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Alternative Splicing of *Pax-8* Gene Transcripts Is Developmentally Regulated and Generates Isoforms with Different Transactivation Properties

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***Pax-8*, a member of the paired box-containing gene family, was shown to be coexpressed with *Pax-2* in several human kidney carcinoma cell lines. Four different Pax-8 mRNA isoforms, a to d, were cloned from one of these cell lines by polymerase chain reaction amplification, and the *Pax-8* gene was isolated from a human cosmid library. Analysis of the exon-intron structure of *Pax-8* revealed that the four mRNA isoforms arise by alternative splicing, resulting in inclusion or exclusion of exon 7 and/or exon 8 sequences. All four Pax-8 proteins retain the paired domain as their DNA-binding motif and recognize DNA in the same manner as do the closely related Pax-2 and BSAP (Pax-5) proteins. The Pax-8a and Pax-8b isoforms end in a serine/threonine/tyrosine-rich sequence, while the C terminus of Pax-8c and Pax-8d is translated in a different, proline-rich reading frame. Transient transfection experiments revealed that Pax-8 isoforms a and b, but not c and d, strongly stimulate transcription from a promoter containing six copies of a paired-domain recognition sequence. The same four mRNA variants were also detected by RNase protection analysis in the mouse embryo and adult kidney, thus indicating evolutionary conservation of Pax-8 mRNA splicing. A different splice pattern was observed in the developing placenta, which expresses two new variants, Pax-8e and Pax-8f, instead of transcripts b to d. Expression of these mRNAs is high at embryonic day 9.5 and is gradually reduced until Pax-8a is the predominant transcript in the 12.5-day placenta. In the embryo, however, the synthesis of mRNAs b to d is initially low and then increases relative to that of Pax-8a. Hence, alternative splicing of *Pax-8* gene transcripts not only generates six different Pax-8 variants but is also temporally and spatially regulated during early mouse development.**

The highly conserved paired box is characteristic of a small family of developmental control genes that were found first in *Drosophila melanogaster* (7, 9, 10) and subsequently in vertebrates (reviewed in reference 30). This protein domain of 128 amino acids specifies a novel DNA-binding activity (12, 62), and some of the paired box-containing proteins have been shown to function as sequence-specific transcriptional activators (1, 12, 70). Nine paired-box genes, *Pax-1* to *Pax-9*, have been identified, and their chromosomal locations have been determined in the human and mouse genomes (references 55 and 66 and references therein). During embryogenesis, these genes are expressed in specific regions of the developing central nervous system (CNS) (1, 28, 35, 44, 45, 65), with the exception of *Pax-1*, which is expressed in the developing vertebral column (15). Mutations in *Pax-1*, *Pax-3*, and *Pax-6* have been associated with the mouse developmental mutants *undulated* (4), *Splotch* (20), and *Small eye* (33), respectively. Moreover, genetic lesions in the human *Pax-3* and *Pax-6* genes cause Waardenburg's syndrome (3, 41, 58, 59) and aniridia (26, 34, 61). This evidence implicates Pax proteins as important regulators of mammalian development.

In the past, we characterized a sea urchin transcription factor, TSAP, which binds to and regulates the promoters of two nonallelic pairs of late histone *H2A-2* and *H2B-2* genes in a developmental stage- and tissue-specific manner (5). Subsequently, the B-cell-specific transcription factor BSAP was identified as a mammalian homolog of TSAP by virtue of its DNA sequence recognition, which is indistinguishable from that of the sea urchin protein (6). BSAP was shown to be involved in the regulation of the *CD19* gene (37), which codes for a B-lymphoid-specific transmembrane protein implicated in signal transduction. Biochemical purification and cDNA cloning revealed that BSAP is encoded by the *Pax-5* gene and is expressed in testis and specific regions of the developing CNS in addition to B-lymphoid cells (1). BSAP (Pax-5) is closely related to Pax-2 and Pax-8 not only in the paired domain (66) but also throughout the entire length of the protein (1). These three proteins therefore constitute a subclass within the Pax family.

The expression patterns of *Pax-2* and *Pax-8* partially overlap during mouse development. The spatial and temporal expression profiles are similar, if not identical, for both genes in the hindbrain and neural tube of the embryo, while *Pax-2* is additionally expressed in the optic stalk and otic vesicle of the developing CNS (44, 45). Both genes are also expressed during kidney organogenesis, which is initiated by inductive interactions between the nephric duct (ureter) and nephrogenic mesenchyme (reviewed in reference 52). *Pax-2* is expressed in both the inducing and responding cells of the developing kidney (16, 17), while *Pax-8* is expressed only in the responding tissues, including the mesenchymal condensations and the derived epithelial structures (45). *Pax-8* expression was also observed in the thyroid gland (45), and in agreement with this finding, the Pax-8 protein was recently shown to bind to and transactivate the promoters of two thyroid-specific genes coding for thyroglobulin and thyroperoxidase (70).

In this study, we investigated the molecular nature of *Pax-8* gene transcripts both in human kidney cell lines and during mouse ontogeny. Polymerase chain reaction (PCR)

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cloning and RNase protection analyses revealed that human kidney cell lines express four different Pax-8 mRNA variants which arise by alternative splicing and give rise to distinct Pax-8 isoforms. All four Pax-8 proteins bind DNA with apparently equal affinity. However, the transactivation properties of Pax-8a and Pax-8b differ from those of Pax-8c and Pax-8d. All four *Pax-8* transcripts are also expressed in the mouse embryo and adult kidney. However, two novel mRNA variants, Pax-8e and Pax-8f, are expressed in the placenta instead of transcripts b to d. Moreover, the relative abundance of the different splice variants changes both in the developing embryo and placenta, thus indicating that alternative splicing of *Pax-8* gene transcripts is regulated during embryogenesis.

MATERIALS AND METHODS

Cell lines. All human cell lines were obtained from American Type Culture Collection (Rockville, Md.). Cell lines 293 and SP2/0 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum; the same medium was further supplemented with sodium pyruvate (1 mM) and nonessential amino acids for culturing A-704, ACHN, and A-498 cells. Cell lines CAKI-1 and CAKI-2 were grown in McCoy's 5a medium containing 10% fetal calf serum.

Isolation and sequencing of a human Pax-8 cosmid clone. A human cosmid library constructed in the vector pcos2-EMBL (19) was screened with randomly primed human Pax-8a cDNA according to standard procedures (50). The isolated cosmid clone pcos-hPax8 was directly used to determine the DNA sequence of each exon-intron junction on an automated sequencer (Applied Biosystems model 373A) with primers derived from the human Pax-8a cDNA sequence.

Oligonucleotides. Oligonucleotides and their sequences were as follows: a, GCGGAATTCGGCGATGCCNCAPyA APyTCNATNAG (5′ human Pax-8); b, CGCAAGCTTCAA CTANAGPuTGPuTCPuAANGCNGT (3′ human Pax-8); c, GCGATCGATCCTGAAGTTGAGTTTGAGAGG (5′ mouse Pax-2); d, GCGTCTAGACTGCCTGAAGCTTGATGTGGT (3′ mouse Pax-2); e, GCGTCTAGACTTGAGTGCCCGTTT GAGCGG (5′ Pax-8); f, GCGAAGCTTACGTGGGCCCC ACCATCTCTCG (3′ Pax-8); g, GCGGGATCCCAGATGC ACAGCAGGAAGCAC (5′ human WT-1); h, GCGAAGCT TAATGCATGTTGTGATGGCGGAC (3′ human WT-1); i, CTTACTAGTCACGATTGGAACCGTTCCGCTCTAGAT ATCTCG (PRS-5); k, TAAGCGAGATATCTAGAGCGGA ACGGTTCCAATCGTGACTAG (PRS-5).

