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Distinct *cis*-Essential Modules Direct the Time–Space Pattern of the *Pax6* Gene Activity

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***Pax6* is a regulatory gene with restricted expression and essential functions in the developing eye and pancreas and distinct domains of the CNS. In this study we report the identification of three conserved transcription start sites (P0, P1, α) in the murine *Pax6* locus. Furthermore, using transgenic mouse technology we localized independent *cis*-regulatory elements controlling the tissue-specific expression of *Pax6*. Specifically, a 107-bp enhancer and a 1.1-kb sequence within the 4.6-kb untranslated region upstream of exon 0 are required to mediate *Pax6* expression in the lens, cornea, lacrimal gland, conjunctiva, or pancreas, respectively. Another 530-bp enhancer fragment located downstream of the *Pax6* translational start site is required for expression in the neural retina, the pigment layer of the retina, and the iris. Finally, a 5-kb fragment located between the promoters P0 and P1 can mediate expression into the dorsal telencephalon, the hindbrain, and the spinal cord. The identified *Pax6*/*cis*-essential elements are highly conserved in pufferfish, mouse, and human DNA and contain binding sites for several transcription factors indicative of the cascade of control events. Corresponding regulatory elements from pufferfish are able to mimic the reporter expression in transgenic mice. Thus, the results indicate a structural and functional conservation of the *Pax6* regulatory elements in the vertebrate genome.**

***Key Words: Pax6;* regulatory sequence; enhancer; transgenic mouse.**

INTRODUCTION

Molecular and genetic analyses of development of various animal phyla provided evidence that conserved regulatory proteins control the morphogenesis in evolutionarily distant species. Members of the *Pax* gene family that encode conserved transcription factors in vertebrates and in invertebrates are a remarkable example for such regulators (reviewed in Walther *et al.*, 1991; Chalepakis *et al.*, 1993; Noll, 1993).

The vertebrate *Pax6* gene is related to the *Drosophila* pair-rule gene, *paired* (Walther and Gruss, 1991), and encodes two DNA-binding domains, a *paired* (Bopp *et al.*, 1986; Treisman *et al.*, 1991) and a paired-like homeo domain (Frigerio *et al.*, 1989). In different species including man, the *Pax6* gene shows a complex spatiotemporal expression, exclusively confined to the developing eye, the central nervous system, and the pancreas (reviewed in Mansouri *et al.*, 1998; Macdonald and Wilson, 1996; Callaerts *et al.*, 1997).

Pax6 plays a key role in the eye morphogenesis. The most striking consequence from homozygocity for mutations of *Pax6* homologues in *Drosophila* (*eyeless,* Quiring *et al.*, 1994), mice (*Small eye* mouse, *Sey,* Hogan *et al.*, 1986; Hill *et al.*, 1991), rat (*rSey;* Fujiwara *et al.*, 1994), and human (*aniridia,* Jordan *et al.*, 1992; Glaser *et al.*, 1992, 1994) is the lack of eyes or a variety of ocular abnormalities in heterozygous conditions with a strong gene dosage effect (Glaser *et al.*, 1994). Developmental eye defects are seen not only in *Pax6* loss-of-function mutations, but also after overexpression of the gene by introducing additional copies of the *Pax6* locus into the mouse (Schedl *et al.*, 1996). The *Drosophila* and the mouse *Pax6* genes can induce formation of ectopic eye structures when induced in the imaginal disc primordia (Halder *et al.*, 1995), demonstrating the conservation of the function of *Pax6* during evolution. In *Xenopus,* the *Pax6* ectopic expression results in the induction of lenses only (Altmann *et al.*, 1997), supporting the view that the product of *Pax6* has a pivotal role for lens determination

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in the head surface ectoderm (Grindley *et al.,* 1995). In differentiating lens *Pax6* is involved in the lens-specific transcription of the αA-crystallin gene, δ1-crystallin gene, and ζ-crystallin gene (reviewed in Cvekl and Piatigorsky, 1996).

