

## ***Pax8*, a murine paired box gene expressed in the developing excretory system and thyroid gland**

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### **Summary**

Several mouse genes designated 'Pax genes' contain a highly conserved DNA sequence homologous to the paired box of *Drosophila*. Here we describe the isolation of *Pax8*, a novel paired box containing clone from an 8.5 day *p.c.* mouse embryo cDNA library. An open reading frame of 457 amino acids (aa) contains the 128 aa paired domain near the amino terminus. Another conserved region present in some other paired box genes, the octapeptide Tyr-Ser-Ile-Asn-Gly-Leu-Leu-Gly, is located 43 aa C-terminal to the paired domain. Using an interspecies backcross system, we have mapped the *Pax8* gene within the proximal portion of mouse chromosome 2 in a close linkage to the *surf* locus. Several developmental mutations are located in this region. *In situ* hybridization was used to determine the pattern of *Pax8* expression during mouse embryogenesis. *Pax8* is expressed transiently between 11.5 and 12.5 days of gestation along the rostrocaudal axis extending from the

myelencephalon throughout the length of the neural tube, predominantly in two parallel regions on either side of the basal plate. We also detected *Pax8* expression in the developing thyroid gland beginning at 10.5 days of gestation, during the thyroid evagination. In the mesonephros and metanephros the expression of *Pax8* was localized to the mesenchymal condensations, which are induced by the nephric duct and ureter, respectively. These condensations develop to functional units, the nephrons, of the kidney. These data are consistent with a role for *Pax8* in the induction of kidney epithelium. The embryonic expression pattern of *Pax8* is compared with that of *Pax2*, another recently described paired box gene expressed in the developing excretory system.

Key words: paired box, Pax cDNA, mouse chromosome 2, kidney development, thyroid development, mouse embryogenesis.

### **Introduction**

The molecular mechanisms governing mammalian development are poorly understood. The investigation of these mechanisms is hampered mainly due to the lack of mutants in which the genes involved in the developmental control are affected. However, a number of developmental mutants have been identified in *Drosophila* (Lewis, 1978; Nüsslein-Volhard and Wieschaus, 1980), facilitating the isolation of the corresponding genes (for review see Akam, 1987). A complex network of molecular interactions leading to the establishment of segmentation and segment identity of the *Drosophila* embryo is being revealed (for review see Scott and Carroll, 1987; Ingham, 1988).

One approach used to identify the molecules involved in mammalian development takes advantage of the fact that certain protein domains such as the homeobox, a common motif among the *Drosophila* developmental genes, are strongly conserved in evolution (McGinnis *et al.* 1984). Thus, numerous murine

homeobox-containing genes have been identified by screening with *Drosophila* homeobox probes and the expression of these genes in the developing embryo has been studied (for review see Holland and Hogan, 1988).

Another conserved domain of 128 amino acids, the paired box, was identified in the *Drosophila* segmentation genes *paired* (Kilchherr *et al.* 1986; Frigerio *et al.* 1986), *gooseberry-distal* and *gooseberry-proximal* (Baumgartner *et al.* 1987; Côté *et al.* 1987) suggesting a functional role for this domain in the process of *Drosophila* segmentation (Bopp *et al.* 1986). Subsequently, two additional *Drosophila* paired box genes of unknown function, *Pox meso* and *Pox neuro*, were cloned (Bopp *et al.* 1989). The paired box domain is also conserved in the evolution of fly, mouse and man (Dressler *et al.* 1988; Burri *et al.* 1989). Because various segmented structures, such as somites and mesonephric tubules, appear during the mouse development (Hogan *et al.* 1985), an intriguing possibility is that paired box genes are also involved in the process of mammalian segmentation. Indeed, *Pax1*, a murine paired box gene,

is expressed in a segmented manner in the intervertebral disk anlagen along the entire vertebral column (Deutsch *et al.* 1988). Furthermore, a point mutation in the highly conserved region of the paired box of *Pax1* is associated with the *undulated* mutant, characterized by malformations in the vertebral column (Balling *et al.* 1988). Another recently described murine paired box gene, *Pax2*, is expressed in restricted regions along the entire developing spinal cord as well as in the mesonephros and metanephros (Dressler *et al.* 1990; Nornes *et al.* 1990). The mesonephric nephrons constitute a segmented structure. Hence, the expression of *Pax2* in this tissue supports a possible role for this gene in the segmentation of the mouse embryo.

Several paired box sequences have been detected in the mouse genome by hybridization (Dressler *et al.* 1988). As a first step to understanding the underlying mechanisms by which members of the Pax gene family function, we have sought to identify additional paired box genes of the mouse. In this report we describe the isolation and sequence of a novel paired box gene, *Pax8*. We show that *Pax8* is closely linked to the *surfeit* (*surf*) gene cluster near the centromere of chromosome 2. Using *in situ* hybridization, we demonstrate that *Pax8* is expressed in restricted regions of the developing mouse embryo. The expression pattern is compared with that of the *Pax2* gene, whose paired domain shows an extensive homology to the paired domain of *Pax8*. Comparison of the spatial pattern of expression of both genes in the developing metanephros is especially instructive. The morphogenesis of the metanephros results from mutual inductive interactions between the ingrowing ureteric bud and the metanephrogenic mesenchyme. During this process the branching ureter induces in the mesenchyme the cellular condensations that develop shortly thereafter to the S-shaped bodies, the precursors of the nephrons (for review see Potter, 1972; Saxén, 1987). We report here that *Pax8* is expressed predominantly in the induced tissues, mesenchymal condensations and the S-shaped bodies. *Pax2* is expressed in the ureter, condensations, and S-shaped bodies. The results suggest a function for both genes in the inductive process of kidney development.

## Materials and methods

### cDNA library screening

Initially, the cDNA clone c960 was isolated from an 8.5 days *p.c.* embryonic C57BL/6 mouse  $\lambda$ gt10 cDNA library (Fahrner *et al.* 1987) in a low-stringency screen (hybridization in  $7\times$ SSC at  $60^\circ\text{C}$  with a subsequent washing in  $2\times$ SSC/ $0.2\%$  SDS at  $42^\circ\text{C}$ ) using a mixture of *Pax1*, *Pax2* and *Pax3* paired box probes (Deutsch, unpublished). DNA sequencing revealed that this clone had part of a novel paired box. A 112 bp fragment containing the partial paired box sequence of c960 was labeled by random priming (Feinberg and Vogelstein, 1983) and used to screen  $6\times 10^6$  clones of the same cDNA library under high-stringency conditions (hybridization in  $500\text{ mM NaPi}$ , pH 7.2/ $7\%$  SDS/ $1\text{ mM EDTA}$  at  $65^\circ\text{C}$ ; washing several times in  $40\text{ mM NaPi}$ , pH 7.2/ $1\%$  SDS at  $65^\circ\text{C}$ ).

### DNA sequencing

The overlapping restriction fragments of the *Pax8* cDNAs were cloned into the plasmid vector Bluescript KS (Stratagene) and sequenced from both strands by the dideoxy method (Sanger *et al.* 1977) using commercial sequencing kits (Sequenase, US Biochemicals; T7, Pharmacia).