RNA preparation and PCR cloning. Mouse embryos were dissected under the microscope into embryo proper, extraembryonic membranes (yolk sac and amnion), and placenta. Total RNA was prepared from this embryonic material as well as from adult mouse tissues and human kidney cell lines by the method of Chirgwin et al. (13). Polyadenylated RNA was selected by three runs of oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA (~5 μg) from adult mouse kidney and human CAKI-2 and A-704 cells was transcribed into cDNA by Moloney murine leukemia virus reverse transcriptase (SuperScript; Bethesda Research Laboratories), and 100 ng of cDNA was used for 35 cycles of PCR amplification (1 min at 94°C, 2 min at 55°C, 4 min at 72°C). The different human Pax-8 isoforms were amplified from CAKI-2 cDNA with oligonucleotides a and b, mouse Pax-2a and Pax-2b cDNAs were amplified from mouse kidney cDNA with oligonucleotides c and d, and mouse Pax-8a cDNA was amplified from kidney cDNA with the two specific oligonucleotides described by Adams et al. (1). All of these cDNAs were cloned into the polylinker of the eukaryotic expression vector pKW10 (1). The sequences of both DNA strands of these cDNA inserts were determined by automated DNA sequencing.

RNase protection analysis. *Pax-2* and *Pax-8* gene transcripts were detected in total RNA (10 μg) from human kidney cell lines and from mouse embryos and adult tissues by RNase protection analysis, which was carried out as described in detail elsewhere (64). The human Pax-8 paired-domain probe (Fig. 1B) was obtained by cloning a blunt-ended 139-bp *Nco*I fragment of Pax-8a cDNA into the *Hin*dII site of pSP64. A 133-bp-long fragment of human Pax-2 cDNA was amplified from RNA of A-704 cells with the two degenerate oligonucleotides derived from the BSAP peptides 1 and 2 (1). This cDNA fragment was cloned into the *Sal*I and *Eco*RI sites of pSP64 to generate the human Pax-2 paired-domain SP6 probe (Fig. 1B). The human and mouse C-terminal Pax-8 SP6 probes (see Fig. 7 and 8) were generated by subcloning 405- and 423-bp *Xba*I-*Hin*dIII fragments, respectively, into the polylinker of pSP64. These two fragments were obtained by PCR amplification from human or mouse Pax-8a cDNA with oligonucleotides e and f. A 294-bp *Bam*HI-*Hin*dIII fragment containing the three extra codons of the +KTS form was amplified by PCR from A-704 cDNA with oligonucleotides g and h and cloned into the polylinker of pSP64 to generate the human WT-1 SP6 probe (Fig. 1B). The murine S16 probe was previously described (1).

Construction of the reporter gene OVEC-PRS5. Oligonucleotides i and k containing the PRS-5 sequence (12) were annealed and ligated to a 6-mer followed by filling in the 5′ ends with Klenow DNA polymerase and cloning into a *Sma*I site which was engineered into OVEC-1 (67) upstream of the TATA box of the rabbit β-globin gene.

Transactivation assays. The different Pax-8 cDNA expression plasmids (2 μg), the test gene OVEC-PRS5 (5 μg), the control plasmid OVEC-Ref (1 μg), and pSP64 carrier DNA (3 μg) were transfected into 293 cells by calcium phosphate coprecipitation and into SP2/0 cells by electroporation. Cytoplasmic RNA was extracted 48 h later, and 10 μg of RNA was analyzed by RNase protection assay with a rabbit β-globin SP6 probe as described previously (6).

EMSA. cDNA expression plasmids directing the synthesis of Pax-2, Pax-8, BSAP, and the *Drosophila* Paired (Prd) protein were transiently transfected into COP-8 cells by the DEAE-dextran method, and cell extracts were subsequently prepared as described previously (1). These extracts were used for electrophoretic mobility shift assays (EMSA) as described by Barberis et al. (5) except that 10 μg of bovine serum albumin was included in the binding reaction mixture.

Western blot (immunoblot) analysis. Nuclear extract (40 μg) of CAKI-2 cells and extracts (10 μg) of COP-8 cells transiently transfected with human Pax-8 expression plasmids were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently processed for immunoblotting with affinity-purified anti-BSAP paired-domain antibodies as described previously (1).

Nucleotide sequence accession number. The entire Pax-8a cDNA sequence has been submitted to GenBank and assigned sequence number L19606.

RESULTS

***Pax-2* and *Pax-8* gene expression in human kidney cell lines.** Earlier we screened different mammalian cell types for homologs of the sea urchin transcription factor TSAP by EMSA using the TSAP-binding site of the histone *H2A-2.2* gene as a probe. This search led to the identification (6) and subsequent cloning of the B-cell-specific transcription factor BSAP, which is encoded by the *Pax-5* gene (1). In nuclear extracts of human kidney carcinoma cell lines (A-704, CAKI-1, CAKI-2, A-498, and ACHN), we detected an additional DNA-binding activity, which specifically recognizes the H2A-2.2 TSAP-binding site (Fig. 1A) but differs in electrophoretic mobility from BSAP (36a). We therefore reasoned that this kidney-specific activity might be due to a different member of the Pax protein family that would also use the highly conserved paired domain as its DNA-binding motif. Indeed, polyclonal antibodies raised against the *Drosophila* Prd protein blocked DNA binding of the kidney-specific activity by recognizing conserved epitopes in its paired domain (Fig. 1A).

Two *Pax* genes, *Pax-2* and *Pax-8*, have previously been shown to be expressed in the developing kidney of the mouse (16, 45). As the paired-domain sequences of Pax-2, Pax-8, and BSAP are almost identical (1), it seemed likely that the DNA-binding activity described above might correspond to Pax-2, Pax-8, or a mixture of the two proteins. The DNA-binding experiment in Fig. 1A did not permit us to distinguish between Pax-2 and Pax-8, so we investigated the expression of these two genes in the same human kidney cell lines by RNase protection analysis. For this purpose, we generated riboprobes by PCR cloning of short cDNA fragments corresponding to part of the human Pax-2 and Pax-8 paired domains (see Materials and Methods). As shown in Fig. 1B, all five kidney carcinoma cell lines expressed high levels of *Pax-8* transcripts, while only very little Pax-8 mRNA was detected in the adenovirus-immortalized embryonal kidney cell line 293. Equivalent amounts of RNA from all cell lines were, however, analyzed, as judged from the mapping of the glyceraldehyde phosphate dehydrogenase control mRNA. The *Pax-2* gene was weakly expressed in A-704 and ACHN cells compared with *Pax-8*. Even lower expression levels were observed in all other cell lines (A-498, CAKI-1, CAKI-2, and 293). Therefore, the expression pattern of *Pax-8*, and not that of *Pax-2*, correlates with the DNA-binding activity seen in Fig. 1A. More importantly, these data demonstrate for the first time that the *Pax-2* and *Pax-8* genes are both expressed within the same cell, as the kidney cell lines analyzed are of clonal origin.

