GENOMICS **42,** 452–461 (1997)
ARTICLE NO. GE974735

The Genomic Organization of the Murine Pax 8 Gene and Characterization of Its Basal Promoter

Olga Okladnova,*[,1] Andrej Poleev,*[,2] Judy Fantes,†
Muriel Lee,† Dimitrij Plachov,*[,3] and Jürgen Horst*[,4]

*Institut für Humangenetik, Universität Münster, Vesaliusweg 12-14, 48149 Münster, Germany; and
†MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, United Kingdom

Received October 25, 1996; accepted March 24, 1997

λ phage clones containing the murine Pax 8 gene were isolated from a C57BL/6 kidney genomic mouse library using mouse cDNA fragments as probes. A clone encompassing about 16 kb of the 5′ untranslated region of the murine Pax 8 gene was isolated from a mouse embryonic stem cell (D3) library. The murine Pax 8 gene has a size of approximately 26 kb and contains the coding sequence for mRNA in 12 exons. The major and several minor transcription initiation sites were identified. Position +1 is located 488 nucleotides upstream of the ATG initiation codon and 24 bases downstream of a TATA-like sequence, ATAAAA. The translation initiation and termination sites are located in exons 2 and 12, respectively. Further analysis of 570 bases of the 5′ flanking sequence revealed AP2, SP1, PEA3, zeste, NF-κB, and CCAAT consensus binding sites. Ribonuclease protection assays with a probe spanning the first two exons of mouse Pax 8 cDNA on total RNA samples isolated from different tissues of newborn mice show that the murine Pax 8 gene is predominantly expressed in kidney tissue. Low levels of Pax 8 gene expression were also found in the liver, spleen, lung, brain, and heart. The same transcription initiation sites are utilized in different tissues of newborn mice and embryo at Day 10.5 postconception. A FISH assay shows that the murine Pax 8 gene is located on chromosome 2, map position B.

INTRODUCTION

Pax 8 is a paired box-containing gene and a member of the Pax gene family (Walter *et al.*, 1991; Pilz *et al.*, 1993). Nine paired box-containing genes have been isolated from vertebrates by their homology to *Drosophila* genes that regulate pattern formation and contain conserved DNA-binding domains, such as the homeobox or paired box domain (Gehring *et al.*, 1990; Treisman *et al.*, 1991; Lewis, 1978; Nüsslein-Volhard and Wieschaus, 1980; Stapleton *et al.*, 1993; Holland and Hogan, 1988). Genes of this group are highly conserved throughout evolution. Each member of this family shows temporally and spatially restricted expression patterns during embryonic development (Tremblay and Gruss, 1994). The murine Pax genes are located on five different chromosomes and are not arranged in clusters. This fact and numerous other lines of evidence that these genes have different expression patterns, suggest that the functions of Pax genes are independent. Pax genes 2, 5, and 8 encode the paired box, octapeptide, and strongly reduced parts of the homeodomain; Pax 1 and Pax 9 encode the paired domain and octapeptide; Pax 7 and Pax 3 encode the full homeodomain in addition to the paired domain and octapeptide; and Pax 4 and Pax 6 genes lack the octapeptide but encode the paired box and homeodomain (for review, Stuart *et al.*, 1993). It was shown recently that Pax 1, 2, 5, and 8 proteins are transcriptional activators (Chalepakis *et al.*, 1991; Fickenscher *et al.*, 1993; Adams *et al.*, 1992; Zannini *et al.*, 1992). The developmental importance of these genes is also suggested by developmental disorders in mice and humans, found to be associated with Pax gene mutations (for review, Gruss and Walter, 1991; Tremblay and Gruss, 1994; Stuart and Gruss, 1995).

The Pax 8 gene is expressed in the adult thyroid and during mouse embryogenesis in the intermediate zone of the myelencephalon and metencephalon up to the midbrain–hindbrain boundary (Nornes *et al.*, 1990), in induced mesenchymal condensations and their epithelial derivatives of the excretory system, as well as in the thyroid (Plachov *et al.*, 1990; for review, Kessel and Gruss, 1990). It has also been shown that the human Pax 8 gene is expressed at high levels in Wilms tumors (Poleev *et al.*, 1992). In addition Pax 8 protein binds

Sequence data from this article have been deposited with the EMBL Data Library under Accession Nos. X99592–X99598.

[1] Present address: Universität Würzburg, Psychiatrische Klinik, 97080 Würzburg, Germany.
[2] Present address: Institut für Zellbiologie, GBK Haus, Arbeitsgruppe 3, Universitätsklinikum, 45147 Essen, Germany.
[3] Present address: Tresckowstrasse 10, 48163 Münster, Germany.
[4] To whom correspondence should be addressed. Telephone: (+49) 251-835401. Fax: (+49) 251-835431.

0888-7543/97 $25.00

to similar consensus sequences and transactivates two thyroid-specific genes, thyroglobulin and thyroperoxidase (Zannini *et al.*, 1992). In both promoters, the binding site of Pax 8 overlaps with the binding site of TTF-1, which is a homeodomain-containing protein involved in the activation of thyroid-specific transcription (Sinclair *et al.*, 1990). The Pax 8 gene is also subjected to an alternative splicing that may modify the coding region (Poleev *et al.*, 1992, 1995; Kozmic *et al.*, 1993).

We have previously cloned and sequenced the cDNA of the mouse Pax 8 gene (Plachov *et al.*, 1990). Here we report the genomic organization of the mouse Pax 8 gene and describe its organization. This gene spans about 26 kb of genomic DNA and is organized into 12 exons. In addition, we mapped the gene promoter of Pax 8. The mouse Pax 8 gene has multiple transcriptional initiation sites and contains TATA- and CCAAT-like boxes. We show that the putative promoter region contains consensus sequences for the binding of various transcription factors. We also demonstrate that the murine Pax 8 gene is expressed at low levels in tissues of newborn mice such as liver, lung, brain, spleen, and heart, as well as kidney, thyroid and pituitary glands, and entire embryo at Day 10.5 postconception, in which expression is predominant.

MATERIALS AND METHODS

Screening of a mouse genomic DNA library and phage DNA analysis. Clones l8B, l10B, and l5B were isolated from a C57BL/6 mouse kidney genomic *Mbo*II partial-digest library cloned into the EMBL 3 vector. This library was constructed by I. Koseki (Japan) (Koseki *et al.*, 1989) and kindly provided by Kenji Imay. It was screened with a 294-bp cDNA probe (position 182–476 according to the cDNA sequence). Clone l12C was isolated with a 1635-bp murine Pax 8 cDNA probe (position 893–2528) (Plachov *et al.*, 1990). Clone λStu27, containing the first two exons of Pax 8, was isolated with a murine Pax 8 cDNA probe *Eco*RI/*Sty*I (182 bp) from a mouse embryonal stem cell genomic (D3) library in the lambda Dash II vector (Stratagene) (Kaestner *et al.*, 1993) and was kindly provided by Klaus Kaestner. All DNA fragments used as specific probes were radioactively labeled by random priming with the Random Priming kit (Pharmacia) and [α-^{32}P]dCTP (Hartmann Analytics). Libraries were screened under high-stringency hybridization conditions (500 m*M* sodium phosphate, pH 7.2, 7% SDS, 1 m*M* EDTA at 65°C and wash with 40 m*M* sodium phosphate, pH 7.2, 1% SDS at 65°C). A total of 1×10^6 plaques were screened.