### Embryos and tissues

Embryos for RNA isolation and *in situ* analysis were obtained from natural matings of female NMRI mice. The day of the vaginal plug was designated as day 0.5 *p.c.* Tissues were isolated from adult NMRI mice.

### RNA isolation and Northern blot analysis

Total RNA was isolated by homogenizing tissues and embryos in guanidinium thiocyanate (Chirgwin *et al.* 1979) followed by centrifugation through a  $5.7\text{ M CsCl}$ ,  $25\text{ mM sodium acetate}$  pH 5.0 gradient in a Beckman SW40 rotor spun at  $30\,000\text{ revs}\cdot\text{min}^{-1}$  for 24 h. Poly (A)<sup>+</sup> RNA was isolated using oligo(dT)-cellulose columns.  $5\text{ }\mu\text{g}$  of the respective RNA samples were electrophoresed through  $1\%$  agarose gels containing  $3.7\%$  formaldehyde and Mops buffer ( $20\text{ mM morpholine propane sulfonic acid}$ ,  $50\text{ mM sodium-acetate}$ ,  $10\text{ mM EDTA}$ , pH 7.0). RNA was blotted onto nylon membranes (Hybond-N) with  $10\times$ SSC and hybridized overnight in  $50\%$  formamide,  $5\times$ SSC at  $42^\circ\text{C}$ . The membranes were then washed twice in  $0.1\times$ SSC,  $1\%$  SDS at  $65^\circ\text{C}$  for 15 min each.

### In situ hybridization

The cDNA fragments chosen as templates for *in vitro* transcription were cloned into the Bluescript KS vector (Stratagene) and the resulting plasmids were linearized with restriction endonuclease. These templates were transcribed using T3 or T7 RNA polymerase (Promega Biotech),  $^{35}\text{S-UTP}$  and  $^{35}\text{S-CTP}$  (each approximately  $100\text{ }\mu\text{Ci}$ ) and then degraded by DNase digestion. RNA probes were precipitated with  $10\%$  trichloroacetic acid, collected on nitrocellulose filters (Millipore), eluted by brief boiling, and incubating at  $65^\circ\text{C}$  in  $20\text{ mM EDTA}$  pH 8/ $0.1\%$  SDS. After ethanol precipitation, the probes were partially degraded in  $0.2\text{ N NaOH}$  on ice for 30 min, neutralized with  $1\text{ M acetic acid}$  and ethanol precipitated. The probes for *in situ* hybridization were resuspended at  $5\times 10^4\text{ cts}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$  in hybridization buffer ( $50\%$  formamide;  $2\times$ SSC;  $10\text{ mM Tris}$ , pH 7.5;  $10\text{ mM NaPi}$ , pH 6.8;  $5\text{ mM EDTA}$ ;  $10\%$  dextran sulfate;  $10\text{ mM DDT}$ ;  $10\text{ mM }\beta$ -mercaptoethanol;  $1\text{ mM ADP-}\beta\text{-S}$ ;  $0.1\text{ mM UTP}$ ;  $10\text{ }\mu\text{M S-ATP}$ ;  $150\text{ }\mu\text{g}\cdot\text{ml}^{-1}$  salmon sperm DNA;  $150\text{ }\mu\text{g}\cdot\text{ml}^{-1}$  yeast t-RNA).

Preparation of sections and *in situ* hybridization was done as described by Hogan *et al.* (1986) with modifications. Embryos were frozen in isopentane on dry ice and  $8\text{ }\mu\text{m}$  cryosections were cut at  $-19^\circ\text{C}$ . Sections were dried at  $55^\circ\text{C}$ , fixed in  $4\%$  freshly dissolved paraformaldehyde, dehydrated in graded ethanol series, air dried and stored at  $-20^\circ\text{C}$  until use. Prior to hybridization, slides were treated successively in water, 1 min;  $2\times$ SSC, 30 min at  $70^\circ\text{C}$ ;  $\text{H}_2\text{O}$ , 1 min;  $0.125\text{ mg}\cdot\text{ml}^{-1}$  pronase, 10 min at room temperature;  $0.2\%$  glycine, 30 s; PBS, 1 min;  $4\%$  paraformaldehyde, 20 min; PBS, 1 min;  $0.1\text{ M triethanolamine}$  with  $1/400$  volume acetic acid, 10 min; PBS, 1 min; followed by dehydration in graded ethanol and air drying. Sections were treated overnight with  $8\text{ }\mu\text{l}$  of boiled (2 min) hybridization mix under siliconised coverslips in a humidified atmosphere at  $42^\circ\text{C}$ , washed 2 h in  $50\%$  formaldehyde/ $2\times$ SSC/ $10\text{ mM }\beta$ -mercaptoethanol at  $37^\circ\text{C}$ , treated with  $50\text{ }\mu\text{g}\cdot\text{ml}^{-1}$  RNase for 15 min, washed again overnight, and dehydrated in graded ethanol. Slides

were dipped in Kodak NTB-2 emulsion, autoradiographed for about 10 days at 4°C, developed in Kodak D-19 for 3 min, washed in 1% acetic acid for 1 min and fixed in 30% sodium thiosulphate for 3 min. Sections were stained with Giemsa and visualized using a Leitz Labovet bright-field/dark-field microscope.

## Results

### Isolation and structure of the Pax8 cDNAs

*Pax8* was identified by sequencing c960, a short cDNA isolated from an 8.5 day *p.c.* embryonic cDNA library (Fahrner and Hogan, 1985) using a low-stringency screen. This clone contained part of a new paired box sequence. A 112 bp *EcoRI*-*NciI* fragment of c960 (indicated as probe 1 in Fig. 1) was used to screen the above cDNA library under high-stringency conditions. A 552 bp cDNA clone, c2A, was isolated. It contained more of the paired box as well as sequences downstream. The entire c2A sequence was found to be present in the corresponding region of the genomic clone (data not shown). To isolate full-length *Pax8* cDNA, the above cDNA library was screened again using the whole c2A clone as a probe. Two largely overlapping cDNAs, c3B and c27B, isolated in this screen, are shown schematically in Fig. 1. Together they span 2528 bp and include the entire 1371 bp coding region of *Pax8* (Fig. 2).

The clone c3B has an AAUAAA polyadenylation signal (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981) near its 3' end, followed 12 nucleotides further downstream by a stretch of 14 adenosine residues. Hence, c3B most likely represents the authentic 3' end of *Pax8* mRNA. Because the Northern blot analysis indicates a *Pax8* transcript of 3.1 kb, approximately 600 nucleotides of the 5' untranslated region are still lacking in the full length *Pax8* cDNA sequence.

Comparison of the nucleotide sequence of the paired box of *Pax8* with those of published paired box genes indicates that the *Pax8* and *Pax2* paired domains are

very similar and constitute a separate class of paired domains (Table 1).