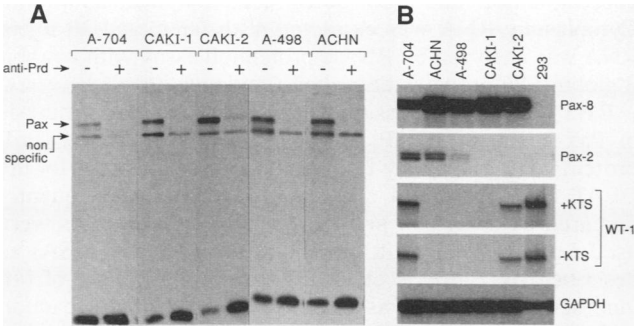

FIG. 1. Expression of *Pax-2* and *Pax-8* genes in human kidney cell lines. (A) DNA-binding experiment. Nuclear extracts of the indicated human kidney cell lines were analyzed by EMSA using the TSAP H2A-2.2 probe described by Barberis et al. (6). A polyclonal rabbit anti-Prd serum (1 μl of a fivefold dilution) was added to the DNA-binding reactions where indicated. (B) RNase protection analysis. Transcripts of the human *Pax-2*, *Pax-8*, *WT-1*, and glyceraldehyde phosphate dehydrogenase (*GAPDH*) genes were mapped in total RNA (10 μg) of human kidney cell lines with RNA probes described in detail in Materials and Methods. The Pax-2, Pax-8, and WT-1 signals can be directly compared, as the corresponding autoradiographs were exposed for the same time period.

The Wilms' tumor suppressor gene *WT-1* codes for a transcription factor which is also expressed during kidney development, similar to Pax-2 and Pax-8 (11, 25). The zinc finger region of this gene is known to be alternatively spliced so that the major transcript encodes a protein with three extra amino acids (+KTS) (31). RNase protection analysis indicated that both alternatively spliced *WT-1* transcripts (−KTS and +KTS) are expressed in a subset of our kidney cell lines (Fig. 1B). Interestingly, we could not find any correlation between the expression patterns of *Pax-2*, *Pax-8*, and *WT-1*, suggesting that there is no cross-regulation between these transcription factors in these cell lines.

Cloning of alternatively spliced transcripts of the human *Pax-8* gene. We next used poly(A)$^+$ RNA isolated from CAKI-2 cells and degenerate oligonucleotides derived from N- and C-terminal sequences of the mouse *Pax-8* gene (45) to isolate and clone human Pax-8 cDNA by PCR. Several different cDNA clones that could be categorized into four different size classes were isolated (see below). The DNA and deduced amino acid sequences of the longest Pax-8 cDNA (referred to as Pax-8a) are shown in Fig. 2A, while the structural organization and evolutionary conservation of Pax-8a are diagrammed in Fig. 2B. The N-terminal sequences comprising the paired domain are completely conserved between the human and mouse Pax-8 proteins. In contrast, eight amino acid substitutions and two insertion-deletions are scattered throughout the central and C-terminal sequences. Thus, Pax-8 appears to be less stringently conserved than Pax-6 and BSAP, which differ between human and mouse by only one and three amino acid substitutions, respectively (1, 61, 66). The central part of Pax-8 contains three regions with a high proportion (39, 47, and 39%) of serine and threonine residues. Moreover, Pax-8 ends in a sequence that is rich in serine, threonine, and tyrosine (48% in total).

The exon-intron structure of *Pax-8* was determined by isolating the human *Pax-8* gene (pcos-hPax8) from a cosmid library and by sequencing its exon-intron junctions (Fig. 3B). This analysis revealed that the protein-coding sequence of Pax-8 is split into 10 exons. The positions of these exons relative to the Pax-8a cDNA sequence are indicated in Fig. 2A and 3B. We also determined the sequence of Pax-8 cDNA clones representing each cDNA size class. Sequence comparison of these Pax-8 cDNAs with the *Pax-8* gene led to the identification of four alternatively spliced transcripts (Fig. 3A). The Pax-8a mRNA contains the coding information of all 10 exons and directs the synthesis of a full-length Pax-8 protein. The Pax-8b transcript arose by skipping the entire exon 8, thus leading to in-frame fusion of exon 7 to exon 9. The Pax-8c mRNA lacks exons 7 and 8. As a consequence, fusion of exon 6 to exon 9 leads to a shift in reading frame and to premature termination of translation, giving rise to a Pax-8 isoform with a proline-rich C terminus (26% proline). A consensus 3′ splice sequence is present in the middle of exon 8. Use of this internal site results in splicing out of the 5′ part of exon 8 in transcript Pax-8d, leading to the same shift in reading frame as for Pax-8c. The existence of all four alternatively spliced Pax-8 mRNA