Pax6 has an important function for the development of the brain and spinal cord. Recent evidence indicates that the *Pax6* loss-of-function causes distortion of the cortical plate (Schmahl *et al.,* 1993) and migration defects of the cortical neurons (Caric *et al.,* 1997) that are most probably due to a failure of the radial glia cell differentiation (Götz *et al.,* 1998). Pax6 is required for correct forebrain patterning, as indicated by the defects in the establishment of morphological and expression boundaries, axonal pathfinding, and differentiation of diencephalon in the *Small eye* mutant (Stoykova *et al.,* 1996, 1997; Mastick *et al.,* 1997; Grindley *et al.,* 1997; Warren and Price, 1997) and in human probands of *aniridia* (Glaser *et al.,* 1994).

A restricted expression of *Pax6* has been detected in the developing pancreas (Walther and Gruss, 1991). In later embryonic stages and after birth the expression becomes localized to the endocrine α, β, γ, and δ cells of the islands (Turque *et al.,* 1994), producing the pancreatic hormones glucagon, insulin, somatostatin, and pancreatic polypeptide PP, respectively (Slack, 1995). Mice with targeted disruption of *Pax6* lack glucagon-producing α cells, indicating an essential role for *Pax6* for the differentiation of this cell type (St-Onge *et al.,* 1997; Sander *et al.,* 1997). In support of this genetic evidence, biochemical studies demonstrate that the human PAX6 binds to a common element in the glucagon, insulin, and somatostatin promoter (Sander *et al.,* 1997).

Little is known about the molecular mechanisms that control the expression of the *Pax6* gene. Results from studies on the primary structure of *Pax6* in quail and *Caenorhabditis elegans* suggest that the expression of *Pax6* is under the control of different regulators through alternate promoters (Dozier *et al.,* 1993; Plaza *et al.,* 1995a; Zhang and Emmons, 1995). Recent analysis of the human *PAX6* promoter in transient transfection assays identified multiple *cis*-regulatory elements with distinct function in different cell lines (Xu and Saunders, 1997). To understand the molecular mechanisms that govern the roles of *Pax6* in distinct regions, organs, and cells of the developing embryo, it is necessary to define the control elements that direct the complex spatiotemporal expression of the gene. This approach will allow a first insight into the regulatory cascade required for the *Pax6* activity.

In this study we report the identification of individual control elements that regulate the *Pax6* gene activity in the lens, neural retina, pancreas, and the telencephalon, together with the hindbrain and the spinal cord. These elements are individual modules located 5′ and 3′ to the *Pax6*-coding region. This extends the findings of a recent parallel report on the identification of a lens element (Williams *et al.,* 1998). We further identified a distinct element located downstream of the initiation codon between exon 4 and exon 5 that directs the *Pax6* expression in the retina. Our results revealed a high structural and functional conservation between the *Pax6* control elements in pufferfish, mouse, and human. Moreover, we noticed among these highly conserved regulatory sequences several potential DNA-binding sites of transcription factors, which might be involved in the regulation of *Pax6* expression.

MATERIAL AND METHODS

RNA Extraction and Mapping the Transcription Initiation Sites

RNA was isolated by the guanidinium thiocyanate method of Chomczynski and Sacchi (1987).

For the primer extension experiment, a 5′ ^{32}P-labeled 18-base primer nt 113–191 complementary to the 5′ end of the *Pax6* cDNA (Walther *et al.,* 1991) was hybridized to mouse embryo mRNA (day 12.5) and extended by reverse transcription.

For the RT-PCR we used the First-Strand cDNA synthesis kit (Pharmacia). Following primers were used for the PCR: (exon 2, 3′ AGGCATCCTCTCTTTCGTCGTTGT 5′), (exon 1, 5′ GCGCAGGAGGAAGTGTTTTGCT 3′), (exon α, 5′ CGTTGACATTTAAACTCTGGGGC 3′). The obtained PCR products were subcloned into TA vector (Invitrogen, Pharmacia) and sequenced.