Several positive clones were purified, and phage DNA was isolated by the PEG precipitation method according to Sambrook *et al.* (1989). The restriction map of each clone was constructed by appropriate digests. Restriction endonuclease mapping of fragments was carried out by established protocols (Sambrook *et al.*, 1989). Clones were digested with different enzymes and probed with Pax 8 cDNA or with different probes corresponding to different Pax 8 exons. The fragments containing exons were identified by Southern blot analysis and subcloned into the pBluescript (KS$^+$) phagemid (Stratagene).

DNA sequencing and analysis. (1) Double-stranded DNA was sequenced by the dideoxy chain-termination method with [α-^{35}S]dATP (Sanger *et al.*, 1977) using the T7-sequencing kit (Pharmacia), universal and reverse primers, and gene-specific oligonucleotides as internal primers. The intron–exon boundaries were sequenced directly from subcloned genomic fragments or overcloned smallest fragments (exon 1 and 2—from 5.6-kb fragment *Bam*HI; exon 3—from 1.2-kb *Eco*RI fragment; exon 4—from 560-bp *Eco*RI fragment; exons 5, 6, and 7—from 2.7-kb *Hin*dIII fragment; exons 8 and 9—from 1.8-kb *Eco*RI/*Bam*HI fragment; exon 10—from 1.65-kb *Eco*RI/*Hin*dIII fragment; exon 11—from 375-bp *Xba*I fragment; and exon 12—from 625-bp *Xba*I fragment and from 1.8-kb *Xba*I/*Eco*RI fragment).

(2) The sizes of the two introns, one between exons 2 and 3 and the other between exons 11 and 12, were defined by the polymerase chain reaction (PCR) with oligonucleotides of two adjacent exons as primers and mouse genomic DNA clones as templates. The sequences for those PCR primers were derived from the cDNA sequence (Plachov *et al.*, 1990) and were as follows: for intron 2, forward—5′-ATGCCTCACAACTCGATCAGATC-3′ and reverse—5′-CAGAGG-CCTGCCATTCAC-3′ and for intron 11, forward—5′-GAAGTGAAT-ATTCTGGCAATG-3′ and reverse—5′-CTACAGATGGTCAAAGGC-TGT-3′.

PCR were performed with *Taq* polymerase (Boehringer Mannheim) in the supplied buffer for 30 cycles at 94°C (0.5 min), annealing temperature according to the melting temperature of the appropriate set of primers, and 72°C (1 to 5 min, depending on intron size).

RNA isolation. Total RNA was isolated from different tissues of newborn NMRI mice (1–2 days of age), i.e., kidney, liver, lung, spleen, brain, and heart. Total RNA from thyroid and pituitary glands was purified from 1-month-old adult NMRI mice by the guanidinium thiocyanate–phenol–chloroform technique (Chomczynski and Sacchi, 1987) using RNAzol (Cinna/Biotecx).

Nuclease S1 analysis. S1 protection assays were performed using a modified procedure of the method of Green (1987). Briefly, the 1.7-kb *Xba*I (from position −494 to +1193 with respect to the first nucleotide of exon 1) was subcloned into the *Xba*I site of the pBluescript (KS$^+$) and designated pBX1.5. This construct was linearized with *Hpa*II, dephosphorylated with calf intestinal phosphatase, and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol). The labeled DNA was then digested with *Sca*I, and a 319-bp fragment was isolated from a 6% DNA-sequencing gel. Hybridizations were carried out in 80% formamide in the buffer 40 m*M* Pipes [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 1 m*M* EDTA, pH 8.0, 400 m*M* NaCl with 30 μg total RNA and 165,000 cpm of a labeled probe at 80°C for 15 min and then at 48°C overnight. Then, 300 ml of S1 buffer (250 m*M* NaCl, 30 m*M* sodium acetate, pH 4.5, 1.5 m*M* $ZnSO_4$) and 300 U of S1 nuclease (Pharmacia, Biotech) per milliliter were added. The reaction mixtures were incubated at room temperature for 1 h. The protected fragments were precipitated with ethanol and analyzed on a 6% polyacrylamide sequencing gel. A pBluescript digested with *Hpa*II and labeled with T4 polynucletide kinase and [γ-^{32}P]ATP was used as a size marker. After electrophoresis the gel was dried and exposed to a Kodak X-OMAT AR film.

Primer extension analysis. The 36-nt primer (5′-GGATCTGAT-CGAGTTGTGAGGCATCGCCGGGGAGTC-3′) from the 3′ end of exon 2 was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. Total RNA from different tissues of NMRI mice was used as template for reverse transcription, and 5 pmol of 5′-labeled primer was hybridized with 50 μg total RNA according to the standard protocol. The hybrid mixture of total RNA and the 5′-labeled primer was then precipitated with ethanol, washed with 70% ethanol, dried, and dissolved in 20 ml of the hybridization buffer (250 m*M* KCl, 10 m*M* Tris–Cl, pH 7.5, 1 m*M* EDTA, pH 8.0). Afterward it was incubated for 10 min at 85°C and 2 h at 65°C. The hybridization mixture was then ethanol-precipitated, and the pellet was resuspended in 100 ml of reverse transcription buffer. The primer was extended by incubation with 600 units of Superscript RNase H$^-$ reverse transcriptase (Life Technologies) at 42°C for 30 min. The reaction was terminated by adding 5 ml of 0.5 *M* EDTA, pH 8.0, and 0.5 ml of 10 mg/ml RNase A1. After 30-min incubation at 37°C, the transcribed fragments were purified by phenol–chloroform extraction, precipitated with ethanol, and analyzed on 6% acrylamide/8 *M* urea gels. Primer extension products were sized against the sequencing reaction of mouse Pax8 cDNA template, using the same reverse primer utilized for primer extension and T7 DNA polymerase (Pharmacia).

Riboprobe construction. A *Xba*I–*Bam*HI fragment encompassing 494 bp upstream of exon 1, the entire exon 1, and 147 bp downstream of the relevant donor splice site was subcloned into pBluescript (KS$^+$) (Stratagene), and the resulting construct was linearized with

*Bam*HI. A Pax 8 gene-specific fragment *Eco*RI/*Nco*I (blunt end) from Pax 8 cDNA was subcloned into *Eco*RI/*Hin*dIII (blunt end) pBluescript (KS^+) (Stratagene), and the resulting construct was linearized with *Eco*RI. Antisense riboprobes were synthesized with T3 RNA polymerase (812 and 251 bp, respectively) using the Riboprobe kit (Promega) and [α-^{32}P]UTP under conditions specified by the supplier (Promega). The quality of the probes was checked on a 6% polyacrylamide/8 *M* urea gel.

Ribonuclease protection assay. The RNase protection assay was essentially carried out as described previously (Melton *et al.*, 1984). Briefly, 50 mg of total RNA, isolated from kidney, liver, spleen, lung, brain, and heart of newborn mice (1–2 days of age) and from entire embryos at Day 10.5 postconception, was heated with the riboprobe (2×10^5 cpm) in 10 ml of 80% formamide hybridization buffer (80% formamide, deionized; 40 m*M* Pipes, pH 6.4; 0.4 *M* NaCl; 1 m*M* EDTA) at 85°C for 5 min to denature the RNA, and samples were incubated overnight at 37°C. Then 300 ml of cold RNase digestion solution (10 m*M* Tris–HCl, pH 7.5; 5 m*M* EDTA; 300 m*M* NaCl; 2 mg/ml RNase T1 and 40 mg/ml RNase A) was added, and samples were incubated at 37°C for 60 min. To terminate the RNase digestion, 20 ml of 10% SDS and 10 ml of proteinase K (10 mg/ml in H_2O) were added and incubated at 37°C for 30 min. Reaction mixtures were then extracted with phenol–chloroform and precipitated with ethanol. Samples were washed with 70% ethanol and air-dried, and pellets were carefully resuspended in 6 ml of formamide loading buffer (80% formamide, deionized; 10 m*M* EDTA; 1 mg/ml xylene cyanol; 1 mg/ml bromphenol blue). After heating to 95°C for 5 min and chilling in an ice bath, samples were subjected to electrophoresis on a denaturing 6% polyacrylamide/8 *M* urea gel followed by autoradiography.

