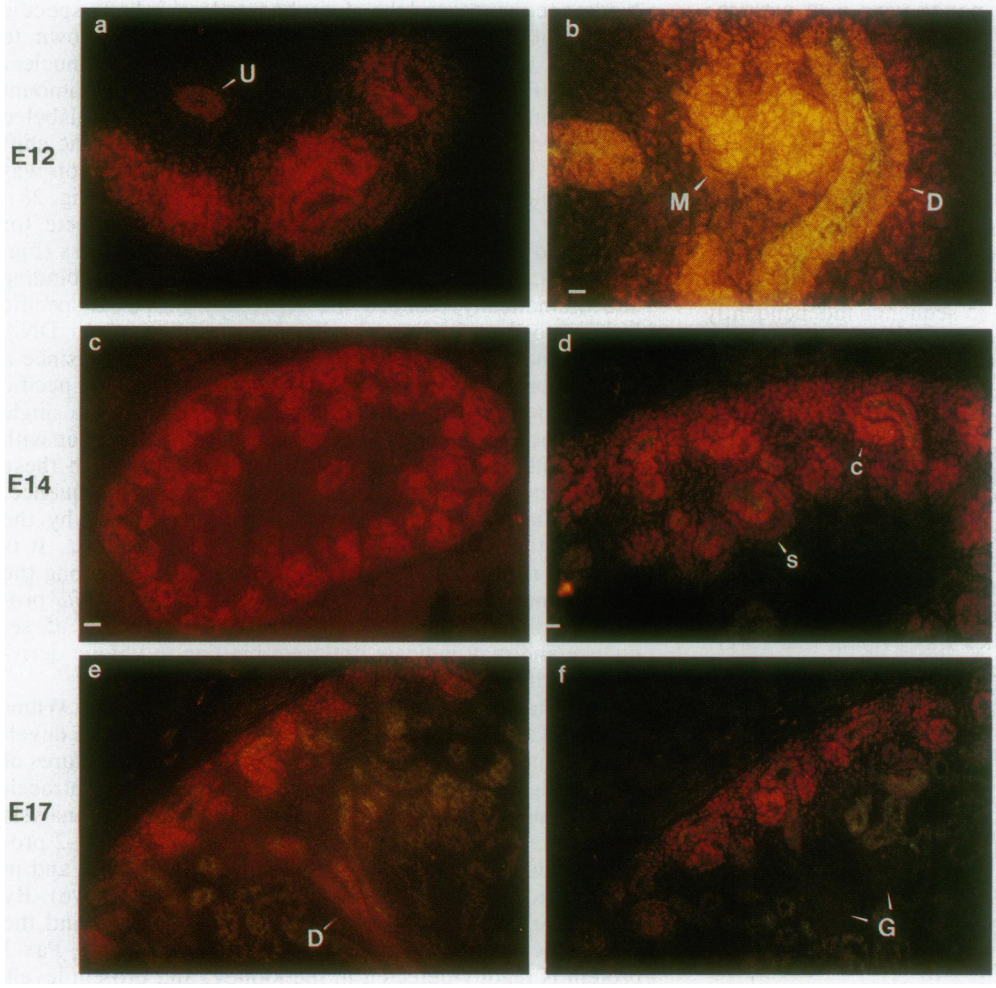

FIG. 3. Localization of Pax-2 protein in mouse kidney development. Pax-2 is stained with rhodamine in all micrographs, and uvomorulin is stained with fluorescein in *b*, *d*, *e*, and *f*. (*a*) Para-sagittal section through an E12 embryo showing the metanephros. The ureter (U) is indicated. (*b*) Higher magnification of epithelial duct (D) and condensed mesenchyme (M). (Bar = 13 μm.) (*c*) Para-sagittal section through an E14 embryo showing *Pax-2* expression in the kidney. (Bar = 50 μm.) (*d*) E14 kidney showing a typical S-shaped body (S) and a comma-shaped body (C). (Bar = 25 μm.) (*e*) Para-sagittal section through E18 embryo showing the nephrogenic zone, kidney perimeter, and major collecting duct (D). (*f*) Different section of E18 embryonic kidney. Note *Pax-2* expression is still in mesenchymal condensations of the nephrogenic zone but is clearly absent in mature glomeruli (G). (*a*, *d–f*, ×140; *b*, ×280; *c*, ×70.)

serial sections stained with the Pax-2 antibodies. In the tumor LO, the expression of Pax-2 appears to be restricted to an epithelial-like component of the tumor, whose structure is poorly defined (Fig. 4 *a* and *b*). The stromal cells are clearly negative, and the small blastemal-like cells also do not express Pax-2. The tumor AB consists of random patches of more tubule-like epithelium separated by densely packed stroma (Fig. 4*c*). Clearly, the epithelial component of tumor AB stains positive for Pax-2 expression (Fig. 4*d*). Although this tumor consists of predominantly disorganized tubular epithelium that expresses Pax-2, some areas show only small clusters of tubular epithelium within a more undifferentiated epithelial component, not expressing Pax-2 (Fig. 4*e*). Higher magnification of more differentiated tubular epithelium shows nuclear localization of Pax-2 staining (Fig. 4*f*). The higher percentage of Pax-2 expressing cells found in tumor AB, relative to tumor LO, is consistent with the increased Pax-2 protein levels observed by Western blotting.