Transgene Construction for the Identification of Mouse Pax6 Regulatory Elements

Five overlapping genomic DNA clones containing sequences extending further 5′ of the published *Pax6* sequences (Walther, 1992) were obtained by screening a λ-EMBL3A library from C57Bl/6 mice using a 1.7-kb *Sal*I/*Eco*RI *Pax6* genomic DNA fragment located 5′ to the translation start site as a probe. A composite restriction map of part of the genomic region isolated is shown in Fig. 3.

All constructs used in this study were generated by standard molecular cloning techniques (Sambrook *et al.,* 1989) and are shown in Figs. 3–5. The reporter transgene vector 406 (construct 1, Fig. 3) contains a 3.7-kb genomic *Eco*RI fragment including exon 0 with the most 5′ *Pax6* promoter (P0) and *lacZ* gene with its own ATG and SV 40 polyadenylation signal. For cloning of construct 1 the 3.7-kb *Eco*RI fragment was partially linearized with *Bgl*II which cuts twice in this fragment and was blunt ended. One *Bgl*II site cuts into exon 0 in which the *lacZ* poly(A) cassette was inserted. The transcriptional initiation site is shown as E0 in red.

Construct 2 (406/*Sal*I) is an elongation of a 5-kb genomic fragment located further upstream of the *Eco*RI fragment of construct 1.

Construct 3 (406/*Spe*I) was generated by removing the *Sal*I–*Spe*I fragment from construct 2.

Construct 4 (TK-1) was generated by cloning a 2.4-kb *Not*I–*Asp*718 fragment from the 7-kb *Sal*I subclone mentioned above (see construct 2), into the vector pax-L680 which contains a minimal TK promoter and a *lacZ* gene SV40–poly(A) cassette.

Construct 5 (406/*Hinc*II) was generated by removing *Sal*I/*Hinc*II fragment respectively from construct 2.

Constructs 6–9 were generated by ligating the following blunt-ended genomic DNA fragments into construct 11: *Hinc*II to *Eco*RI; (construct 6), *Eco*RI–*Eco*RI (construct 7), *Acc*I–*Acc*I (construct 8), and *Bgl*II–*Eco*RI (construct 9). Construct 8 contains two copies of

the fragment in 5′–3′ orientation, while construct 9 has three copies in 3′–5′ orientation.

As a negative control, construct 10 containing the *Pax6* minimal promoter P0 was generated by deleting the *Sal*I–*Xba*I fragment from construct 2. This construct only contains the *Pax6* minimal promoter P0 and the *lacZ*–poly(A) cassette.

In construct 11 the promoter P0 was further shortened by deleting the upstream *Xba*I–*Bam*HI fragment and subsequently the *Hin*cII–*Eco*RI fragment from construct 6 was subcloned into it in 5′ to 3′ orientation.

The construct 12/*Fugu* (Fig. 4) carrying pufferfish control elements was generated as follows. A 12-kb genomic *Sal*I fragment was subcloned into pBSKS+. A blunt-ended internal ribosome entry site (IRES)/lacZ/poly(A) fragment was ligated into the blunt-ended *Kpn*I restriction site in the polylinker of the 12-kb genomic subclone. The IRES was added upstream to the *lacZ* gene to facilitate its cap-independent translation.

For construct 13, the lacZ–poly(A) cassette was inserted into the *Bam*HI site of exon 4. A total of 13 kb of upstream DNA sequence was added in multiple steps to generate the final construct 2118/14P (Fig. 4).

For construct 14, the lacZ–poly(A) cassette was inserted into the *Nar*I site of exon 1. Subsequently, the 5-kb upstream genomic sequence was added (Fig. 4).

Construct 15 was generated by inserting the *lacZ*–poly(A) cassette into the *Xba*I site of the exon α present in the 1.2-kb *Eco*RI–*Xba*I clone (Fig. 5).