Computer analysis and sequence comparison. Analysis of the 5′ upstream region was performed using the HUSAR program (Heidelberg GENIUS computer centrum; Heidelberg, Germany).

Fluorescence in situ hybridization. Probe Stu27, containing Pax 8 genomic DNA in a phage vector (Dash II) was nick-translated with biotin-16– or digoxigenin-16–dUTP (Langer-Safer *et al.*, 1982).

Metaphase chromosome preparations were made from an exponentially growing culture of mouse embryonic stem cells incubated for 1 h in 0.1 mg/ml colcemid before harvesting. The cells were treated with 0.5% trisodium citrate and 0.25% KCl hypotonic solution for 10 min at 37°C before fixing three times in 3:1 methanol:acetic acid. Slides were prepared and stored for 3–7 days under vacuum.

Specific probes were hybridized to mouse metaphase chromosomal preparations as described previously (Fantes *et al.*, 1992), using mouse cot-1 DNA to suppress the hybridization of repeated sequences. For detection biotin label was visualized using successive layers of fluorescein isothiocyanate–avidin D (avidin–FITC DCS) (cell sorter grade) from Vector Laboratories (Burlingame, CA), biotinylated anti-avidin, and avidin–FITC DCS. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images were collected on a Zeiss Axioplan microscope, using a Chroma Pinkel No. 1 filter set with a Photometrics charge-coupled device camera and Digital Scientific Software. Most of the mouse chromosomes could be identified after enhancing the DAPI image allowing the position of hybridization signals to be determined.

RESULTS

Isolation and Mapping of Genomic Clones

Using a mouse cDNA probe from Pax 8 (Plachov *et al.*, 1990), we screened a mouse genomic library and isolated five λ phage clones, designated l5B, l10B, l8B, l4.1, and l12C. Restriction analysis and hybridization assay revealed that these clones contain the complete Pax 8 gene sequence. Figure 1 shows the genomic organization of the mouse Pax 8 gene. The genomic clone l8B is about 28 kb long and includes sequences that correspond to exons 3 to 10, and it also contains sequences of unknown origin at the 3′ end. Two other genomic clones are identical (l5B and l10B), about 25 kb in length, and include exons 3 to 12, the polyadenylation signal, and a part of the 3′ untranslated region. Clone λ4.1 contains exons 1 to 3 and has sequences of unknown origin at the 5′ and 3′ ends. The genomic clone λ12C has a length of about 30 kb which includes exons 11 and 12 and a large 3′ flanking region. The 16-kb genomic clone λStu27, which hybridized with the probe that has the exon 1 and 2 sequence, was isolated from the mouse genomic embryonal stem cells (D3) library. This clone includes about 14 kb of the 5′ untranslated part of the promoter region and the first two exons.

The identified clones were selected for further detailed analysis. Intron sizes were derived from the gene map or from PCR products by sequencing. The compiled sequencing data revealed that the murine Pax 8 gene is approximately 26 kb in length and is composed of 12 exons. The positions of the intron–exon boundaries were determined by comparison with consensus sequences for intron–exon splice junctions and were also compared by the existing high degree of homology with the human Pax 8 gene, for which intron–exon boundaries have been determined for exons 3 to 12 (Kozmic *et al.*, 1993). The sequences of the intron–exon boundaries are presented in Table 1.

The site of cleavage and polyadenylation of the primary transcript was determined by comparison to the full-length murine Pax 8 cDNA sequence (Plachov *et al.*, 1990). There is a GT-rich sequence approximately 20 bp downstream of the poly(A) signal AATAAA found in many genes which is thought to be involved in cleavage and/or processing of primary transcripts (Birnstiel *et al.*, 1985; McLauchlan *et al.*, 1985). The genomic structure of the murine Pax 8 gene is presented in Fig. 1.

Analysis of the 5′ Flanking DNA Sequence of the Murine Pax 8 Gene

To identify potential *cis*-acting regulatory elements in the 5′ region flanking the murine Pax 8 gene, 570 bp upstream of exon 1 were sequenced. The TATA-like consensus ATAAAA was found 43 bases upstream of the first nucleotide of exon 1. The CCAAT box-like sequence GCCAATCC was identified 53 bases upstream of ATAAAA. Some other consensus sequences for transcription factors in the putative promoter region were found by sequence analysis with the HUSAR programm (Heidelberg). A computer-assisted search revealed a number of potential regulatory elements: two putative SP1-binding sites, two AP2 consensus sequences, three zeste-like sequences (two of which are in opposite orientation), and one site for TFIID-EIIa transcriptional activation factor. Two PEA3 consensus sequences, one in opposite orientation, are placed on the boundary of the first exon (Fig. 2). The respective

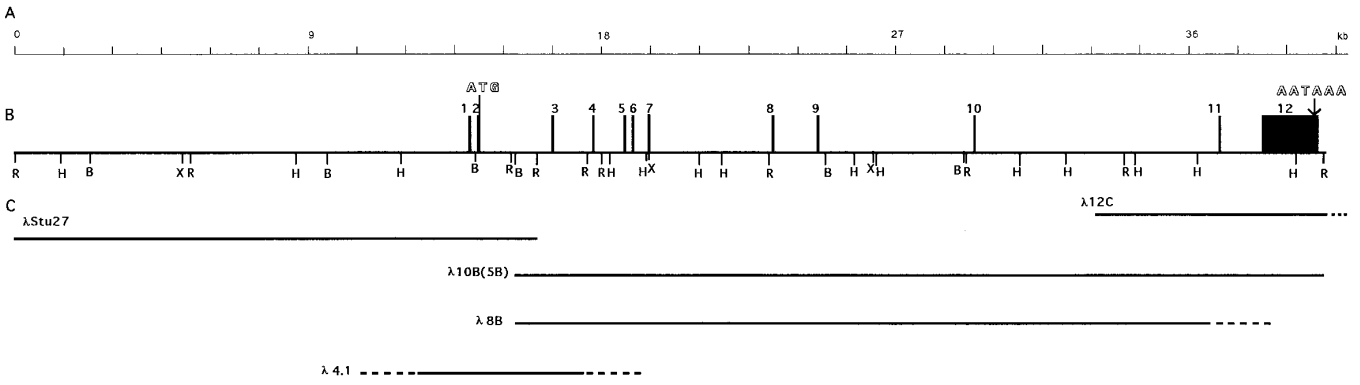

FIG. 1. Genomic organization of the murine Pax 8 gene. (**A**) The scale of sizes. (**B**) Restriction map of the murine Pax 8 gene. The restriction map incorporates information from the λ gDNA clones described in the text. The positions of exons 1–12, restriction recognition sites [*Eco*RI (R), *Hin*dIII (H), and *Xho*I (X)], and predicted translational initiation (ATG codon) and polyadenylation sites (AATAAA) are indicated by vertical bars. (**C**) Genomic clones used for the analysis of the murine Pax 8 gene. Clone λ8B is recombinat at the 3′ end; clone λ4.1 contains exons 1, 2, and 3 and respective introns and is recombinant at 5′ and 3′ ends; in addition to the indicated genomic Pax 8 region, clone λ12C contains additional DNA, which was not characterized (dashed line).

positions of all regulatory elements found in the sequence are shown in Fig. 2.