### The open reading frame of the Pax8 gene

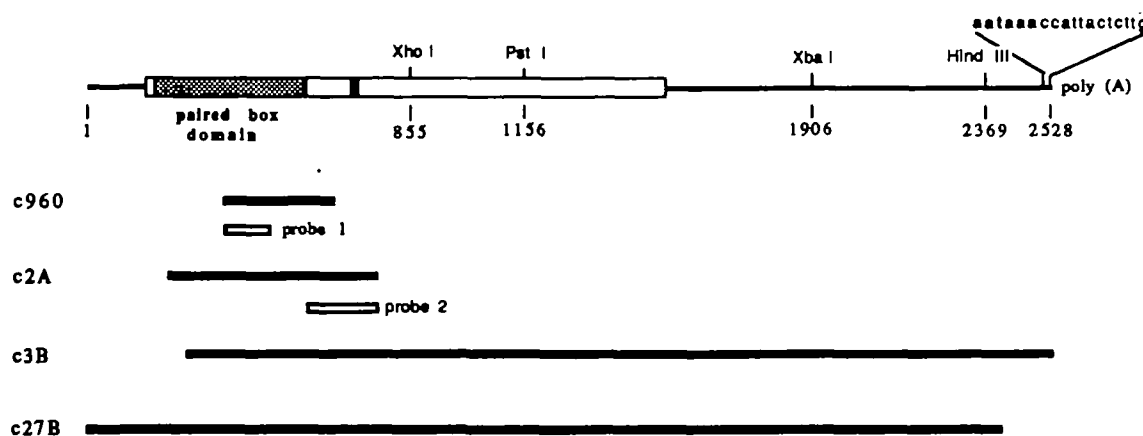
The *Pax8* cDNA clone c27B contains a large open reading frame that could translate into a protein of 457 amino acids (aa) containing the paired domain near its amino terminus (Figs 1 and 2). This open reading frame begins at nucleotide 156 and ends with a stop codon UAG at position 1527. The sequence surrounding this first AUG codon does not match perfectly with Kozak's optimal mutation consensus (CC(A/G)CCAUGG; Kozak, 1986). However, the occurrence of stop codons in all reading frames upstream of the AUG codon at position 156 strongly indicates that this is the start of an open reading frame. Furthermore, the second AUG codon, at position 459, occurs in the paired domain, making it an unlikely candidate for a translational start site.

The conservation of *Pax8* paired box sequences at the amino acid level is even stronger than at the nucleotide level, suggesting a conservation of protein domain

**Table 1.** Nucleotide (ntd) and amino acid (aa) identities between the paired domain of Pax8 and published paired box sequences (in %)

Gene	ntd 1-384	aa 1-128
<i>Pax1</i>	67.7	71.9
HuP48	68.7	71.9
<i>Pox meso</i> (P29)	66.7	70.3
<i>Pox neuro</i> (P4)	64.3	71.1
<i>Pax2</i>	80.2	89.8
HuP1	67.2	71.1
HuP2	65.9	71.1
<i>prd</i>	61.2	64.8
<i>gsb-p</i> (BSH4)	55.7	60.9
<i>gsb-d</i> (BSH9)	57.8	62.5

The published genes are *Pax1* (Deutsch *et al.* 1988), *Pax2* (Dressler *et al.* 1990), *Pox neuro*(P4)/*Pox meso*(P29) (Bopp *et al.* 1989), HuP48/HuP1/HuP2 (Burri *et al.* 1989), *prd* (Frigerio *et al.* 1986; Bopp *et al.* 1986) and *gsb-p*(BSH4)/*gsb-d*(BSH9) (Baumgartner *et al.* 1989).



**Fig. 1.** The composed structure of *Pax8* cDNA. The coding region is represented by an open box. The paired box domain and the conserved octapeptide are indicated as dotted and filled boxes, respectively. The polyadenylation site is shown with its adjacent sequences immediately preceding the poly-A tail. The *Pax8* cDNA clones and probes used for Northern analysis and *in situ* hybridization are outlined underneath.

function (Table 1). Similar to Pax1 (Deutsch *et al.* 1988) and Pax2 (Dressler *et al.* 1990), Pax8 protein contains no paired type homeodomain, a characteristic of *paired* and *gooseberry* proteins (Bopp *et al.* 1986). Another protein region conserved among several paired box

genes, the octapeptide, was recently described by Burri *et al.* (1989). The octapeptide region is found 43 amino acids downstream from the paired domain in Pax8. The spacing between the paired domain and the octapeptide in the Pax2 protein (41aa) is very similar. The Pax8