Construct 16 (TK-2, Fig. 5) was generated by cloning a 1.8-kb genomic *Acc*I fragment containing exon α into the vector pax-L680 which contains a minimal TK promoter and a *lacZ* gene SV40–poly(A) cassette. To test further DNA sequences for the retina-specific element we used the minimal promoter P0 of *Pax6* with the *lacZ* gene (see constructs 7–11). DNA sequences tested in the reporter transgenes (construct 17, 19, and 20, Fig. 5) were isolated from the 1.8-kb *Acc*I genomic fragment, end filled using the Klenow fragment of DNA polymerase I and subcloned into the minimal promoter oriented 5′ to 3′ with respect to the *lacZ*. The upstream DNA sequences contained in constructs 17, 19, and 20 are *Acc*I–*Acc*I (1.4 kb), *Bgl*II–*Xba*I (0.6 kb), and *Dra*III–*Xba*I (0.29 kb), respectively.

To generate construct 18 (406/Fugu, Fig. 5) we used a 600-bp *Eco*RV/*Sal*I fragment with genomic sequences from the pufferfish *Pax6* locus, which was cloned 5′ to the minimal promoter P0.

Transgenic sequences were always purified from vector sequences by appropriate restriction enzymes prior to microinjection.

Cloning the Pufferfish Pax6 Locus

For the isolation of the *Fugu Pax6* homologue a genomic λ-DASHII library of the pufferfish (*Fugu rubripes*) was screened with a 320-bp *Eco*RI fragment of the murine *Pax6* cDNA. Three *Pax6* phage clones were isolated and subcloned for sequence analysis. The fugu and mouse sequences were aligned with the program BESTFIT and FASTA of the GCG package.

Production and Genotyping of Transgenic Mice

All *lacZ*-fusion gene constructs were linearized and microinjected into the pronuclei of fertilized FVB mouse oocytes by using standard procedures (Hogan *et al.*, 1994). Genomic DNAs prepared either from yolk sac or from tail biopsies were digested with the restriction endonuclease *Eco*RI for Southern blot analysis, using a labeled 1.4-kb *Ava*I DNA fragment of the *lacZ* gene as a probe to confirm the integration of the transgene.

Whole Mount β-Galactosidase Staining and Histological Analysis

β-Galactosidase activity was determined as described in (St-Onge *et al.*, 1997). After staining, tissues were embedded in paraffin and 10-μm sections were prepared. The sections of the transgenic embryos were counterstained with haematoxylin–eosin or neutral red.

Immunohistochemistry

Primary antibodies mouse anti-insulin (Sigma) and mouse anti-glucagon (Sigma) were applied on paraffin sections after β-galactosidase staining and detected with a secondary horseradish peroxidase antibody as previously described (Sosa-Pineda *et al.*, 1997).