Determination of the Transcription Start Site

We showed that TATA- and CCAAT-like boxes are located 43 and 97 nucleotides upstream, respectively, of the first exon of the murine Pax 8 gene. To map the transcriptional start site, primer extension, S1-nuclease, and RNase protection experiments were performed with total RNA isolated from different tissues of newborn 1- to 2-day-old mice and also from thyroid and pituitary glands of 1-month-old mice. Primer extension utilizing an antisense 36-bp oligonucleotide (Fig. 3) complementary to nucleotides 144 to 179 of the murine Pax 8 cDNA revealed multiple transcription initiation sites, extending from 130 to approximately 600 nucleotides upstream of the predicted translational initiation codon at position +155 located in exon 2 (Fig. 4A). Sequencing reactions using the same oligonucleotide for a sequencing primer served as size markers. Five major transcription initiation sites were identified, which altogether account for more than 95% of the total signal. The most intensive signal corresponds to the −19 nucleotide position, counting the first nucleotide of exon 1 as +1. This nucleotide is A, a common initiation site, which is designated with an arrow and numbered in the promoter sequence +1 (Fig. 4A). Only a few of these initiation sites coincide with consensus recognition motifs for transcription factors mapped by our computer analysis. For example, three extension products coincide with consensus motifs for the TATA-box and SP1 sites, and two with the AP2 factor and CCAAT-box motifs, respectively.

The results of the primer extention analysis show also that banding patterns for RNA isolated from differ-

TABLE 1

Intron–Exon Splice Junction Sites of the Murine Pax 8 Gene

Exon	Size (bp)	Splice donor	Splice acceptor	Intron size (bp)
1	72	AGCCGGCCAG**gt**atgtcacctaggggctag. . .	cgccctgggtctatatgca**g**GGTAGCTGCG	304
2	107	ATCAGATCCG**gt**aaggaccgcggaggggc. . .	tgacaatttggtctgttttt**ag**GCCATGGAGG	~2500
3	266	TCCTTGGCAG**gt**aagcacgaaattcaccaca	gggggttctctattggagt**ag**GTACTACGAG	~778
4	270	CCATCAACAG**gt**gagagacagcagttattgg	cagtccttctgcttccccac**ag**AATCATCCGG	~740
5	88	CACACACTGA**gt**gagtgtttagggatttggct	atcctctccttattcctttg**cag**TCCCCAGCTC	388
6	126	ATGGATGATA**gt**gagtgctcaggggacctg	cttcctgcctgggggcacta**g**GTGACCAGGA	262
7	275	AGGGGAGCAG**gt**aagaagctgggatgcaag	gctgacttccctttgttctcca**g**GGGCTGTACC	~4100
8	59	GTGGTGGCAG**gt**aggatgcctagaggctcc	gttgtttccctccctccggca**g**ATCCTCATTC	476
9	306	CTCCTCTCAG**gt**cgacagggacgtccccgg	gactctttggtgtcttttacca**g**GGCGAGAGAT	~5100
10	101	ATGGTGGCAG**gt**aagggtcgaggggctggg	tcttgcctgtgtgctccaaca**g**GAAGTGAATA	~8200
11	87	AGCCTGCTGA**gt**aacttcctggggttatccag	cgtgtgtgtttaatgtgtcca**g**GTTCTCCATA	1359
12	1059	ATTACTCTTG**gt**ctcaggtttatcttgggatgg	—	—

Note. The sequences were determined from clones λ8B, λ10B (λ5B), and λ12C (see Fig. 1). Exon sequences are represented by uppercase letters, while intron sequences are represented by lowercase letters. Consnesus gt-at (5′ → 3′) splice site sequences are underlined. Intron sizes were determined by direct sequencing (introns 1, 5, 6, and 8), estimation from restriction mapping analysis of genomic clones (intron 3, 4, 7,8, 9, and 10), or size estimation of PCR products (intron 2 and 11). Intron–exon boundaries and intron sequences, EMBL Accession Nos. X99592 through X99598.

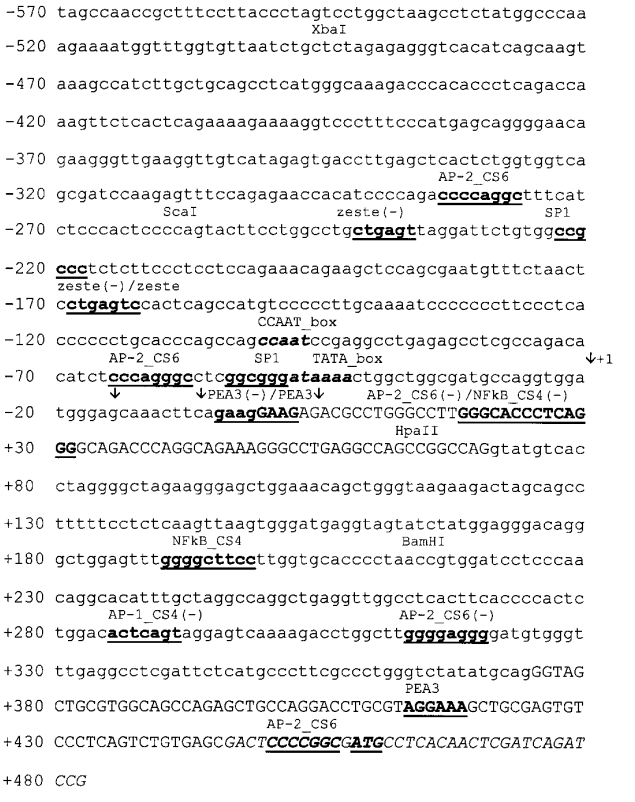

FIG. 2. Genomic sequence and computer analysis of the 5′ end (nt −570 to +480) of the murine Pax 8 gene. Sequences of exons are indicated by uppercase and sequences of the 5′-flanking region and intron 1 by lowercase letters. Potential TATA and CCAAT sequences are shown in italics and boldface; sequences exhibiting similarity to transcription factor-binding sites are in boldface and underlined. Restriction sites for *Xba*I, *Hpa*II, and *Bam*HI are marked, oligonucleotides used for primer extension analysis are in italic letters. The five major transcription start sites are given with vertical arrows. The main start site is designated +1.

ent tissues are identical, although the relative and overall intensity of the bands may vary in different samples. The most intensive pattern of radioactive bands is produced by kidney RNA. These results indicate that the same start sites of the murine Pax 8 gene are used in distinct tissues and in different developmental stages (newborn and adult mice).

To confirm the results obtained in primer extension experiments, S1-nuclease assays were performed. A *Sca*I–*Hpa*II fragment covering the first exon of the murine Pax 8 gene and extending 260 bp upstream was used (Fig. 3). The S1-nuclease mapping analysis indicated the presence of several transcriptional initiation sites (Fig. 4B), most of which correspond to the sites determined by the primer extension analysis. The most intensive band (Fig. 4B) corresponds to the nucleotide that was designated +1 and is located 24 nucleotides downstream of the TATA-like box. Patterns of protected products are similar in different tissues, but their relative intensity is different. Analyses of kidney and embryonal RNA specifically resulted in very intensive signals. The negative control (yeast tRNA) did not provide any protection fragments (Fig. 4B, lane 6).