Pax8 cDNA	
1	GAAGAGACGCTGGGCTTGGCACCCCTCAGGGGAGACCCAGGCAGAAAGGGCTGAGGCCAGCCGGCCAGGGTAGCTGCGTGGCAGCC 90
91	AGAGCTGCCAGGACCTGCGTAGGAAAGCTGCGAGTGTCCCTCAGTCTGTGAGGCACTCCCCGGCGATGCTCACAACCTCGATCAGATCGG 180
1	M P H N S I R S G 9
181	GCCATGGAGGGCTGAATCAACTAGGAGGGGCTTTGTGAATGGCAGGCCTCTGCCAGAAGTTGTACGTCAACGCATTGTGGACTTGGCCC 270
10	H G G L N Q L G G A F V N G R P L P E V V R Q R I V D L A H 39
271	ACCAGGGGGTGGAGCCCTGTGATATTTCTGCCAGCTCCGTGTGAGCCATGGCTGTGAAGCAAGATCCTTGGCAGGTACTACGAGACTG 360
40	Q G V R P C D I S R Q L R V S H G C V S K I L G R Y Y E T G 69
361	GCAGCATCCGGCTGGAGTGATAGGGGGCTCCAAGCCCAAGGTGGCCACCCCAAGGTGGTGGAGAAGATAGGAGACTACAAGCGGCAGA 450
70	S I R P G V I G G S K P K V A T P K V V E K I G D Y K R Q N 99
451	ACCCTACCATGTTTCTTGGGAGATCCGGGACCGGCTCCTGGCAGAAGGGCTTTGTGACAATGACACTGTCCCCAGTGTGAGTCCATCA 540
100	P T M F A W E I R D R L L A E G V C D N D T V P S V S S I N 129
541	ACAGATCATCCGGACCAAGTTCAGCAGCCATTCAACCTCCCATGGATAGCTGTGTGGCCACCAAGTCTCTGAGCCAGGACACACAC 630
130	R I I R T K V Q Q P F N L P M D S C V A T K S L S P G H T L 159
631	TGATCCCCAGCTCAGCTGTAAACCCCCAGAGTACCCCAAGTGGATTCCTTGGGCTCTACCTACTCTATCAACGGGCTCCTAGGAATTG 720
160	I P S S A V T P P E S P Q S D S L G S T Y S I N G L L G I A 189
721	CTCAGCTGGCAATGACAACAAGAGAAAGATGGATGATAGTGACCAGGACAGCTGTGGCTAAGCATCGACTCACAGAGCAGCAGCTG 810
190	Q P G N D N K R K M D D S D Q D S C R L S I D S Q S S S S G 219
811	GTCCTCGAAAGCACCTTCGTACGGACACCTTCAGCCAGCACCATCTCGAGGCCCTTGAGTGCCCGTTTGGCGGCAGCATTACCCGGAGG 900
220	P R K H L R T D T F S Q H H L E A L E C P F E R Q H Y P E A 249
901	CCTATGCTCCCCAGCCACACCAAGGGGAGCAGGGGCTGTACCCACTGCCCTTACTCAACAGTGCCCTGGATGATGGGAAGGCCACCC 990
250	Y A S P S H T K G E Q G L Y P L P L L N S A L D D G K A T L 279
991	TGACATCTTCCAATACACCTCTGGGACGCAACCTCTCGACTCACCAGACCTACCTGTGGTGGCAGATCTCTATTACCCCTTCGCCATAA 1080
280	T S S N T P L G R N L S T H Q T Y P V V A D P H S P F A I K 309
1081	AGCAGGAAACCCAGAGCTCTCCAGTTCTAGCTCCACCCCTTCTCTTTATCTAGCTCCGCTTTTGGATCTGCAGCAAGTCGGCTCTG 1170
310	Q E T P E L S S S S S T P S S L S S S A F L D L Q Q V G S G 339
1171	GGGGCCAGCAGGTGCCCTCGGTCCACCCCTTCAATGCCCTTTCCCATGCTGCTCCGTGTATGGGAGTTACGGGCCAGGCCCTCCTCT 1260
340	G P A G A S V P P F N A F P H A A S V Y G Q F T G Q A L L S 369
1261	CAGGGCAGAGATGGTGGGGCCACGCTGCCGTGGATACCCACCCACATCCCCACCAAGTGGACAGGGCAGCTATGCTCTTCTGCTATCG 1350
370	G R E M V G P T L P G Y P P H I P T S G Q G S Y A S S A I A 399
1351	CAGGCATGGTGGCAGGAAGTGAATATTCTGGCAATGCCATACAGCCACACCCCGTATTCTCTACAGTGAGGCTGGCGCTTCCCAACT 1440
400	G M V A G S E Y S G N A Y S H T P Y S S Y S E A W R F P N S 429
1441	CCAGCTGCTGAGTTCTCCATATTATTACAGCTCTACATCAAGGCCAAGCGCACCCACCTCTGCCACAGCCTTTGACCATCTGTAGT 1530
430	S L L S S P Y Y Y S S T S R P S A P P T S A T A F D H L * 457
1531	TGTCATGGGGACAGTGGGAAGAACCAGGCAAGAATCAGGAGGACTTGGCTGAGACAGGCCAGAGAGTCAACAAAGGAATCTTTATT 1620
1621	TATTACATGAAAAATAACCAATTCAGCATTTGCCGCTCACTCCCTGTGTGGTCAATTAAATGAACCATGAAGTCAGGATGACCTTGG 1710
1711	AGAAGGCCAACTGTCTCCAGGATTCATTTGTGAGTGACAGGAGTTCAGGAAACAAGAACCCCATTTCAAAGAGACACAGAGGA 1800
1801	GAAATGTACCGAAGCTGGCACCCACCAAGAGAGAGAAAGGGTTACGAAGAACTTAGAGGGAGATGAGTCTGAGGGTGTACAGGGCCA 1890
1891	CCTGTTTGGTCTTCACTAGACAACCTCAGCAATATGAAACAGTATGCAAGTCTCTTGGCTTACAATCAGAGCTAGGGCCAAAGCTCT 1980
1981	ACGTGTCAGTCACTAGCAGTTACCACTTGAAGCTCTACTACCTGCATCTTCTGGGATTCAGCTGACCTTCTATTTCAGATTGCTG 2070
2071	TGCAATTTACAAGCACCCCTTAGCCTCTCAACCAAGTGGCTACCCCTTGGCAGCAGGCAAGCTGTCCCTGGAAAGTTCAAGTGGCTCTA 2160
2161	CGTAGAACATAGGAAGCACACAGATGGGACAGCCATGTCTCTCTCGGCAGCCTTGCCAAATCAAGTGTGCTTCTGCAAGTTCACAGC 2250
2251	CGCGCAGCCACTGAAGAGACCCCAAGGCCAGCAAGATTCCTCTCTGCTCTGCTCTGCTCTGCTCTGCTCTGCTCTGCTCTGCTCTGCTCT 2340
2341	ATAACGTAATGAAACTCTGTTTGGTCAAGCTTTCTCTTTTCATCCCCAGTCTTTGGTCTCTGAAATATCTCCCTACTATGTTGGG 2430
2431	TTTTCTCTTCACTCTTTTCTTTTAAAAAAGTCTTCCCTCCATTACCATGACTCAATAAACATTACTCTTGAATAA 2520
2521	AAAAAAA 2528

Fig. 2. Nucleotide and predicted amino acid sequences of Pax8 gene. The paired domain sequences are boxed. A stop codon (TAG) preceding the putative ATG initiation site in the same frame, the octapeptide and the polyadenylation site (AATAAA) are indicated by the solid underlines.

Hup1	His	Ser	Ile	Asp	Gly	Ile	Leu	Gly
Hup2	—	—	—	—	—	—	—	Ser
<i>gsb-d</i> (BSH9)	—	—	—	—	—	—	—	—
<i>gsb-p</i> (BSH4)	Tyr	Thr	—	Asn	—	—	—	—
<i>Pax2</i>	Tyr	—	—	Asn	—	—	—	—
<i>Pax8</i>	Tyr	—	—	Asn	—	Leu	—	—

Consensus Tyr Ser Ile Asp Gly Ile Leu Gly

**Fig. 3.** Compilation of homologous octapeptide sequences found in *Pax8* and several other published paired box containing genes. The genes Hup1 and Hup2 are from man (Burri *et al.* 1989), *gsb-p* and *gsb-d* are from *Drosophila* (Baumgartner *et al.* 1987). *Pax2* is from mouse (Dressler *et al.* 1990). The consensus sequence is as proposed by Burri *et al.* (1989).

octapeptide differs from that of *Pax2* by only one conservative amino acid substitution (Fig. 3). *Pax8* protein has a repeat of leucine residues at aa positions 265, 272, 279 and 286. However, occurrence of two proline residues within this repeat makes it unlikely that this repeat forms the  $\alpha$ -helix, needed for a 'leucine zipper' configuration (Landschulz *et al.* 1988).