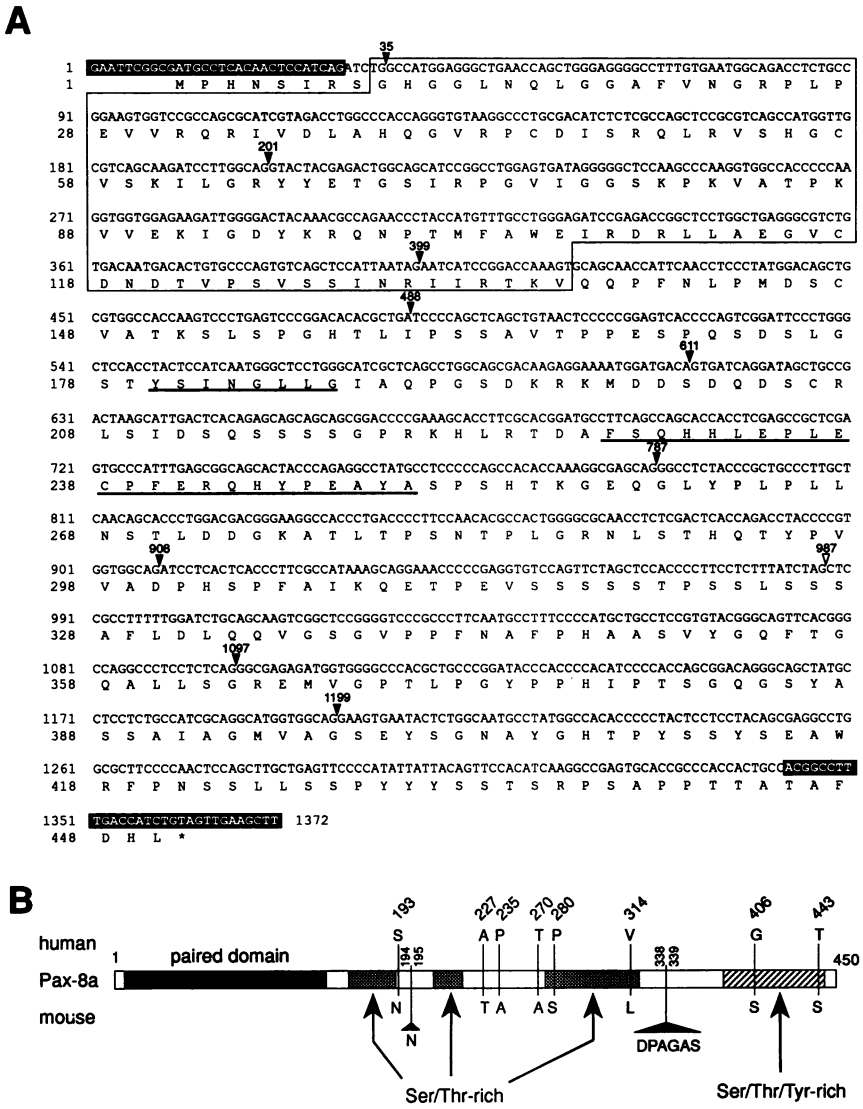

FIG. 2. cDNA sequence of the human Pax-8a splice variant. (A) Nucleotide and deduced amino acid sequences of the human Pax-8a cDNA clone. The paired domain is boxed, and the octapeptide (amino acids 180 to 187) and the homeobox homology region (amino acids 228 to 250) are underlined. Filled arrowheads indicate consensus exon-intron splice junctions that were identified by sequencing the *Pax-8* gene of cosmid pcos-hPax8 (see Fig. 3B). The open arrowhead indicates a consensus 3′ splice site in exon 8 that is used to generate the splice variant Pax-8d (Fig. 3A). The terminal sequences highlighted by black overlay originate from the two oligonucleotides used for PCR cloning. The Pax-8a cDNA sequence is identical from positions 6 to 1366 with the recently published human Pax-8 sequence of Poleev et al. (46) except for two nucleotides (178 and 1262). Codon 418 of their Pax-8 sequence codes for glycine (G: GGC), while our sequence predicts an arginine (R: CGC), which is conserved in all other Pax-8, Pax-2, and BSAP proteins at this position (see reference 1). (B) Structural organization and evolutionary conservation of human and mouse Pax-8a. The different subdomains and corresponding amino acid positions of human Pax-8 are shown together with the amino acid sequence differences observed between the human and mouse proteins. The three serine/threonine-rich regions are indicated by hatched boxes and extend from amino acids 146 to 181, 201 to 217, and 277 to 327, while the sequence from amino acids 380 to 445 is rich in serine, threonine, and tyrosine. The mouse Pax-8 sequence was taken from Plachov et al. (45) with the corrections described by Adams et al. (1).

species in human kidney cell lines was directly verified by RNase protection analysis (see below).

DNA-binding properties of Pax-8. The different Pax-8 cDNAs all contain an intact paired domain, and their proteins are therefore expected to bind known paired-domain recognition sequences. To demonstrate this, we recloned the Pax-8 cDNAs into the expression vector pKW10 (1) and analyzed their expression in the mouse fibroblast cell line COP-8 (63) by transient transfection assay. All four Pax-8 isoforms were efficiently expressed in COP-8 cells, as shown by Western blot analysis of nuclear extracts (Fig. 4). Moreover, they all bound with apparently equal affinity to the TSAP recognition sequence of the sea urchin *H2A-2.2* gene (Fig. 4). Comparison of the individual Pax-8 proteins with ithe complex binding pattern of CAKI-2 nuclear extracts indicated that Pax-8a is the most prevalent isoform in this kidney cell line. We repeatedly noticed that expression of Pax-8 isoforms c and d gave rise to a broad distribution of

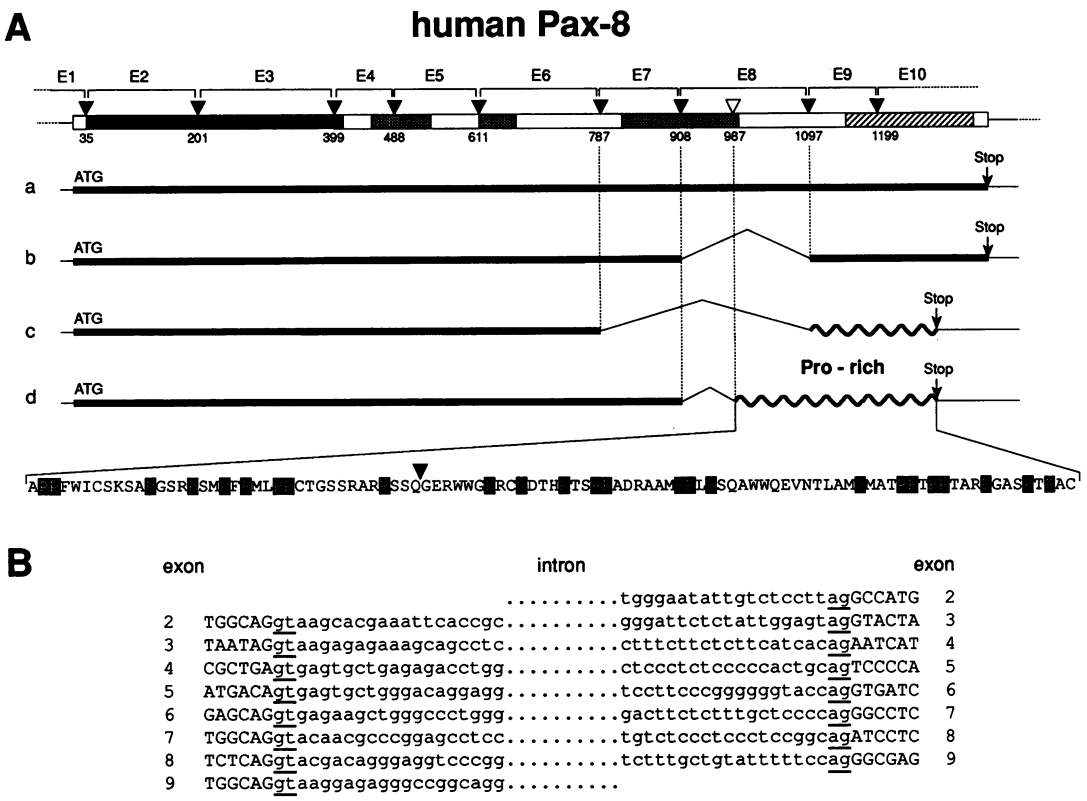

FIG. 3. Schematic diagram of the human Pax-8 splice variants. (A) The exon-intron structure of the human *Pax-8* gene is schematically shown together with the corresponding domain organization, nucleotide positions, and symbols as described in the legend to Fig. 2. The open reading frame of Pax-8a is indicated by a black bar. A shift in reading frame relative to Pax-8a is indicated by a wavy line, and the corresponding amino acid sequence is shown underneath. (B) DNA sequences of the exon-intron junctions. Exon and intron sequences were determined on cosmid pcos-hPax8 and are shown in upper- and lowercase letters, respectively. The invariant GT and AG dinucleotides of the 5′ and 3′ splice sites are underlined. Exons 1 and 10 are absent from pcos-hPax8.

protein-DNA complexes in the EMSA analysis, which may be indicative of protein degradation (also see the Western blot in Fig. 4). It is therefore conceivable that the high proline content at the C terminus of these two variants may be responsible for decreased protein stability in COP-8 cells.

We have recently demonstrated that BSAP (Pax-5) and Pax-1, which are distantly related to each other in their paired domains, differ in DNA sequence recognition (1). In contrast, the paired domains of Pax-2, Pax-8, and BSAP are almost identical (1, 66). This high degree of sequence conservation is reflected at the level of DNA binding, as shown by the experiment in Fig. 5. Paired-domain recognition sequences originating from the promoters of the sea urchin *H2A-2* and *H2B-2* (5), the human *CD19*, and the *Drosophila evenskipped* genes (62) were used as probes for EMSA analysis with the *Drosophila* Prd and mouse Pax-2, Pax-8a, and BSAP proteins, all of which were expressed in COP-8 cells. Pax-2, Pax-8a, and BSAP bound to these sequences in an almost indistinguishable manner, indicating that these three proteins are virtually identical with respect to DNA sequence recognition. In contrast, the distantly related Prd protein interacted only with a subset of these recognition sequences.