A ribonuclease protection analysis was performed, using a *Xba*I–*Bam*HI fragment (see Materials and Methods and Fig. 3). An 812-nt *in vitro* transcript spanning the entire first exon detected multiple protected fragments ranging in size from approximately 60 to 400 bp, corresponding to transcripts that initiated 1 to 350 bp upstream of exon 1 accordingly. The most intensive and specific pattern of protected product corresponded to kidney RNA samples (Fig. 5, lane 1). The strongest band is indicative of a nucleotide +1 transcript and was obtained in kidney and embryonal RNA. This band is also present as a weaker signal in analyzed RNA preparations from other tissues (Fig. 5).

Thus, primer extension, S1-nuclease mapping, and ribonuclease protection analyses gave similar results, indicating that there are multiple initiation sites for transcription of the murine Pax 8 gene with one major transcription start site in kidney. RNA from other tissue samples also show the presence of this start site. The size of the extension products revealed by the primer extension analysis is slightly larger than the size of protected fragments seen by S1-mapping and ribonuclease protection assay. The specificity of transcriptional initiation sites in RNA preparations from different tissues in the primer extension experiments is not as obvious as with S1-nuclease mapping and RNase protection assays, because of the different sensitivities of the methods, primer extension analysis being the most sensitive.

Expression Analysis of the Murine Pax 8 Gene in Mouse Tissues

We performed ribonuclease protection experiments to identify the Pax 8-expressing tissues of newborn mice. The murine Pax 8 cDNA corresponding to the first two exons was subcloned and used for generation of the single-stranded riboprobe (Fig. 3). This probe is highly specific for the Pax 8 gene, because it does not include the sequence of the paired box. Our results indicate that the murine Pax 8 gene is almost exclusively expressed in kidney (Fig. 6, lane 1) but is also expressed at low levels in other tissues.

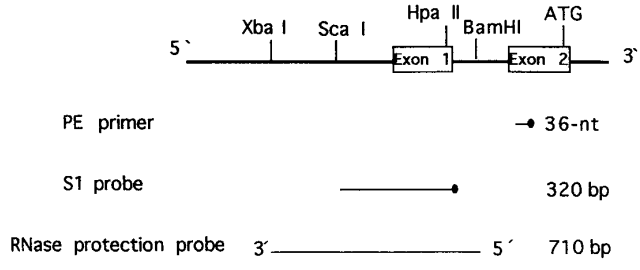

FIG. 3. (**Top**) Partial restriction map of exons 1 and 2, intron 1, and the 5′ flanking regions of the murine Pax 8 gene. (**Bottom**) PE is the 36-nt end-labeled primer used for primer extension analysis. End-labeled S1 nuclease probe (the *Sca*I/*Hpa*II fragment) encompasses the entire exon 1 and 257 nucleotides of the Pax 8 promoter region. The RNase protection probe is 710 bp (*Xba*I–*Bam*HI fragment) encompassing 494 bp upstream of exon 1, the entire exon 1, and 147 bp downstream of the donor splice site.

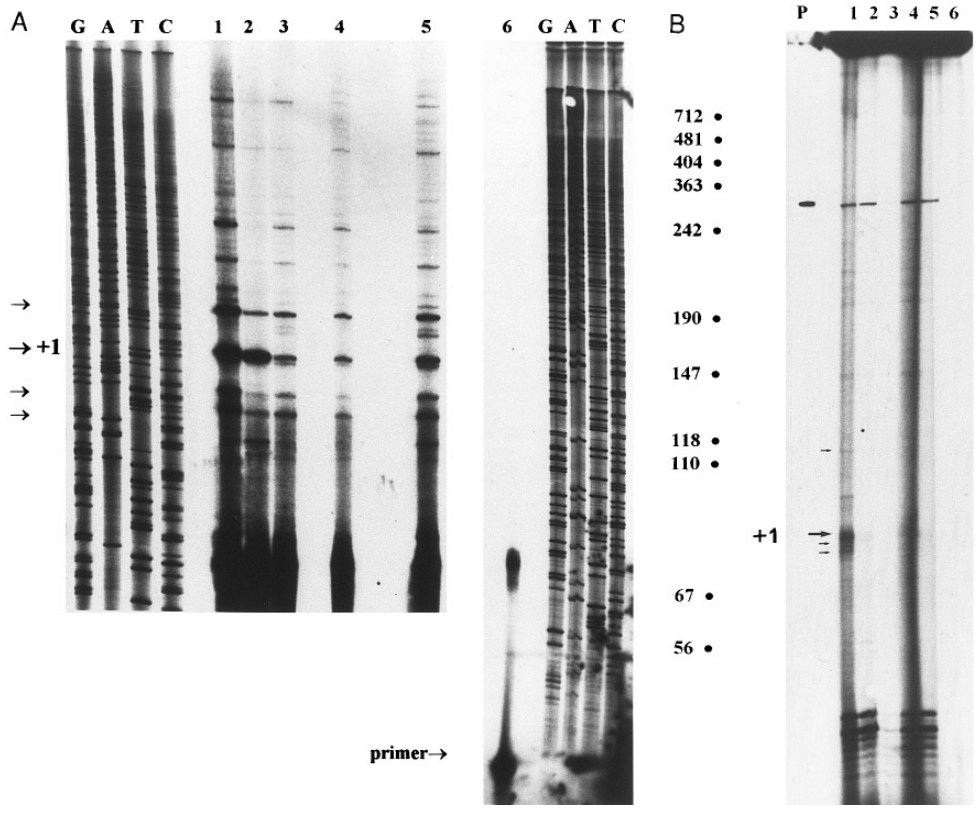

FIG. 4. Determination of the transcription initiation sites by primer extension and S1-nuclease mapping and sequence of the 5′ end of the gene with promoter region. (**A**) The primer-extended products together with standard sequencing reaction of the Pax 8 cDNA, utilizing the same primer as for extension reactions. Numerous primer extension products are obtained, and the four major products are indicated by arrows. The position corresponding to the main start site of the Pax 8 gene is marked with +1. Lanes of primer extension reactions correspond to preparations of total RNAs (50 μg for each reaction) isolated from kidney (lane **1**), lung (lane **4**), and liver (lane **5**) of newborn mice (1–2 days of age) and from thyroid (lane **2**) and pituitary glands (lane **3**) of adult species. Lane **6** shows yeast tRNA used as a negative control. The position of the primer is marked with an arrow. (**B**) S1-nuclease analysis revealed several protected fragments, indicating transcriptional initiation sites in the same region as was obtained by primer extension. Four protected fragments, the same as those generated by primer extension, are shown (arrows). The position of the major start site is designated +1. pBluescript (KS$^+$) digested with *Hpa*II and ^{32}P-end-labeled was used as a size marker. Numbers on the left indicate the ladder fragment sizes. As with primer extension analysis, total RNA preparations (5 μg for every reaction except lane **3,** where only 1 μg of total RNA was used) from kidney (lane **1**), liver (lane **2**), lung (lane **3**), embryo at Day 10.5 postconception (lane **4**), brain (lane **5**), and yeast tRNA (lane **6**) were used. Lane **P** shows the input probe.

Chromosomal Mapping of Genomic Phage Clones

The probe λStu27, containing genomic DNA of the murine Pax 8 gene gave a signal close to the centromere of a chromosome that was clearly identified as chromosome 2 by its banding pattern, as shown in Fig. 7a. The map position, determined on 14 long chromosomes, was 2B according to the mouse idiogram (Fig. 7b).

DISCUSSION

Here we report the molecular cloning of the murine Pax 8 gene, describe its organization, and give an initial characterization of the Pax 8 promoter.