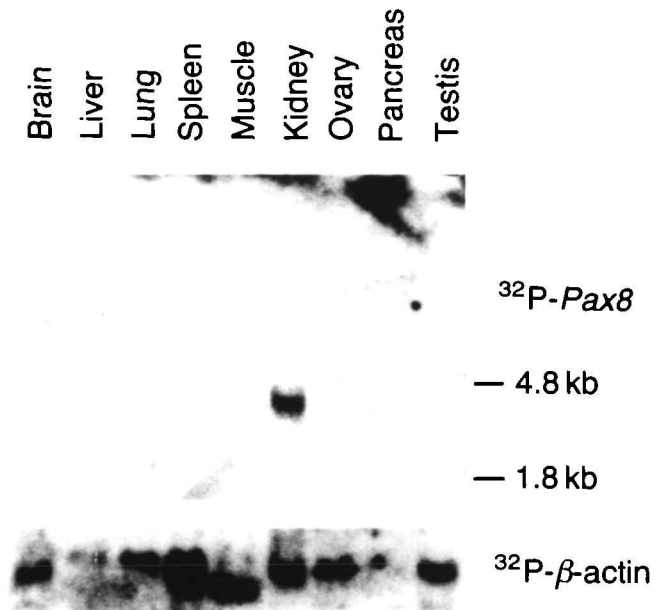
#### The chromosomal localization of the *Pax8* gene

To determine the chromosomal location of *Pax8*, we have used the mouse interspecies backcrosses (Guenet, 1986). Using an approx. 700 bp genomic *HindIII*-*NcoI* fragment from the *Pax8* locus as a probe, we have demonstrated a *Sau3A* restriction fragment length polymorphism (RFLP) between C57BL/6 mice and an inbred *Mus spretus* line SPE/Pas (data not shown). This RFLP was used to probe DNAs from a panel of backcross progeny of (C57BL/6  $\times$  SPE/Pas) $F_1$  females  $\times$  C57BL/6 males. The segregation pattern of *Pax8* alleles was compared to the segregation patterns of all other genes analyzed in the same panel of backcrosses. *Pax8* is closely linked to the surfeit (*surf*) (0/30 recombinants scored) and HOX-5 (4/28 recombinants scored, linkage distance is approx. 14 cM organs) gene clusters within the proximal portion of chromosome 2 (Stubbs *et al.* 1990).

#### Expression pattern of *Pax8* RNA in the mouse embryo and adult tissues

To determine the tissue specificity, temporal and spatial expression pattern of *Pax8* RNA, we used Northern blot and *in situ* hybridization analyses. Two different cDNA fragments of *Pax8* were used as probes; the 112 bp *EcoRI*-*NciI* fragment from clone c960 containing the paired box sequences and the *Pax8* specific 184 bp *NcoI*-*EcoRI* fragment from clone c2A (Probe 1 and 2, respectively; Fig. 1). Both probes revealed the same pattern of *Pax8* expression. Only results obtained with probe 2 are presented here.

Northern blot analysis of poly(A)<sup>+</sup> RNA samples from various adult tissues revealed a 3.1 kb *Pax8* message in kidney (Fig. 4). No *Pax8* transcripts were detected in brain, liver, lung, spleen, muscles, ovary, pancreas or testis. Furthermore, no *Pax8* transcripts could be detected on the Northern blots of poly(A)<sup>+</sup>



**Fig. 4.** Northern blot analysis of *Pax8* transcripts in the adult tissues of the mouse. Approximately 5  $\mu$ g of polyadenylated RNA from corresponding tissues were hybridized with a unique *Pax8* probe depicted in Fig. 1 as probe 2. The presence and integrity of RNA on the blots was confirmed by the hybridization with a  $\beta$ -actin probe.

RNAs from 10 to 17 day *p.c.* embryos, even after prolonged exposure (data not shown). However, *Pax8* mRNA may be scarce in samples of whole embryo poly(A)<sup>+</sup> RNA because its expression is restricted to small regions of the embryo and the level of this expression is low.

To precisely localize the spatial and temporal distribution of *Pax8* RNA, *in situ* hybridization analysis of tissue sections from mouse embryos of various developmental stages was done. Sections were hybridized to the <sup>35</sup>S-UTP/CTP-labeled antisense RNA probes (material and methods). Sense RNA probes were used as negative controls.

Fig. 5 illustrates the expression of *Pax8* in the developing excretory system. Because *Pax2* gene is also expressed in this system (Dressler *et al.* 1990), the patterns of expression of both genes were compared using parallel sections. The 527 bp *BamHI*-*EcoRI* fragment unique for the *Pax2* gene (Dressler *et al.* 1990) was used to produce RNA probes. Both *Pax2* and *Pax8* are expressed in the nephrogenic cord and in the more anterior mesonephric tubules of 10.5 day *p.c.* embryo (Fig. 5A-D). The mesonephric tubules are induced in the nephrogenic cord mesenchyme by the nephric (Wolffian) duct during its growth in the posterior direction. Only *Pax2* was detected in the nephric duct (Fig. 5C,D).

During the next stage of kidney development, the ureteric bud emerges from the most caudal portion of the nephric duct, invades the metanephrogenic mesenchyme and branches. These branches induce the mesenchymal cellular condensations (Saxén, 1987).

Only *Pax2* transcripts are detected in the branching ureter at 13.5 days gestation, whereas both *Pax2* and *Pax8* are expressed in the mesenchymal condensations and in the epithelial structures forming from the condensations (Fig. 5E–H). Thus, the inducing part of the secretory system, the nephric duct and the ureter, has no detectable *Pax8* expression, whereas the responding part, the mesenchymal condensations and ultimately the S-shaped bodies, express *Pax8*. This is demonstrated more clearly in Fig. 5M,N; only the mesenchymal cells that have condensed around the ureteric duct express *Pax8*. The ureteric duct itself and the non-condensed mesenchymal cells show no *Pax8* expression.

At day 16.5 of gestation morphogenesis proceeds in the cortex of the metanephros where *Pax8* and *Pax2* are expressed strongly (Fig. 5I–L). At this stage of gestation, *Pax2* transcripts are also detected in the pancreas (Fig. 5K,L).

Fig. 6 shows the expression of *Pax8* in the developing thyroid gland. *Pax2* expression was not detected in this organ at any stage in development (data not shown). *Pax8* transcripts first appear in the area of thyroid evagination from the floor of the pharynx at day 10.5 *p.c.* (Fig. 6A,B) and remain visible as the thyroid vesicle buds off from the floor of the pharynx and migrates caudally (Fig. 6C–H). Eventually, a two-lobed gland is formed. At this stage *Pax8* is expressed in the lobes (Fig. 6I,J) and in the isthmus, which connects the lobes (Fig. 6K,L). No *Pax8* expression is seen in the parathyroid gland, which develops independently from two pairs of pharyngeal pouches and subsequently embeds itself in the thyroid tissue (Fig. 6I,J).

*Pax8* is expressed transiently in the myelencephalon (Fig. 7I,J) and through the entire length of the neural tube at day 11.5 of gestation (Fig. 7A,B,E,F). The expression subsides at day 12.5 and is not detectable at day 13.5 of gestation (data not shown). The pattern of *Pax8* expression in the neural tube is very similar, if not identical, to that of *Pax2* at this stage of development (Fig. 7C,D,G,H). However, the genes differ in their temporal expression patterns in the CNS, because *Pax2* expression in the spinal column continues at least to day 18.5 of gestation (Nornes *et al.* 1990).