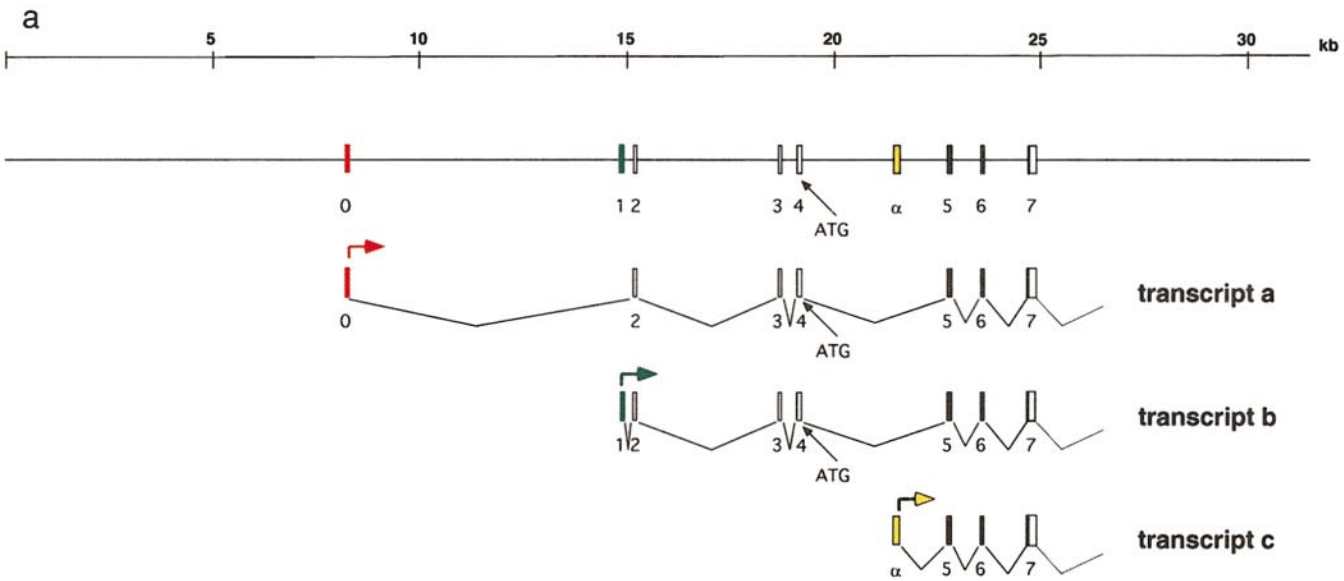
RESULTS

Localization of Three Transcription Start Sites and Sequence Analysis of the Pax6 Promoter Regions in the Mouse

In order to delineate the *cis*-essential elements required for the spatial and temporal activity of the *Pax6* gene we first attempted to localize the transcriptional start sites assuming that at least some control elements are located 5′ of these sites.

The mouse *Pax6* promoter region was identified using a combination of primer extension, RT-PCR, and genomic DNA sequencing. To localize the transcription start sites we used a 18-base primer (nt 113–191) complementary to the 5′ end of the *Pax6* cDNA (Walther *et al.*, 1991) and obtained two primer extension products of 400 and 600 nt in length suggesting that the 5′ end of the published *Pax6* cDNA does not contain the initiation site for mRNA transcription (data not shown). This is compatible with the *Pax6* mRNA size of 3 kb (Walther *et al.*, 1991). Recently, two transcripts with alternative 5′UTR of the *Pax6* gene in quail (Pax-QNR) which are under the control of two promoters (P0,P1) have been described (Dozier *et al.*, 1993; Plaza *et al.*, 1995a). We were interested to know whether