Genomic Organization of the Murine Pax 8 Gene

The exon structure of the murine Pax 8 gene was determined by sequencing of all genomic regions complementary to the murine Pax 8 cDNA, evaluating the exon–intron boundaries by breakpoint of homology with the Pax 8 cDNA and the presence of consensus splice sequences. The murine Pax 8 gene spans approximately 26 kb and consists of 12 exons and 11 introns, similar to the human Pax 8 gene (Kozmic *et al.*, 1993). Human and mouse Pax 8 genes show a high level of homology between their sequences at intron–exon boundaries. For example, 36 nucleotides (the 18 bases 3′ of exon 2 and 18 bases 5′ of exon 3) of the boundary areas of the second and third exon differ by only 2 nucleotides. It has been shown previously that the Pax 8 coding regions of both species show a very high degree of homology (Poleev *et al.*, 1992). Different isoforms generated by alternative splicing have been detected for both mouse and human Pax 8 genes in different tissues (Kozmic *et al.*, 1993; Poleev *et al.*, 1995). The first intron spans 362 bp of genomic DNA for the human and 304 bp for the mouse gene. The high level of homology of both genes also includes the 5′ untranslated region and the first exon (unpublished data), and

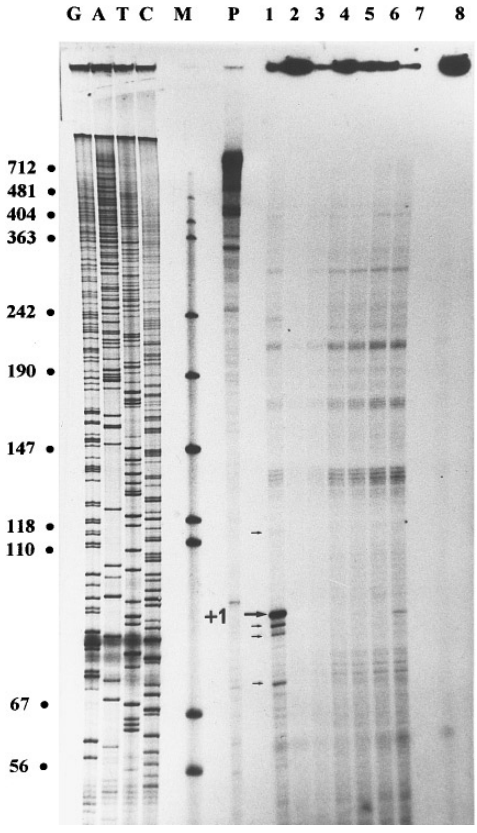

FIG. 5. Ribonuclease protection analysis. A single-stranded antisense riboprobe (see Materials and Methods) was hybridized to 50 μg total RNA isolated from kidney (lane **1**), liver (lane **2**), spleen (lane **3**), lung (lane **4**), brain (lane **5**), and brain (lane **6**) of newborn mice (1–2 days of age) and from entire embryo at Day 10.5 postconception (lane **7**). Yeast tRNA (50 μg) was used as a negative control (lane **8**). Lane **P** indicates the input probe. Probes were digested with RNase A and RNase T1, and digests were run on a 6% sequencing gel with ^{32}P-end-labeled fragments of pBluescript (KS$^+$) *Hpa*II digest as a size marker (lane **M**). The same sequence reaction as was used in primer extension analysis was also used as a marker. Numbers on the left indicate the sizes of the pBluescript (KS$^+$) *Hpa*II fragments. Arrows indicate the position of transcriptional initiation sites, obtained by the primer extension and S1-nuclease mapping analyses. The main start site in kidney is designated +1.

our results indicate that the genomic organization of these two genes is highly conserved.

Expression of Pax 8 mRNA in Mouse Tissues

Previous *in situ* hybridization studies demonstrated that the mouse Pax 8 mRNA is present in developing brain, kidney, thyroid, and in adult kidney tissues (Plachov *et al.*, 1990). Northern blot analysis of Pax 8 transcripts in rat (Zannini *et al.*, 1992) and mouse (Plachov *et al.*, 1990) adult tissues indicated Pax 8 gene expression in thyroid, kidney, and anterior pituitary. No transcripts could be detected in lung, liver, brain, spleen, thymus, intestine, muscle, ovary, and pancreas from adult species. Our results of the RNase protection assay, however, indicated expression of this gene in kidney (predominantly), liver, spleen, lung, brain, and heart of newborn 1- to 2-day-old mice in contrast to other studies (Poleev *et al.*, 1992; Plachov *et al.*, 1990; Zannini *et al.*, 1992). It seems likely that the choice of probes, the age of the mice (e.g., adult versus newborn), and the sensitivities of techniques used might be the reasons for the differences with results obtained in previous studies.

Definition of Start Sites for the Murine Pax 8 Gene Expression and Potentially Important Sequences within the 5′ Flanking Region

As a first step toward elucidating the mechanisms involved in the control of mouse Pax 8 gene expression, the promoter region with its TATA-like sequence, ATAAAA, and CCAAT-box was isolated and characterized by primer extension analysis, S1-nuclease assay, and ribonuclease protection assay to determine the transcription start sites of the murine Pax 8 gene. All experimental results indicated that the 5′ termini of murine Pax 8 mRNA(s) are heterogeneous. Transcriptional initiation sites by primer extension analysis were found predominantly in kidney of newborn 1- to 2-day-old mice, but also in lung and liver (newborn mice) and in thyroid and pituitary glands of adult mice;

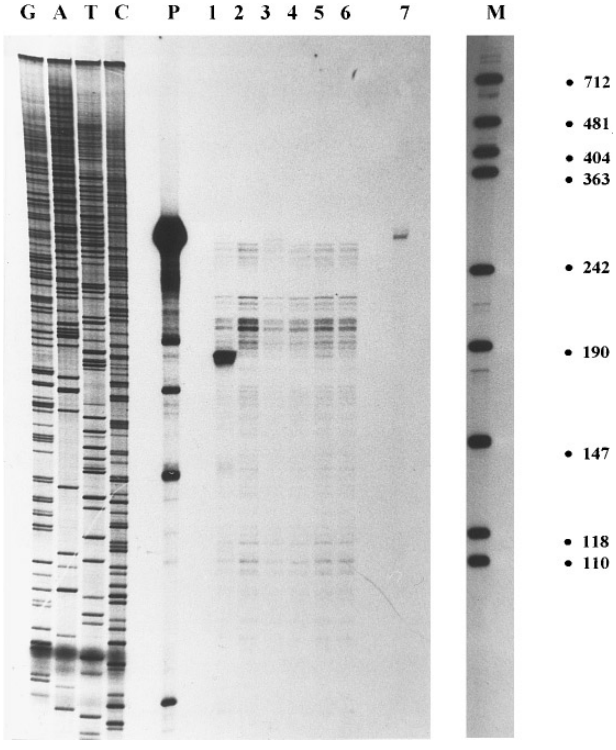

FIG. 6. The murine Pax 8 gene expression. 50 μg total RNA from different tissues of newborn mice (lanes **1–6**) was analyzed by ribonuclease protection assay using an *Eco*RI–*Nco*I fragment spanning the first two exons of the murine Pax 8 cDNA as antisense probe. Lane **P,** input single-stranded riboprobe; lane **1,** kidney; lane **2,** liver; lane **3,** spleen; lane **4,** lung; lane **5,** brain; lane **6,** heart; lane **7,** yeast tRNA; lane **M,** end-labeled pBluescript (KS$^+$) *Hpa*II digest used as a size marker. A sequencing reaction was used as a size marker too. Numbers on the right indicate the sizes of the pBluescript (KS$^+$) *Hpa*II fragments.