Distinct transactivation properties of alternative Pax-8 splice products. The four Pax-8 mRNA splice variants code for proteins with distinct C termini, thus suggesting that they may possess different transactivation properties. We tested

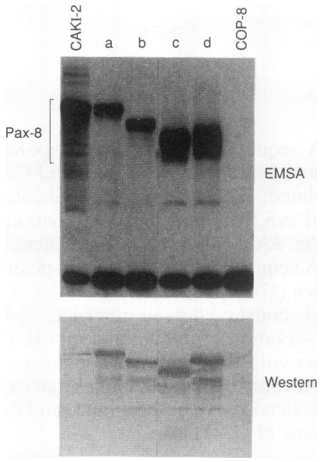

FIG. 4. Expression of the different Pax-8 isoforms in COP-8 cells. The different Pax-8 cDNAs recloned into the expression vector pKW10 were transiently transfected into COP-8 cells, and nuclear extracts were subsequently analyzed for the presence of the different Pax-8 isoforms by SDS-PAGE (10% gel) followed by immunoblotting with affinity-purified anti-paired-domain antibodies (lower panel). The same extracts were analyzed by EMSA for specific binding of the Pax-8 proteins to the H2A-2.2 TSAP-binding site (upper panel). A nuclear extract of the human kidney cell line CAKI-2 was used as the reference. The COP-8 cell extracts, but not the CAKI-2 extract, were diluted 100-fold for the DNA-binding assay as compared with the Western blot.

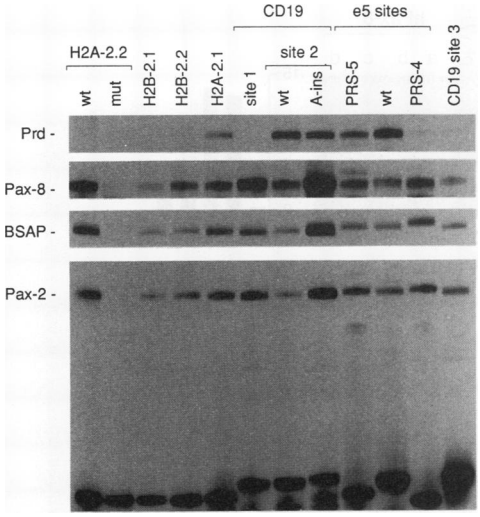

FIG. 5. Similar sequence recognition by Pax-2, Pax-8, and BSAP. The *Drosophila* Prd, mouse Pax-2a, mouse BSAP, and mouse Pax-8a proteins were expressed in transiently transfected COP-8 cells (see Materials and Methods). Nuclear extracts containing equivalent amounts of the four different proteins were analyzed by EMSA with a panel of paired-domain recognition sequences which have been previously described (1, 5, 12).

this hypothesis in transient transfection experiments. Previously, a *CAT* reporter gene containing six copies of the paired-domain recognition sequence PRS-5 upstream of the herpes simplex virus thymidine kinase promoter was successfully used to demonstrate the transactivation potential of Pax-1 by transient transfection assay (12). We inserted six copies of the same PRS-5 sequence upstream of the TATA box of the rabbit β-globin gene in the OVEC-1 vector (67) (Fig. 6C). This test construct (OVEC-PRS5) and a reference gene containing a deletion in the β-globin leader sequence (OVEC-Ref [67]) were transfected into 293 and SP2/0 cells together with expression plasmids directing the synthesis of the different Pax-8 variants. The β-globin transcripts of the test and reference genes were then mapped by RNase protection assay. The human embryonal kidney cell line 293 and the mouse plasma cell line SP2/0 were chosen for these experiments because they express little (293) or no (SP2/0) endogenous Pax protein (Fig. 1) (6). As shown in Fig. 6A and B, only Pax-8a and Pax-8b efficiently transactivated the β-globin promoter containing the PRS-5 sequence. A five- to six-fold-lower stimulation of the same promoter was repeatedly observed with Pax-8 isoforms c and d, even though these proteins were expressed to a similar level as Pax-8a and Pax-8b in the transfected cells, as shown by the EMSA analysis of Fig. 6D. We therefore conclude that Pax-8 isoforms can be distinguished by their transactivation potential, which is determined by the presence of either a serine/threonine- or a proline-rich sequence at the C terminus.

We showed above that Pax-2 and Pax-8 are coexpressed in several human kidney cell lines (Fig. 1) and that the two proteins recognize the same DNA sequences (Fig. 5). The possibility therefore exists that Pax-2 is a transcriptional repressor antagonizing Pax-8 function by binding to the same recognition sequences. Alternative splicing of Pax-2 gene transcripts gives rise to two abundant isoforms which differ by 23 amino acids in the middle of the coding region (16, 17) (Fig. 6E). Transient transfection experiments in 293 cells clearly demonstrated that both Pax-2a and Pax-2b stimulate β-globin transcription of OVEC-PRS5 as efficiently as Pax-8a does (Fig. 6E). Both Pax-2 isoforms are therefore potent transcriptional activators, in agreement with the fact that Pax-2 and Pax-8a share a high degree of homology throughout the entire protein-coding region, including the serine/threonine-rich C-terminal sequences (1).

Evolutionary conservation of alternative Pax-8 mRNA splicing. The existence of four alternatively spliced Pax-8 transcripts in human kidney cells was directly demonstrated by RNase protection analysis (Fig. 7). For this purpose, we used a riboprobe spanning Pax-8 sequences from position 716 in exon 6 to position 1120 in exon 9 (Fig. 2 and 7). Protection of this probe by splice variants a, c, and d gives rise to diagnostic fragments of 405, 72, and 133 nucleotides, respectively, which was verified by mapping each RNA isoform separately following transfection of the corresponding cDNA into COP-8 cells (36a). All kidney cell lines except 293 cells express Pax-8 mRNA isoforms a, c, and d (Fig. 7). The Pax-8b mRNA generates a 193-nucleotide-long RNA fragment which is identical with one of the fragments of the Pax-8d variant. However, the signal of the 193-nucleotide RNA fragment is clearly more intense than the Pax-8d-diagnostic signal of 133 nucleotides, suggesting that the difference must be due to the presence of Pax-8b mRNA. Of the four Pax-8 mRNA isoforms identified, Pax-8a was the most abundant transcript in the human kidney cell lines tested, thus confirming the result of the EMSA analysis in Fig. 4.

The kidney of the adult mouse was previously shown by Northern (RNA) blot analysis to express *Pax-8* gene transcripts (45). We analyzed the structure of these transcripts by RNase protection assay using a mouse Pax-8-specific riboprobe (Fig. 8C) that maps the same exon sequences as does the corresponding human probe (Fig. 7). All four Pax-8 mRNA variants, a to d, are also expressed in the kidney of the adult mouse (Fig. 8B). In this tissue, the Pax-8a transcript appears to be most abundant, as seen with the human kidney cell lines (Fig. 7). This evidence therefore indicates that alternative splicing of *Pax-8* gene transcripts has been conserved between human and mouse. Weak expression of Pax-8a mRNA was in addition detected in the mouse ovary (Fig. 8B).

Two distinct Pax-8 mRNA splice patterns during mouse embryogenesis. The *Pax-8* gene was shown by in situ hybridization to be expressed in the developing CNS, thyroid gland, and kidney of the mouse embryo (45). We used the RNase protection method to analyze the Pax-8 mRNA splice pattern in whole embryos (embryo proper plus extraembryonic membranes plus placenta) at days 9.5 and 10.5, in the embryo proper at day 11.5, and in the head (h) and trunk (t) of embryos at later stages. As shown in Fig. 8A, all four Pax-8 mRNA isoforms a to d are expressed in the embryo proper from days 11.5 to 16.5 in a ratio which is similar to that seen in the adult kidney (Fig. 8B). A different splice pattern was observed in whole embryos at days 9.5 and 10.5. While Pax-8a mRNA was still abundantly expressed, the level of Pax-8 mRNA variants b and d was considerably reduced. Instead, two new RNase-resistant fragments of ~350 and ~120 nucleotides were detected (see legend to Fig. 8A for size determination). Together, these two fragments are clearly longer than the 423-bp Pax-8 sequence present in the riboprobe, thus indicating that they originate from two different transcripts. The partial structure of these two novel splice forms, referred to as Pax-8e and Pax-8f, is shown in Fig. 8C and has been directly verified by the