## Discussion

In this report, we have described a novel murine paired box gene, *Pax8*, which is expressed in a tissue-specific manner during development. The 2528 bp *Pax8* cDNA sequence encompasses the entire coding region for a 457aa protein. A paired domain is located near the amino terminus of this conceptual protein. Interestingly, all the paired box genes described so far in man, mouse and fly contain the paired domain near the amino end of their corresponding proteins. A conservation of this domain among such different species suggests conservation of function. However, this function is at present unknown. It has been shown that the products of the *Drosophila* segmentation gene

*paired*, which contain both paired and homeodomains, can bind specific DNA sequences (Hoey and Levine, 1988; Treisman *et al.* 1989). Not all of these DNA-binding activities can be ascribed to the homeodomain of the paired protein (Treisman *et al.* 1989). Thus, the DNA-binding function of the paired domain remains an attractive hypothesis.

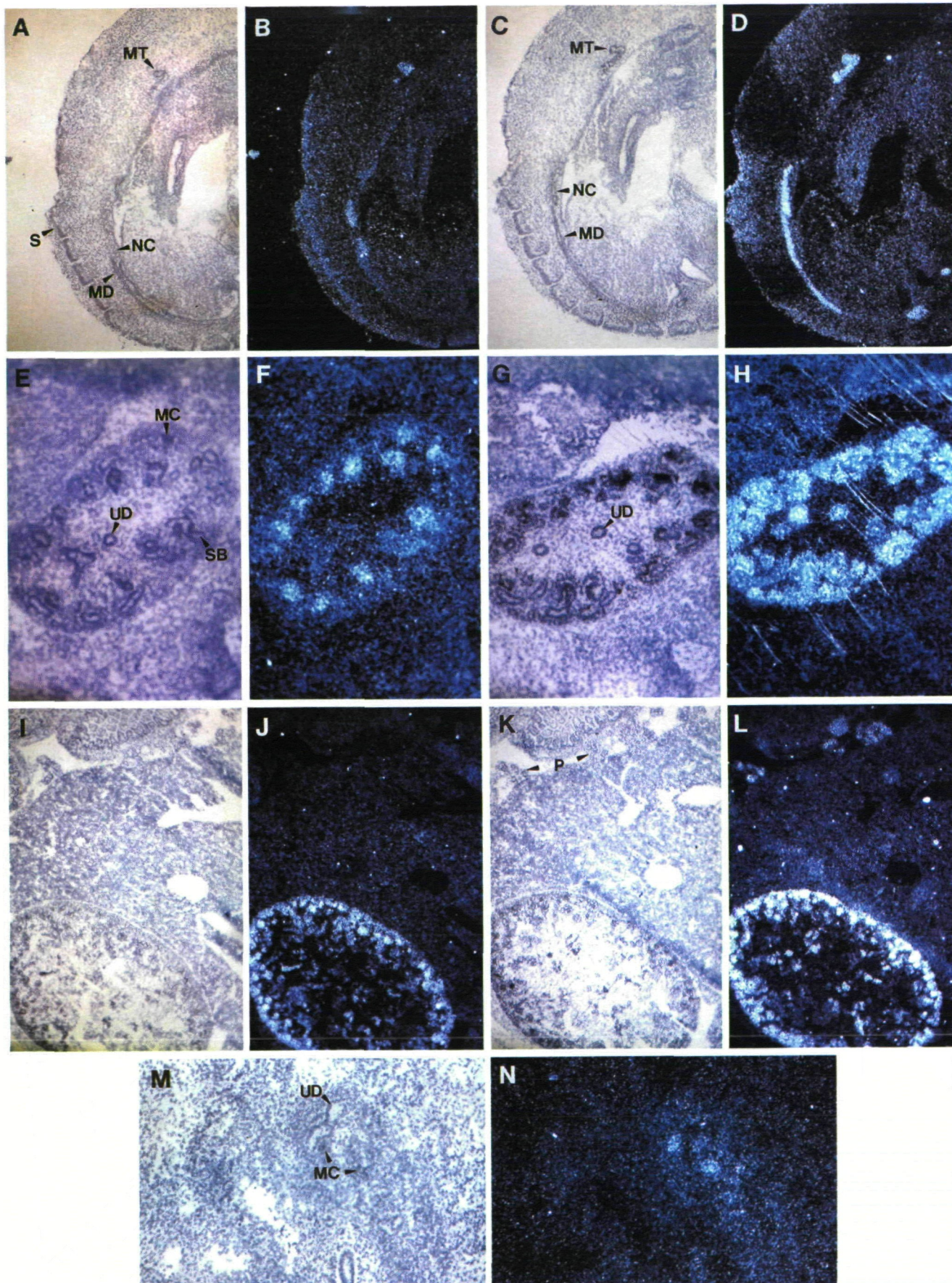
In *Drosophila*, two paired box genes that do not contain a homeodomain have been described (Bopp *et al.* 1989). *Pax8* and two other murine genes, *Pax1* (Deutsch *et al.* 1988) and *Pax2* (Dressler *et al.* 1990), share this characteristic. Furthermore, the paired domains of *Pax8* and *Pax2* have a pronounced similarity. This structural similarity is especially noteworthy because both genes have similar tissue-specific expression. Using *in situ* hybridization, we have compared the expression of *Pax8* and *Pax2* in the developing excretory system. The cellular compartments of this system result from the reciprocal inductive interactions between the growing nephric duct and later ureter, and nephrogenic mesenchyme (for review see Saxén, 1987). The nephrogenic mesenchyme responds to the induction by formation of cellular condensations, which give rise to the epithelial structures of the nephrons. The expression of both genes has parallels with the morphogenetic changes during these inductive processes: whereas *Pax8* expression is restricted to the responding tissues, *Pax2* transcripts can be detected both in the inducing and in the responding tissues of the kidney.

During the first stage of kidney development, the nephric duct induces the formation of the mesonephric tubules where both *Pax8* and *Pax2* are expressed. The tubules are organized segmentally along the rostrocaudal axis. However, this segmental pattern does not correspond to the primary segmentation of the mouse embryo into somites. Thus, it is questionable whether *Pax8* or *Pax2* play the part in primary segmentation analogous to their *Drosophila* homologs. Accordingly, we could not detect *Pax8* or *Pax2* expression by *in situ* hybridization at 8.5 day of gestation when segment determination is occurring (Hogan *et al.* 1985; Hogan *et al.* 1986).