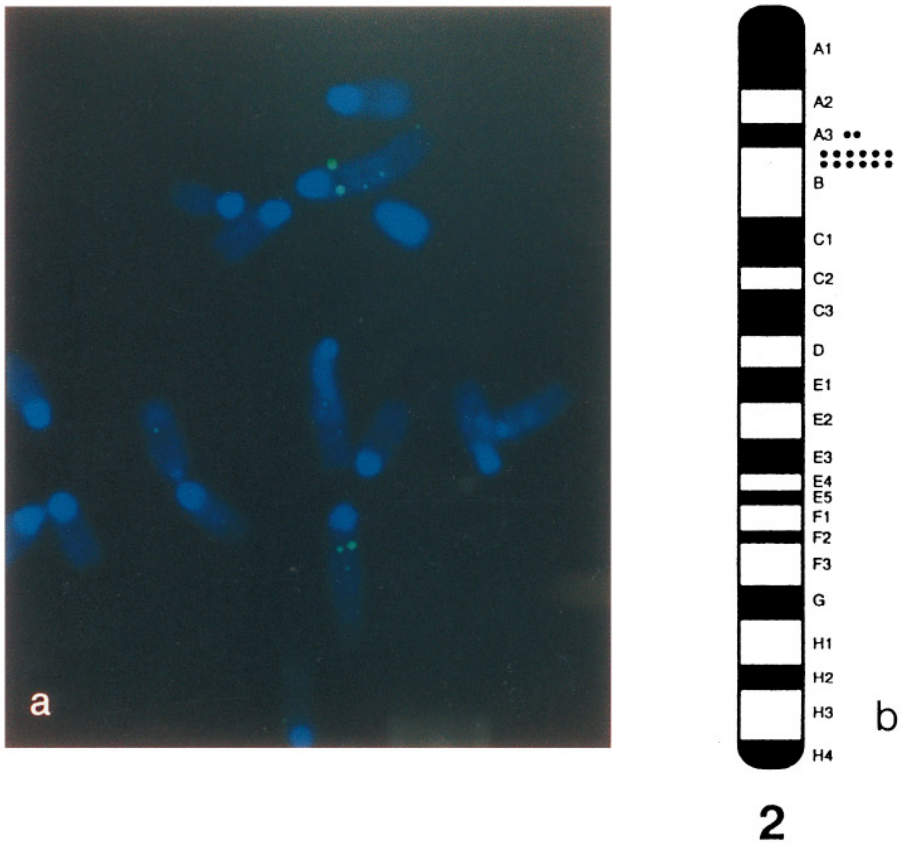

FIG. 7. Mapping the murine Pax 8 gene to chromosome 2. (**A**) Fluorescence *in situ* hybridization was performed using the biotinylated λStu27 as a probe, containing Pax 8 genomic DNA in a phage vector (Dash II). Green hybridization signals are shown. (**B**) Diagram of FISH mapping results. Each dot represents the double FISH signal on mouse chromosome 2.

by S1 mapping predominantly in kidney (newborn mice) and in the entire embryo at Day 10.5 postconception and also in liver, lung, and brain (newborn mice), and by ribonuclease protection assay predominantly in kidney (newborn mice), less intensive signals in mouse embryos and very weak signals in liver, spleen, lung, brain, and heart (newborn mice) were found. Samples of total RNA from newborn mice showed similar patterns. Thus, it can be concluded that the murine Pax 8 gene exhibits a distinct tissue-specific expression pattern. The significance of the low-level expression in several additional organs, at least in newborn mice, is as yet unclear.

Possible binding sites for transcription factors were identified upstream of the first exon in addition to the TATA- and CCAAT-like boxes as well as within the first and second exons and the first intron of the murine Pax 8 gene, which all might be involved in the transcriptional regulation of the murine Pax 8 gene (Fig. 2), i.e., two PEA3 consensus sequences in opposite orientation and several Sp1 and AP2 motifs. Sp1 can interact with other cellular transcription factors including those that bind to AP1 and AP2 sites and can be activated together with TFIID protein expression of genes with or without TATA boxes. Multiple SP1-binding sites have been found in a few promoter genes without TATA boxes, for example, the adenosine deaminase gene, the hypoxanthine phosphoribosyltransferase gene, and the ras family of proto-oncogenes. Some of these genes are ubiquitously expressed, but others are tissue specific. The SP1 factor is ubiquitously expressed, but a detailed analysis of its expression demonstrated a 100-fold difference concerning the amount of protein in various tissues and its association with differentiation processes (Briggs *et al.*, 1987; Mitchell and Tjian, 1989; Rauscher *et al.*, 1988). AP2-binding sites have been reported to mediate cellular response via two different signal-transduction pathways (Imagawa *et al.*, 1987; Medcaff *et al.*, 1990). PEA3 sequences are sites for tissue-specific transcription factors for phorbol esters, epidermal growth factor, and serum response elements (Faisst and Meyer, 1992). Phorbol ester-induced modulation of protein kinase C and upregulation of cAMP have been reported to increase the activity of AP2. The CCAAT box in several genes expressed mainly in the liver has been shown to play a role in the tissue-specific pattern of expression (Lichtsteiner *et al.*, 1987; Gorski *et al.*, 1986). The tissue-specific CCAAT box-binding factors can also be involved in a tissue-specific gene regulation, thus acting inhibitively to prevent the binding of a constitutively expressed activator factor (Akira *et al.*, 1992). The

TFIID-EIIa-binding site found is a member of TFIID consensus sequences. TFIID is one of the necessary cofactors in RNA polymerase II-mediated transcription. For example, in promoters containing TATA boxes, the TFIID factor binds to this element, protecting a region from 35 to 19 bases upstream of the transcription start site. TFIID can also bind to its motifs to participate in upstream regulation of transcription (Nakajiama *et al.*, 1988).

Other noteworthy regulatory elements are the two recognition sites for the zeste protein, which is required for transfection events in *Drosophila* (Chen and Pirotta, 1993), and the NF-κB tissue-specific response element (Faisst and Meyer, 1992).

Chromosomal Localization of the Murine Pax 8 Gene

Using an interspecies backcross, the murine Pax 8 gene has already been mapped to the centromeric region within the proximal portion of chromosome 2 in a close linkage to the *surf* locus (Plachov *et al.*, 1990), and its map position was defined (Frankel *et al.*, 1994). Therefore, the results obtained confirm the localization of Pax 8 to mouse chromosome 2 and localize the gene to band 2B cytogenetically.

ACKNOWLEDGMENTS

We thank Dr. M. Busslinger for critical discussion of the results, and Drs. St. Rand and A. Markoff for reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, the Volkswagenstiftung, the Mildred-Scheel Stiftung, the European Community, and the Alexander von Humboldt Stiftung.

REFERENCES

Adams, B., Dörfler, P., Aguzzi, A., Kozmick, Z., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS and adult testis. *Genes Dev.* **6:** 1589–1607.

Akira, S., Isshiki, H., Nakajima, T., Kinoshita, S., Nishio, Y., Natsuka, S., and Kishimoto, T. (1992). Regulation of expression of the interleukin 6 gene: Structure and function of the transcription factor NF-IL6. *Ciba-Found-Symp.* **167:** 47–62. [discussion 62-7]

Birnstiel, M. L., Busslinger, M., and Strub, K. (1985). Transcription termination and 3′ processing: The end is in site! *Cell* **41:** 349–359.

Briggs, M. R., Kadmaga, J. T., Bell, S. P., and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor, SP1. *Science* **243:** 47–52.