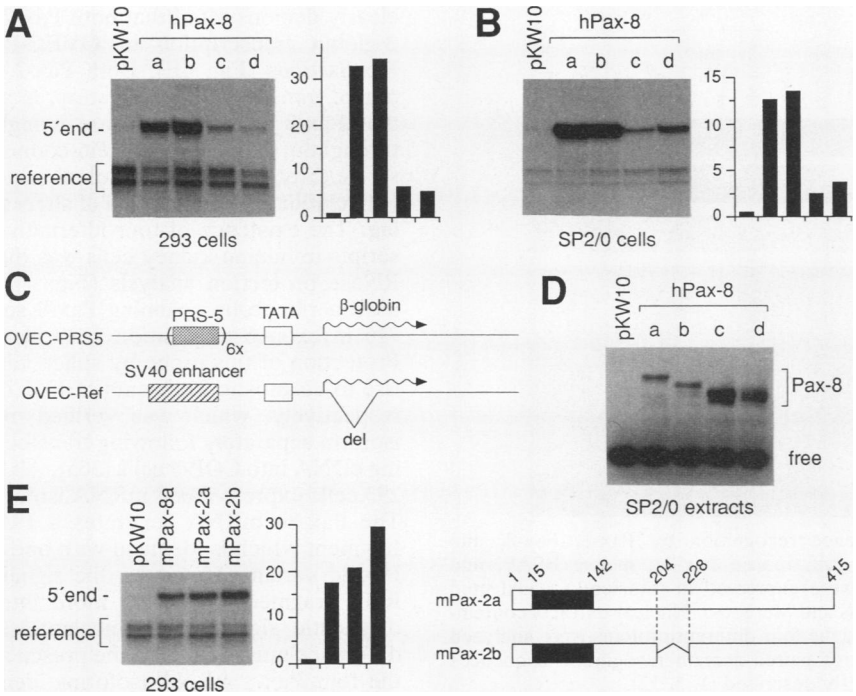

FIG. 6. Pax-8 isoforms differ in transactivation potential. (A and B) Transactivation by different Pax-8 isoforms. Expression plasmids directing the synthesis of the human Pax-8 isoforms were transiently transfected together with the test construct OVEC-PRS5 and the reference plasmid OVEC-Ref into the embryonal kidney cell line 293 and plasma cell line SP2/0 as described in Materials and Methods. Expression of the β-globin reporter gene was detected by RNase protection assay. The autoradiographic signals corresponding to the correctly initiated transcript of the test gene (5′ end) were quantitated on a PhosphorImager (Molecular Dynamics), normalized to the reference gene transcript (reference), and plotted as relative transactivation strength on the right. The β-globin mRNA level obtained with the empty expression vector pKW10 was given a value of 1. (C) Schematic diagram of the OVEC reporter plasmids. Six copies of the paired-domain recognition sequence PRS-5 (12) were cloned upstream of the TATA box of the rabbit β-globin gene present in OVEC-1 (67). OVEC-Ref was described by Westin et al. (67). (D) Pax-8 expression in transfected SP2/0 cells. Nuclear extracts were prepared from the second half of the transfected SP2/0 cells. The same relative amounts of these extracts corresponding to the RNA mapped in panel B were analyzed by EMSA using the H2A-2.2 TSAP-binding site as the DNA probe. (E) Transactivation by Pax-2 splice variants. OVEC-PRS5 and OVEC-Ref were transiently transfected into 293 cells together with expression plasmids directing the synthesis of mouse Pax-2a, Pax-2b, or Pax-8a protein, and transcripts of the β-globin reporter gene were analyzed as described for panel A. The structures of the two mouse Pax-2 isoforms (16, 17) are schematically diagrammed on the right. cDNAs corresponding to these isoforms were isolated by PCR cloning from mouse kidney RNA (see Materials and Methods).

following experiment. Exon 6 was first eliminated from Pax-8a and Pax-8b cDNA by PCR cloning, and the corresponding cDNAs were then transcribed in vitro into RNA. These two transcripts gave rise to the same RNase-protected fragments (e and f) that were seen with day 9.5 whole-embryo RNA (36a). Hence, exon 7 of the Pax-8 splice forms e and f must be linked to sequences other than exon 6. Identification of these sequences will have to await full-length cDNA cloning of *Pax-8* transcripts eand f.

Spatial and temporal regulation of Pax-8 mRNA splicing in early mouse development. The two different Pax-8 mRNA splice patterns observed with RNA from whole embryos versus the embryo proper suggested that the *Pax-8* gene is additionally expressed in tissues other than the embryo proper. We therefore dissected whole embryos from days 9.5 to 12.5 into embryo proper, extraembryonic membranes (visceral yolk sac and amnion), and placenta. As shown by the RNase protection analysis in Fig. 9A, the Pax-8e transcript was predominantly expressed in placenta. Of the other Pax-8 splice forms, only Pax-8a mRNA was abundantly expressed in placenta, while no *Pax-8* transcripts were detectable in the extraembryonic membranes. In the embryo proper, Pax-8 isoforms b and d were coexpressed with Pax-8a mRNA as early as day 9.5. Long-term autoradiographic exposure indicated that Pax-8f mRNA followed the expression pattern of the Pax-8e transcript in the placenta, while Pax-8c mRNA was expressed in the embryo proper along with the isoforms b and d (36a). These data clearly demonstrate that alternative splicing of *Pax-8* gene transcripts is differently regulated in distinct parts of the embryo.

Quantitation of the autoradiographic signals corresponding to the different Pax-8 mRNA isoforms indicated that expression of Pax-8e mRNA steadily declines in the placenta starting with embryonic day 9.5, so that Pax-8a is the only prevalent Pax-8 mRNA still expressed at day 12.5 (Fig. 9A and B). A different temporal pattern of Pax-8 mRNA splicing is observed in the embryo proper. At day 9.5, the Pax-8 isoforms b and d are expressed at a low level relative to Pax-8a mRNA. Their expression then increases during the next 4 days to reach the same abundance as Pax-8a mRNA in the 12.5-day embryo. We conclude that alternative splicing of *Pax-8* gene transcripts is spatially and temporally regulated during mouse embryogenesis.

DISCUSSION

Alternative splicing generates Pax-8 isoforms with different transactivation properties. The *Pax-8* gene was previously

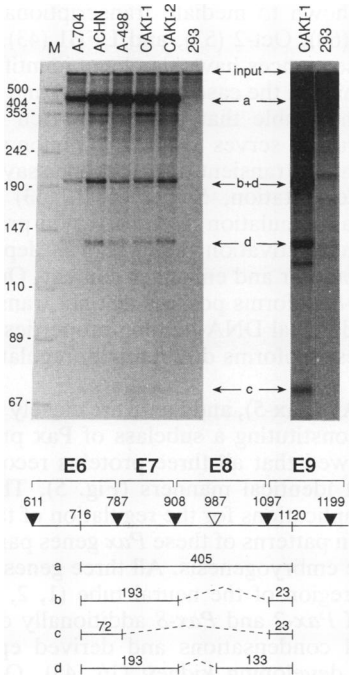

FIG. 7. Expression of alternatively spliced *Pax-8* gene transcripts in human kidney cells. Total RNA (10 μg) of human kidney cell lines was analyzed by RNase protection with an RNA probe mapping human Pax-8 sequences from positions 716 to 1120. RNase-protected fragments were separated on an 8% polyacrylamide sequencing gel together with an end-labeled DNA size marker (lane M; pUC19 digested with *Msp*I; sizes are given in nucleotides). A long-term autoradiographic exposure (CAKI-1 and 293) is shown on the right. The lengths (in nucleotides) of the RNase-protected fragments, which are indicative of the different Pax-8 splice variants, are shown below together with the positions of the splice junctions separating exon 6 to exon 9.