The molecular mechanisms of signal transduction underlying the inductive process of epithelium forma-

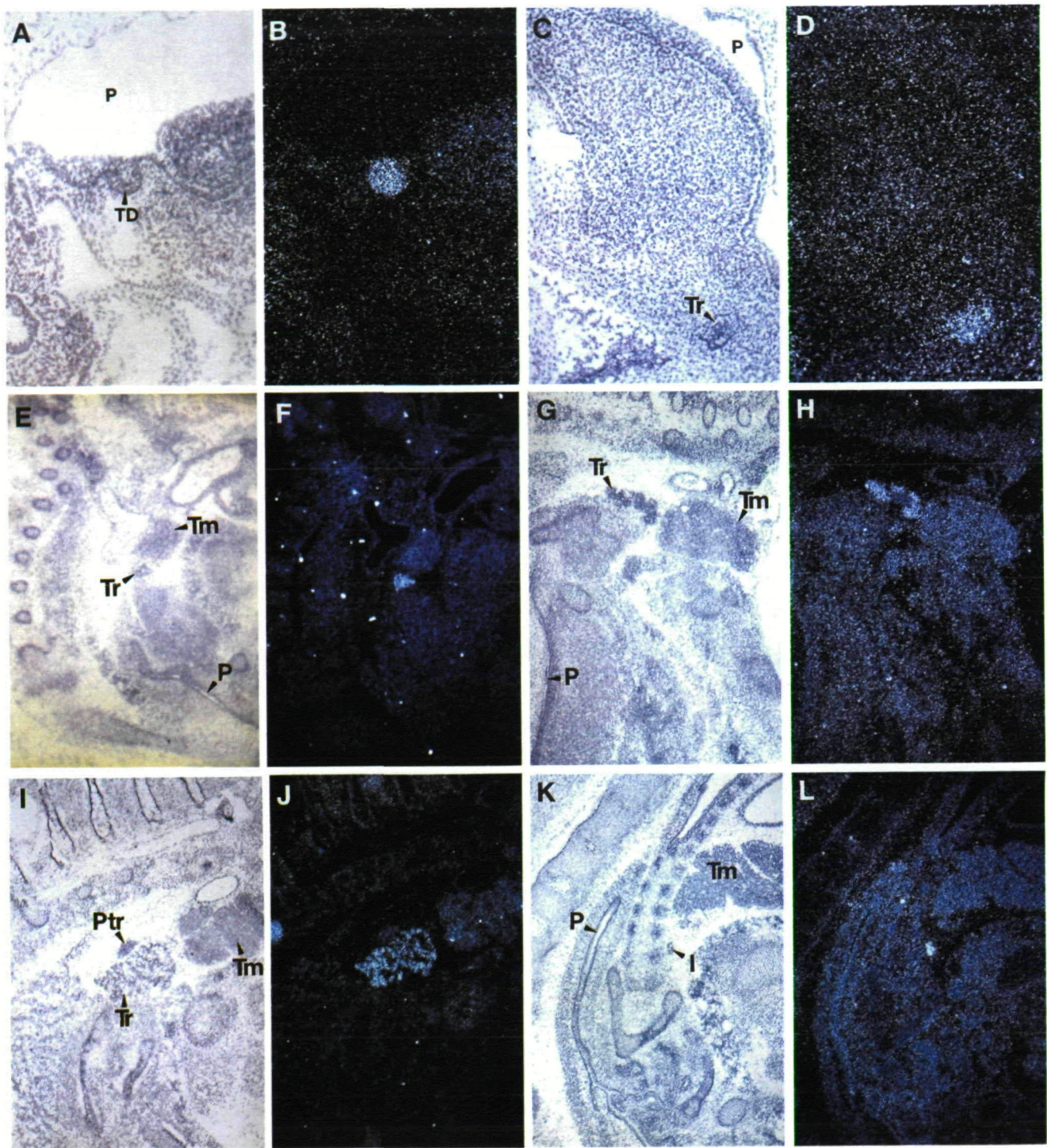
**Fig. 5.** Comparison of expression of *Pax8* and *Pax2* genes in the developing meso- and metanephros. Parasagittal section through 10.5 day *p.c.* embryo, magnification  $\times 40$  (A,B,C,D). MD, mesonephric duct; MT, mesonephric tubules; NC, nephrogenic cord; S, somite. Expression of *Pax8* (A,B) and *Pax2* (C,D). Note the absence of *Pax8* expression in the mesonephric duct. Parasagittal section of 13.5 day *p.c.* embryo, magnification  $\times 100$  (E,F,G,H). UD, ureteric duct; MC, mesenchymal condensation; SB, S-shaped body. Expression of *Pax8* (E,F) and *Pax2* (G,H). Note the absence of *Pax8* expression in the ureter. Parasagittal section of 16.5 day *p.c.* embryo, magnification  $\times 40$  (I,J,K,L). P, pancreas. Expression of *Pax8* (I,J) and *Pax2* (K,L). Note the absence of *Pax8* expression in the medulla of the kidney. Parasagittal section of 13.5 day *p.c.* embryo, expression of *Pax8* (M,N), magnification  $\times 100$ .