DISCUSSION

The expression profile in developing kidney and Wilms tumor suggests that Pax-2 may be required for the early mesenchyme-to-epithelium transition. *Pax-2* expression, already apparent in the ureteric bud, is activated in the condensing mesenchyme and its early epithelial derivatives, the comma-shaped bodies and the S-shaped bodies. However, at or soon after the S-shaped body phase, *Pax-2* expression is abruptly down-regulated. Although there are many extracellular proteins expressed in developing renal tubules, including laminin, desmosomal components, uvomorulin, and syndecan, how the structural genes encoding these proteins are transcriptionally activated remains unclear. As a DNA-binding and potential regulatory protein, perhaps Pax-2 is a transcription factor involved in the activation, but not necessarily the maintenance, of extracellular protein expression. A second potential activating factor is N-myc, expressed in a very similar pattern as Pax-2 in the early condensing mesenchyme (29) and also down-regulated upon further differentiation. Although the precise role of N-myc and Pax-2 in early kidney development is unclear, both of these nuclear factors could mediate the early response to inductive signals and thus activate more epithelial-specific genes directly or through some still unidentified intermediates.

The down-regulation of *Pax-2* expression in the more differentiated renal structures and the failure to properly down-regulate *Pax-2* in Wilms tumor suggest that *Pax-2* shut-off may be required for proper terminal differentiation of kidney epithelial structures. Based on several lines of genetic evidence, Wilms tumor suppressor activity has been mapped to human chromosome 11p13–p15 (30–33), and several genes have recently been cloned from this region (34–37). Interestingly, the Zn finger-containing gene *WT1* (34, 35) is expressed during kidney development but is down-regulated at a later stage compared with *Pax-2*. Expression is detected at the S-shaped body stage, reaches a peak, and decreases in the glomerular capsule (38). In the mouse expression of *WT1* peaks at about 3 days postpartum and decreases to undetectable levels in the adult (39). The *WT1* gene can bind DNA specifically, indicative of transcription regulatory proteins (40). Given the abrupt down-regulation of *Pax-2* and the unique but partially overlapping expression patterns of *Pax-2* and *WT1*, one may speculate whether *Pax-2* is a target for *WT1* repression.

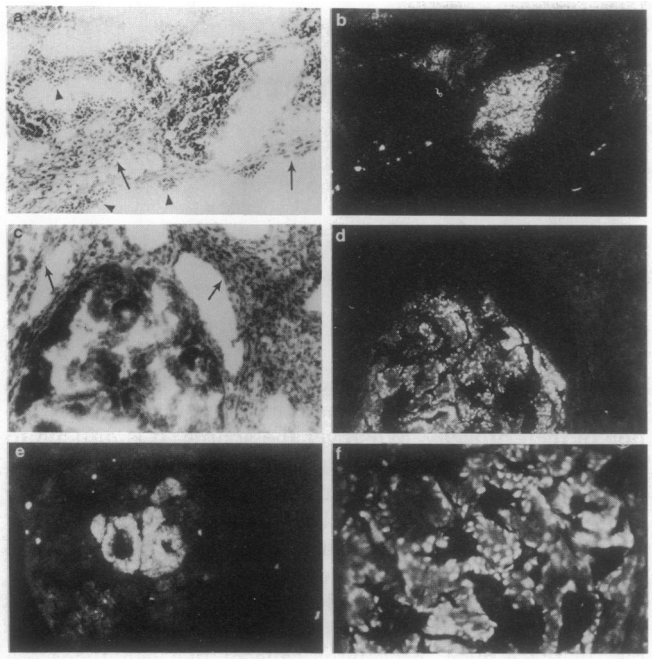

FIG. 4. Localization of Pax-2 in Wilms tumor. (*a*) Hematoxylin-and-eosin-stained cryostat sections of Wilms tumor sample LO. Arrows indicate the stromal cells, and arrowheads point to clusters of small blastemal-like cells. (*b*) Adjacent section to *a* showing expression of Pax-2 predominantly in the epithelial component of the tumor. (*c*) Stained section of tumor AB showing tubular epithelium and stromal cells (arrows). (*d*) Adjacent section to *c* showing Pax-2 expression in the tubular epithelium. (*e*) Different section of tumor AB, near the perimeter of excision, showing Pax-2 expression in more differentiated tubular epithelium, whereas surrounding epithelium is mostly negative. (*f*) Higher magnification showing nuclear localization in epithelial tubules of tumor AB. (*a–e*, ×100; *f*, ×200.)

The binding of Pax-2 to the *Drosophila e5* subsequence is most probably mediated by the paired domain. The mouse Pax-3 protein can bind the complete *e5* sequence with good affinity but binds the paired-domain specific recognition sequence with lower affinity (18). Thus, it is likely that Pax-3 requires both homeo and paired domains for high-affinity binding. The Pax-1 protein also does not contain a homeodomain and binds the *e5* sequence poorly, but specific substitutions flanking the GTTCC core regions result in high Pax-1 binding (26). Although the data presented in this report indicate efficient binding of Pax-2 to the *e5* sequence, it is not clear if both forms of the Pax-2 protein are necessary for binding this sequence or if the two proteins can bind independently. Several other proteins are present in embryonic kidney nuclear extracts that also bind the *e5* sequence but are not recognized by the Pax-2-specific antibodies. Since the *Pax-8* gene contains a paired domain very similar to that of *Pax-2* and is also expressed in embryonic kidney (16), it is likely that at least one of the proteins seen in the *e5* gel shift can be attributed to *Pax-8*. It is also probable that derivatives of the *e5* sequence can bind with even better affinity to the Pax-2 protein.

The induction of the kidney has proved a good model for cellular interactions that result in formation of undifferentiated epithelium and complex epithelium-derived structures. The transcription factors that are activated to drive these morphogenetic events remain to be identified. The *Pax-2* gene product is a prime candidate for such a factor, as defined by its DNA binding capacity and its transient expression pattern. In addition, a more precise immunopathological investigation of Wilms tumor and other renal carcinomas may correlate *Pax-2* expression with the state of differentiation of tumor epithelium and may define a useful diagnostic marker.

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