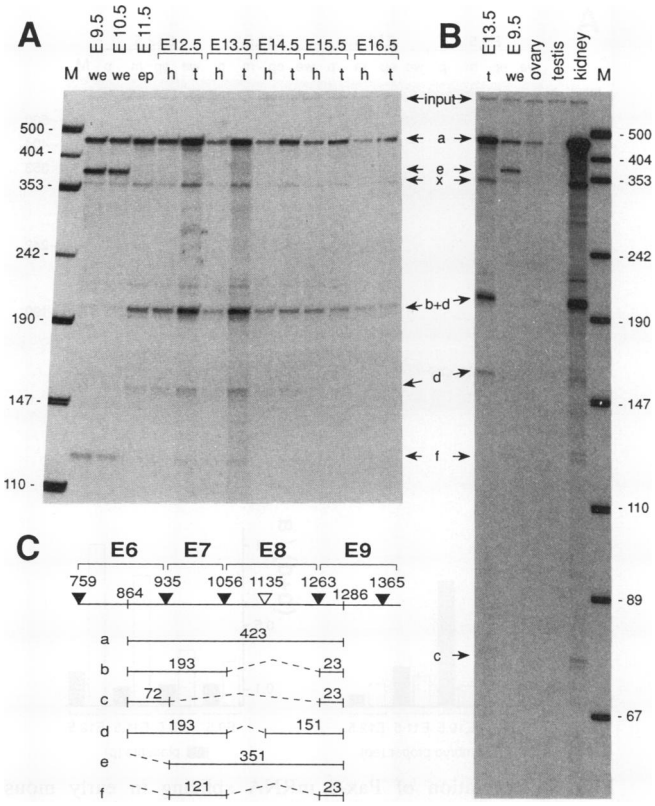

FIG. 8. Pax-8 mRNA splicing in embryonic and adult tissues of the mouse. (A) Pax-8 mRNA splicing during early mouse development. Total RNA was prepared at day 9.5 and 10.5 postcoitum from the whole embryo (we; embryo proper plus extraembryonic membranes plus placenta) and at day 11.5 from the embryo proper (ep). Later embryos were dissected into head (h) and trunk (t) prior to RNA preparation. Total RNA (20 μg) was analyzed by RNase protection assay with an RNA probe mapping mouse *Pax-8* gene sequences from positions 864 to 1286 (45) (see panel C). Note that the migration of the protected RNA fragments is slightly retarded relative to that of the DNA size marker (lane M; end-labeled pUC18 DNA digested with *Msp*I). The length of the RNA fragment (~330 nucleotides) protected by the *Pax-8* gene transcript x predicts that this transcript contains exon 7 sequences spliced to exon 8 (positions 935 to 1263). The relatively low abundance of this transcript furthermore suggests that it may be a splicing intermediate still retaining introns 6 and 8. (B) Pax-8 mRNA splice pattern in tissues of the adult mouse. Total RNA isolated from ovary, testis, and kidney was mapped together with reference RNA of day 9.5 and 13.5 embryos. (C) Schematic diagram of the different Pax-8 mRNA isoforms. The expected protection pattern is indicated for the different Pax-8 mRNA splice variants. The lengths of the protected fragments are given in nucleotides, and the positions of the splice junctions refer to the published mouse Pax-8 cDNA sequence (45).

shown to be expressed in the developing CNS, thyroid gland, and kidney of the mouse embryo (45). In this study, we have investigated the molecular nature of *Pax-8* gene transcripts both in human kidney cell lines and during mouse development. Four different Pax-8 mRNA isoforms were identified in human kidney cell lines by cDNA cloning and RNase protection analysis. Elucidation of the exon-intron structure of the human *Pax-8* gene revealed that these transcripts arise by alternative splicing, thus resulting in inclusion or exclusion of exon 7 and/or exon 8 sequences (Fig. 3). A further consequence of alternative splicing is that the C terminus of isoforms Pax-8c and Pax-8d is translated in a different proline-rich reading frame compared with Pax-8a and Pax-8b, which end in a serine/threonine/tyrosine-rich sequence. All four Pax-8 isoforms contain the conserved N-terminal paired domain as their DNA-binding motif and share two centrally located serine/threonine-rich regions which are reminiscent of transactivation domains of other transcription factors (57, 60). During preparation of this report, Poleev et al. (46) published the recent cloning of two distinct Pax-8 cDNAs from a human kidney cDNA library. These sequences are identical with our Pax-8a and Pax-8b cDNAs except for two nucleotide substitutions (see the legend to Fig. 2).

Alternative splicing of transcripts from a single gene is often used as a mechanism for generating protein variants with diverse functions (reviewed in references 39 and 54). Several genes coding for transcription factors are subject to alternative splicing (reviewed in reference 23). Among them are the genes encoding the CCAAT-binding protein CTF/NF-I (51), the thyroid receptor ErbAα (36), retinoic acid receptor α (38), the helix-loop-helix protein mTFE3 (47), the transcription factor Oct-2 (14, 56, 68), the oncoprotein FosB (42, 69), the zinc finger protein WT-1 (8, 31), the NF-κB subunit p65 (48), and the CRE-binding proteins CREB (49), CREM (21, 22), and CRE-BP (24). In many of these cases, it has been demonstrated that alternative splicing is directly

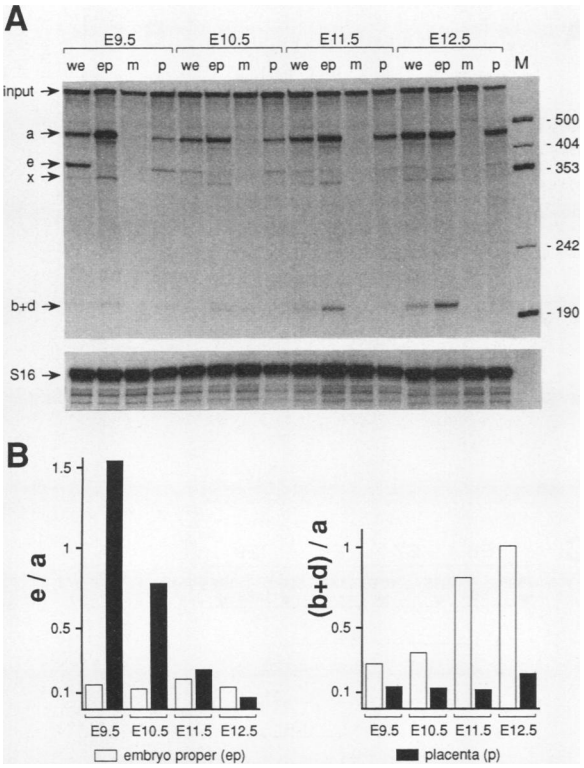

FIG. 9. Regulation of Pax-8 mRNA splicing in early mouse development. (A) RNase protection analysis. Whole embryos (we; embryo plus extraembryonic membranes plus placenta) at days 9.5 to 12.5 were dissected into embryo proper (ep), extraembryonic membranes (m; visceral yolk sac plus amnion), and placenta (p). Total RNA (20 μg) from this dissected material or from whole embryos was mapped with the RNA probe described in Fig. 8C. Only the part of the autoradiogram with the signals corresponding to the *Pax-8* transcripts a, b, d, e, and x is shown. Separate mapping of the mRNA coding for the ribosomal S16 protein indicated that the same amount of total RNA was analyzed in all cases. Sizes are given in nucleotides. (B) Relative abundance of the different *Pax-8* gene transcripts. The autoradiographic signals of the different Pax-8 mRNA isoforms were quantitated on a PhosphorImager (Molecular Dynamics) and corrected both for the length of the protected fragment and for the background obtained with the extraembryonic membrane RNA. The ratio of the different *Pax-8* transcripts relative to Pax-8a mRNA is plotted.

responsible for expression of transcription factors with distinct or even opposing activities (reviewed in reference 23).