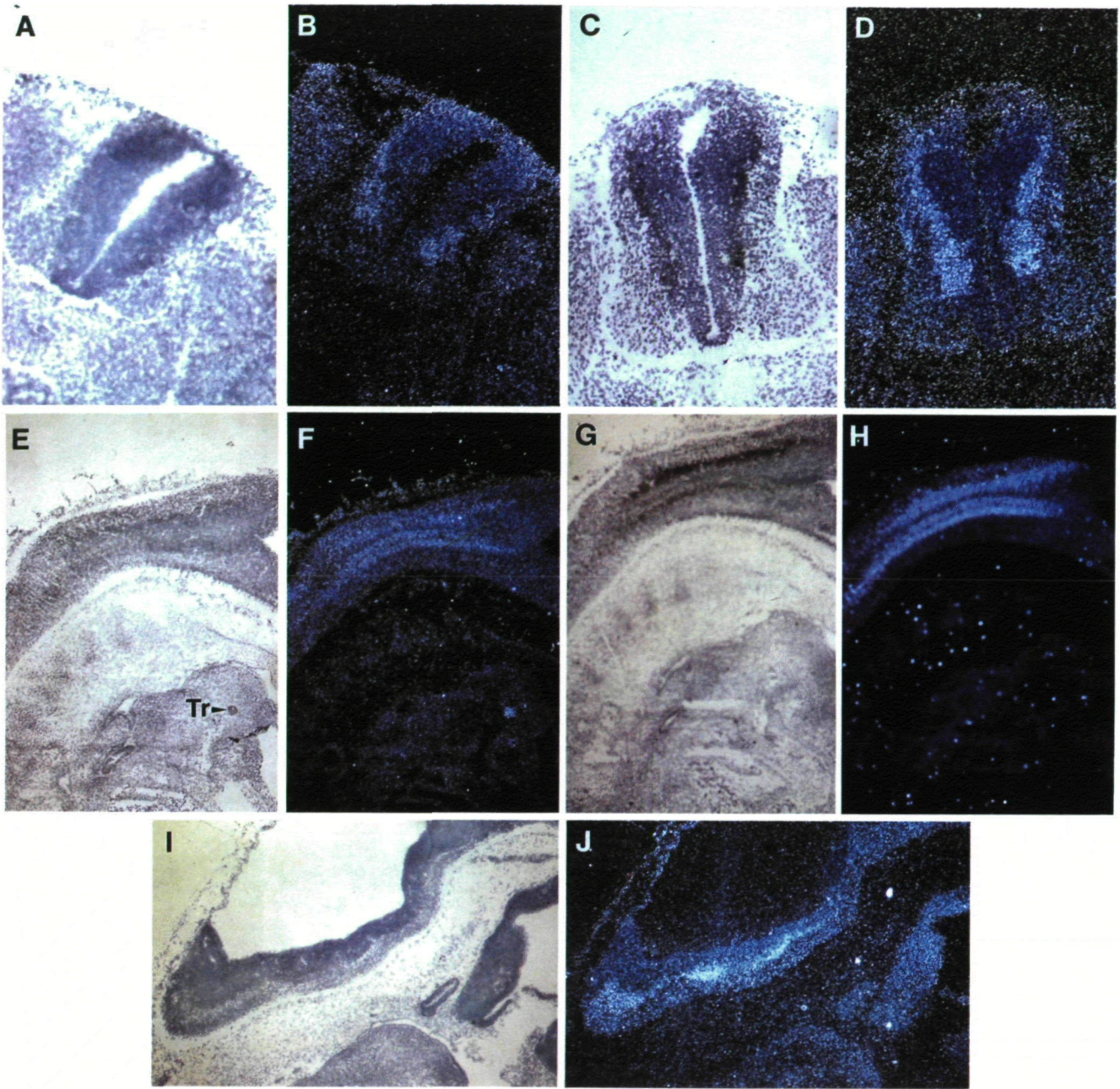






**Fig. 6.** Expression of *Pax8* in the developing thyroid gland. Sagittal section of 10.5 day *p.c.* embryo, magnification  $\times 100$  (A,B). TD, thyroid diverticulum; P, pharynx. Sagittal section of 11.5 day *p.c.*,  $\times 100$  (C,D), 13.5 day *p.c.*,  $\times 40$  (E,F) and 14.5 day *p.c.*,  $\times 40$  (G,H) embryo. Parasagittal section (I,J) and sagittal section (K,L) of 16.5 day *p.c.* embryo, magnification  $\times 40$ . Tr, thyroid; Ptr, parathyroid; I, isthmus; Tm, thymus.





**Fig. 7.** Expression of *Pax8* and *Pax2* in the CNS of 11.5 day *p.c.* embryo. Cross sections, magnification  $\times 100$  (A–D). Sagittal sections, magnification  $\times 40$  (E–H). Expression of *Pax8* (A,B,E,F) and *Pax2* (C,D,G,H). Tr, thyroid. Parasagittal section through the myelencephalon, expression of *Pax8* (I,J), magnification  $\times 40$ .

tion in the metanephric kidney are not known. Two levels of determination can be distinguished in this process. First, the metanephrogenic mesenchyme isolated from an 11 day embryo is already predetermined to respond to the inducing agents by tubule formation (Grobstein, 1955; Saxén, 1970). It may imply that the cells of the metanephric blastema have already reached a state in which only few additional factors are needed to start morphogenesis. Hypothetically, transcription factors, *Pax8* and *Pax2* among them, that are induced in these cells during this period could play a crucial role in this process. Not only ureter but also neural tube can induce epithelialization of the mesenchymal cells (Grobstein, 1955). Interestingly, *Pax2* is expressed in both these tissues. The second level, an actual determination of the mesenchymal cells to become the components of nephron is achieved only after approximately 24 h of contact with the inducer (Saxén and Lehtonen, 1978). The molecular events that take place during this time are completely obscure. Subsequently, irreversible morphogenetic changes occur including the formation of the basal membrane, establishment of the epithelial polarity and an increase in cellular adhesivity (Saxén, 1987).

Profound molecular changes in the extracellular matrix and cell surface molecules during the nephron formation have also been reported recently. The expression of fibronectin and interstitial type I and type III collagens is replaced by the basement membrane components including type IV collagen and laminin chains (Ekblom, 1981; Ekblom *et al.* 1981b; Ekblom *et al.* 1980; Senior *et al.* 1988; Laurie *et al.* 1989; Ekblom *et al.* 1990). Antibodies against laminin A chain can inhibit the polarization of the mesenchymal cells, suggesting a functional role for laminin in this process (Klein *et al.* 1988b). Another extracellular matrix glycoprotein, tenascin, is induced around the mesenchymal condensations (Aufderheide *et al.* 1987). Furthermore, neural cell adhesion molecules (N-CAM) are replaced by uvomorulin, another primary CAM (Vestweber *et al.* 1985; Klein *et al.* 1988a). Another result of induction is that the mesenchymal cells acquire the responsiveness to transferrin (Ekblom *et al.* 1983) and express the desmosomal proteins (Garrod and Fleming, 1990). Being putative transcription factors, *Pax8* and *Pax2* may be involved in the described molecular processes.

It is also worth noting that the developing nephron itself becomes segregated into three segments, i.e., glomerulus, proximal and distal tubules. The markers specific for each of these segments have been described (Ekblom *et al.* 1981a). *Pax8* and *Pax2* are molecular markers characteristic of both early mesenchymal condensations and late epithelial structures resulting from condensations.

Using an interspecies backcross, we have mapped *Pax8* to the centromeric region of mouse chromosome 2 in a close linkage to the *surf* locus. It would be interesting to determine whether the human homolog of *Pax8* is located on human chromosome 9q which reveals a strong synteny to proximal mouse chromo-

some 2 (Yon *et al.* 1989; Stubbs *et al.* 1990). Several mouse developmental mutations including Danforth's short tail (*Sd*), stubby (*stb*), fidget (*fi*), lethargic (*lh*) and rachiterata (*rh*) are linked in the proximal portion of mouse chromosome 2 (Davisson *et al.* 1988). The semi-dominant *Sd* mutation is especially interesting in context of the present report. Recently, the *surf* cluster has been positioned within close proximity of the *Sd* locus (Stubbs *et al.* 1990). Thus, *Pax8*, *surf* and *Sd* map close to each other. The *Sd* mutation is characterized by the abnormalities of the axial skeleton and the reduction or absence of kidneys (Dunn *et al.* 1940). The phenotype of the skeleton and kidneys may both have a common origin from the abnormality of the notochord in the *Sd* mice (Grüneberg, 1958). However, the organ culture studies have revealed a reduction of the tubule formation intrinsic to the mutant metanephrogenic mesenchyme (Gluecksohn-Waelsch and Rota, 1963). Because *Pax8* is expressed in the developing tubules, it would be interesting to investigate its association with the *Sd* mutation.

The expression of *Pax8* in the developing neural tube, secretory system and thyroid gland, which originate from ectoderm, mesoderm and endoderm, respectively, may indicate pleiotropic functions of the *Pax8* gene. Furthermore, we cannot exclude the possibility that *Pax8* is also expressed in the mesenchymal component of the developing thyroid. Both in the mesenchymal condensations of the kidney and in the developing thyroid gland, *Pax8* expression is associated with the zones of changes in cell proliferation (Saxén *et al.* 1983; Smuts *et al.* 1978). Common functions of *Pax8* in the developing kidney and thyroid associated with the appearance and/or maintenance of the cell polarization also cannot be ruled out (Chambard *et al.* 1981).

Because of the overlapping expression pattern of *Pax8* and *Pax2*, it would be interesting to find out whether these two gene products interact with each other at the molecular level.

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