Chalepakis, G., Fritsch, R., Fickinscher, H., Deutsch, U., Goulding, M., and Grus, P. (1991). The molecular basis of the *undulated*/*Pax-1* mutation. *Cell* **66:** 873–884.

Chen, J. D., and Pirotta, V. (1993). Stepwise assembly of hyperaggregated forms of Drosophila zeste mutant protein suppresses white gene expression in vivo. *EMBO J.* **12:** 2061–2073.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.

Faisst, S., and Meyer, S. (1992). Compilation of vertebrate-encoded transcription factors. *Nucleic Acids Res.* **20:** 3–26.

Fantes, J. A., Bickmore, W. A., Fletcher, J. M., Ballesta, F., Hanson, I., and van Heyningen, V. (1992). Submicroscopic deletions at the WAGR locus, revealed by nonradioactive in situ hybridization. *Am. J. Hum. Genet.* **51:** 1286–1294.

Fickenscher, H. R., Chalepakis, G., and Gruss, P. (1993). Murine Pax-2 protein is a sequence-specific trans-activator with expression in the genital system. *DNA Cell Biol.* **12:** 381–391.

Frankel, W. N., Sweet, H. O., and Davisson, M. T. (1994). The strumbler mutation maps to proximal mouse chromosome 2. *Mamm. Genome* **5:** 659–662.

Gehring, W. J., Müller, M., Affolter, M., Percival-Smith, A., Billeter, M., Quian, Y., Otting, G., and Wüthrich, K. (1990). The structure of the homeodomain and its functional implications. *Trends Genet.* **6:** 323–329.

Ghosh, D. (1990). A relational database of transcription factors. *Nucleic Acids Res.* **18:** 1749–1756.

Gorski, K., Caniero, M., and Schibler, U. (1986). Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47:** 767–776.

Green, J. M. (1987). S1 analysis of messenger RNA using single-stranded DNA probes. *In* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds.), pp. 4.6.1–4.6.9, Wiley, New York.

Gruss, P., and Walter, C. (1992). Pax in development. *Cell* **69:** 719–722.

Holland, P. W. H., and Hogan, B. L. M. (1988). Expression of homeo box genes during mouse development: A review. *Genes Dev.* **2:** 773–782.

Imagawa, M., Chiu, R., and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C and cAMP. *Cell* **51:** 251–260.

Kessel, M., and Gruss, P. (1990). Murine developmental control genes. *Science* **249:** 374–379.

Koseki, H., Imai, K., Ichikawa, T., Hayata, I., and Taniguchi, M. (1989). Predominant use of a particular α-chain in suppressor T cell hybridomas specific for keyhole limpet hemocyanin. *Int. Immunol.* **1:** 557–564.

Kozmic, Z., Kurzbauer, R., Dörfler, P., and Busslinger, M. (1993). Alternative splicing of *Pax-8* gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. *Mol. Cell. Biol.* **13:** 6024–6035.

Langer-Safer, P. H., Levine, M., and Ward, D. C. (1982). Immunological method for mapping genes on Drosophila polytene chromosomes. *Proc. Natl. Acad. Sci. USA.* **79:** 4381–4385.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila. Nature* **276:** 565–570.

Lichtsteiner, S., Wuari, J., and Schibler, U. (1987). The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. *Cell* **51:** 963–973.

McKnight, S., and Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. *Cell* **46:** 795–805.

McLauchlan, J., Gaffney, D., Whitton, J. C., and Clements, J. B. (1985). The consensus sequence YGTGTTYY located downstream from the AATAAA signal is required for efficient formation of mRNA 3′ termini. *Nucleic Acids Res.* **13:** 1347–1368.

Medcaff, R. L., Ruegg, M., and Schleuning, W.-D. (1990). A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP. *J. Biol. Chem.* **265:** 14618–14626.

Meeker, T. C., Loeb, J., Ayres, M., and Sellers, W. (1990). The human Pim-1 gene is selectively transcribed in different hemato-lymphoid cell lines in spite of a G+C rich houskeeping promoter. *Mol. Cell. Biol.* **10:** 1680–1688.

Melton, D. A., Krieg, P. A., Rebagliati, M. P., Maniatis, T. M., Zinn, K., and Green, M. R. (1989). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids

containing a bacteriophage SP6 promotor. *Nucleic Acid Res.* **12:** 7035–7056.

Mitchell, P. J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245:** 371–378.

Nakajiama, N., Horikoshi, M., and Roeder, R. G. (1988). Factors involved in specific transcription by mammalian RNA polymerase. II. Purification, genetic specificity and TATA box-promoter interactions of TFIID. *Mol. Cell. Biol.* **8:** 4028–4040.

Nornes, H. O., Dressler, G. R., Knapik, E. W., Deutsch, U., and Gruss, P. (1990). Spatially and temporally restricted expression of *Pax-2* during murine neurogenesis. *Development* **109:** 797–809.

Nüsslein-Volhard, D., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287:** 795–801.

Pilz, A. J., Povey, S., Gruss, P., and Abbott, C. M. (1993). Mapping of the human homologs of the murine paired-box-containing genes. *Mamm. Genome* **4:** 78–82.

Plachov, D., Chowdhury, H., Walter, C., Simon, D., Guenet, J.-L., and Gruss, P. (1990). *Pax 8*, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development* **110:** 643–651.

Poleev, A., Fickenscher, H., Mundlos, S., Winterpacht, A., Zabel, B., Fidler, A., Gruss, P., and Plachov, D. (1992). *PAX8*, a human paired box gene: Isolation and expression in developing thyroid, kidney and Wilms' tumors. *Development* **116:** 611–623.

Poleev, A., Wendler, F., Fickenscher, H., Zannini, M., Katsuyuki, Y., and Plachov, D. (1995). Distinct functional properties of three human paired-box-protein, PAX8, isoforms generated by alternative splicing in thyroid, kidney and Wilms' tumors. *Eur. J. Biochem.* **228:** 899–911.

Rauscher, F. J., III, Sambucetti, L. C., Curran, T., Distel, R. J., and Spiegelman, B. M. (1988). Common DNA binding site for Fos protein complexes and transcription factor API. *Cell* **52:** 471–480.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.

Sinclair, A. J., Lonigro, R., Civitareale, L., Ghibelli, L., and Di Lauro, R. (1990). The tissue-specific expression of the thyroglobulin gene requires interaction between thyroid specific and ubiquitous factors. *Eur. J. Biochem.* **193:** 311–318.

Stapleton, P., Weith, A., Urbánek, P., Kozmic, Z., and Busslinger, M. (1993). Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. *Nature Genet.* **3:** 292–298.

Stuart, E. T., and Gruss, P. (1995). PAX genes: What's new in developmental biology and cancer? *Hum. Mol. Genet.* **4:** 1717–1720.

Stuart, E. T., Kioussi, C., and Gruss, P. (1993). Mammalian Pax genes. *Annu. Rev. Genet.* **27:** 219–236.

Treisman, J., Harris, E., and Desplan, C. (1991). The paired box encodes a second DNA-binding domain in the paired homeodomain containing protein. *Genes Dev.* **5:** 594–604.

Tremblay, P., and Gruss, P. (1994). Pax: Genes for mice and men. *Pharm. Ther.* **61:** 205–226.

Walter, C., Guenet, J.-L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R., and Gruss, P. (1991). Pax: A murine multigene family of paired box-containing genes. *Genomics* **11:** 424–434.

Zannini, M., Francis-Lang, H., Plachov, D, and Di Lauro, R. (1992). A homeodomain- and a paired domain-containing factor show mutually exclusive interaction with the promoters of two thyroid specific genes. *Mol. Cell. Biol.* **12:** 4230–4241.