The transactivation potential of Pax-8 isoforms differed in transient transfection assays. The two isoforms Pax-8a and Pax-8b, which share the serine/threonine/tyrosine-rich C terminus, strongly transactivated a β-globin reporter gene containing multimers of a paired-domain recognition sequence. In contrast, a considerably lower level of transactivation was observed with Pax-8c and Pax-8d, which end in a proline-rich sequence (Fig. 6A and B). This evidence implies that the C terminus of Pax-8a/b harbors a potent transactivation domain. Detailed mutational analysis of the closely related transcription factor BSAP (Pax-5) further supports this conclusion, as the transactivation function of BSAP entirely resides within the C-terminal serine/threonine/tyrosine-rich region (15a), which is conserved between BSAP and Pax-8a/b (1). Moreover, protein domains with a similarly high abundance of hydroxylated amino acids have previously been shown to mediate transcriptional activation of GHF-1/Pit-1 (60), Oct-2 (57), and LFB1 (43). Interestingly, proline-rich sequences have also been identified as transactivation domains in the case of CTF/NF-I and AP-2 (40). It is therefore conceivable that the proline-rich C terminus of Pax-8c and Pax-8d serves a similar function which may not be revealed in our transient transfection assay. In agreement with this interpretation, Seipel et al. (53) observed that transcriptional stimulation by Gal4 fusion proteins containing proline-rich activation domains was dependent on the particular promoter and enhancer context. Our finding, that different Pax-8 isoforms possess distinct transactivation potentials but identical DNA-binding properties, strongly suggests that these isoforms differentially regulate *Pax-8* target genes.

Pax-2, BSAP (Pax-5), and Pax-8 are closely related to each other, thus constituting a subclass of Pax proteins (1, 66). Here we showed that all three proteins recognize DNA in similar if not identical manners (Fig. 5). This finding has interesting implications for the regulation of target genes, as the expression patterns of these *Pax* genes partially coincide during mouse embryogenesis. All three genes are expressed in the same region of the neural tube (1, 2, 44, 45), while expression of *Pax-2* and *Pax-8* additionally overlaps in the mesenchymal condensations and derived epithelial structures of the developing kidney (16, 45). Our analysis of human kidney cell lines has directly demonstrated coexpression of *Pax-2* and *Pax-8* within the same cell (Fig. 1B). The *Pax-2* gene also gives rise to two isoforms by alternative splicing (17). Both proteins, Pax-2a and Pax-2b, are as potent transcriptional activators as Pax-8a in our transfection assay (Fig. 6E), in agreement with the fact that all three proteins share the conserved serine/threonine/tyrosine-rich C-terminal sequences (1). This evidence suggests that Pax-2 and Pax-8 regulate the same target genes in those kidney cells in which both proteins are expressed. It is interesting to note that alternative splicing creates two splice variants, Pax-2b and Pax-8b, which are colinear over their entire length with the transcription factor BSAP (1). Consequently, all three proteins may serve the same redundant function in the neural tube, in which they are coexpressed.

Alternative splicing of *Pax-8* gene transcripts is regulated during early mouse development. One mouse Pax-8 isoform corresponding to Pax-8a has so far been identified by cDNA cloning from an E8.5 cDNA library (45) and by expression analysis (46). Using the sensitive RNase protection assay, we have now detected six different Pax-8 mRNA isoforms which are expressed during mouse ontogeny (Fig. 8 and 9). Four of these splice forms correspond to *Pax-8* transcripts identified in the human kidney cell lines. The mouse Pax-8a mRNA is present throughout early development and in the adult kidney whenever the *Pax-8* gene is transcribed. Splice variants b, c, and d are expressed in the embryo proper (E9.5 to E16.5) and in the adult kidney. The synthesis of these three *Pax-8* transcripts is regulated during embryogenesis, as their abundance relative to Pax-8a mRNA is low in early embryos (E9.5) and increases thereafter to a level seen in the adult kidney. It remains to be seen whether Pax-8 mRNA splicing is not only temporally but also spatially controlled in the embryo proper, i.e., whether the splice pattern differs in the developing CNS, thyroid gland, and kidney.

Using RNase protection analysis, we described for the first time *Pax-8* gene expression in the ovary and developing placenta. The placenta is a complex organ consisting of several well-defined cell layers, and hence in situ hybridization analysis will be required to localize the site(s) of *Pax-8*

expression within this tissue. Two novel mRNA isoforms, Pax-8e and Pax-8f, are expressed in the placenta in addition to Pax-8a. These two transcripts contain exon 7 and downstream sequences but apparently lack exon 6. Mapping of *Pax-8* transcripts with overlapping riboprobes spanning exons 2 to 6 did not reveal any heterogeneity specific for placenta. Instead, the level of transcription through these exons was significantly lower in placenta in comparison with exon 7 (36a). This indirect evidence suggests that exon 7 in transcripts e and f is linked to unique sequences which are absent from Pax-8a. Elucidation of the exact molecular nature of these two transcripts will have to await full-length cDNA cloning. Both mRNAs e and f are abundantly expressed in the day 9.5 placenta. Their synthesis is then gradually shut down, until the placenta of day 12.5 embryos predominantly expresses the Pax-8a mRNA variant. Taken together, these data demonstrate that alternative splicing not only generates six different Pax-8 mRNA isoforms but is also temporally and spatially regulated during early mouse development.

Three mouse developmental mutants, *undulated*, *Splotch*, and *Small eye*, are caused by mutations in *Pax-1* (4), *Pax-3* (20), and *Pax-6* (33), respectively. *Pax-8* was suggested as a candidate gene for a fourth mutation, *Danforth's short-tail* (*Sd*), mainly for three reasons (32, 45). First, *Pax-8* was mapped close to the *Sd* locus on mouse chromosome 2 (45). Second, *Sd* is semidominant, similar to mutations in *Pax* genes. Third, the *Pax-8* expression pattern seemed appropriate to explain the *Sd* phenotype, which is characterized in the heterozygote by a short tail, a reduced number of caudal vertebrae, and a reduction in size of one or both kidneys. Homozygotes are born without any tail and kidneys and die shortly after birth from autointoxication (18, 27). However, all of our attempts to confirm a link between *Pax-8* and *Sd* have failed. The *Pax-8* gene was not deleted in *Sd* mice and gave rise to all six mRNA isoforms in *Sd* embryos (E9.5 and E12.5). Furthermore, sequence analysis of 20 Pax-8a and Pax-8b cDNA clones which were isolated from kidneys of heterozygous *Sd* animals did not reveal any point mutations in the *Pax-8* coding sequence (36a). Our negative evidence is consistent with the interpretation of Grüneberg (29) that abnormal development of the notochord is the primary defect in *Sd* embryos and that the effect on kidney development is only a secondary consequence of this.

The complex regulation of Pax-8 mRNA splicing generates protein variants which may fulfill distinct functions during mouse development by differentially regulating target gene expression. Characterization of the different Pax-8 isoforms will now allow us to design experiments to test this hypothesis by changing the expression ratio of the different isoforms either in transgenic mice or stably transfected kidney cell lines.

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