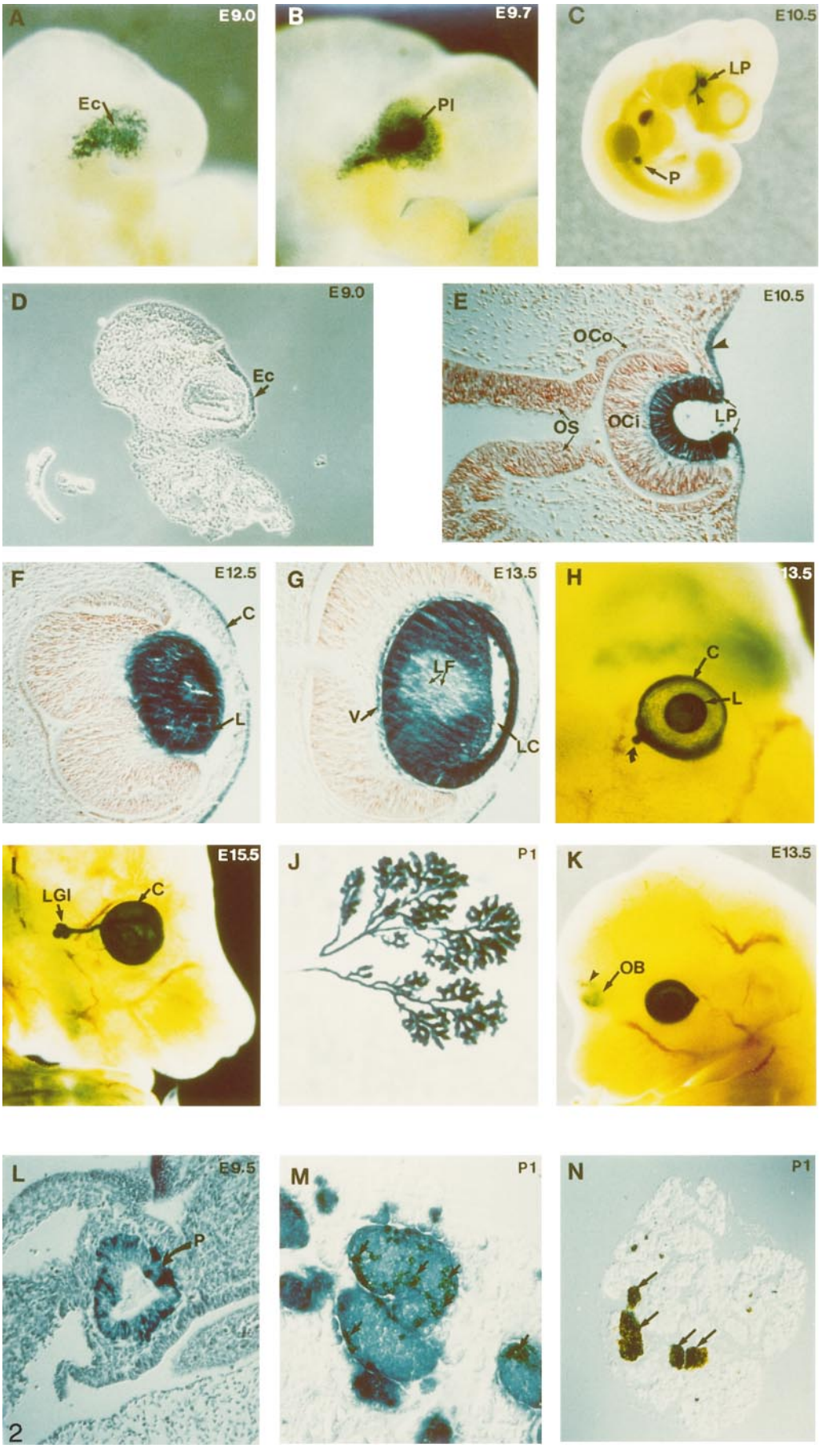
FIG. 1. (a) Structure of the murine *Pax6* transcripts. Arrows indicate the transcriptional start sites of the identified three transcripts, a, b, and c. The translational start site (ATG, thin arrow) is located in exon 4. Exons 5 and 6 and part of exon 7 contain the paired box, marked in black. (b) Sequence comparison between the mouse (M) and the quail (Q) exon sequences. (A, B) Represents mouse RT-PCR products for transcript a and transcript b; (C) mouse genomic DNA sequence corresponding to exon α.



b

```
A M     1 TCTTTTCTTATCGTTGACATTTAAACTCTGGGGCAGGTCCTCGCGTAGAACCCGGTTGTC
  Q     1 ------A--G--A--------------G--T-------T-CG------G-AT--CCGAG-

  M    61 AGATCTGCTACTTCCCCCCCGAGAAGCGGCTTTGAGAAGTGTGGGAACCA
  Q    61 G--G-C--C-GG-G--T--TC-GGC--C-GCGCC-CCG-ACA--t---gg

B M     1 GGAGTGATTAGTGGGTTTGAAAAGCGAACCGTGCTCGGCCTCATTTCCCGCTCTGGTTCA
  Q     1 ---A-------------------------------C-----------------------A-

  M    61 GGCGCA.GGAGGAAGTGTTTTGCTGGAGGATGATGACAGAGGTCAGGCTTCGCTAATGGG
  Q    61 ------G---------------------A----------------------GT---------

  M   121 CCAGTGAGGAGCGGTGGAGGCGACGG..GGCGCCGGCACACACACATTAACACACTTGAG
  Q   121 -------A---------------.--CA--A-.----.-G--TC-----G-G-....-G-

  M   181 CCATCACCAATCAGCATAGgtgtg
  Q   181 G-..-G---------.-G-------

C M     1 CCTGAAAACGCAGCTCCCCTCGAAGGGGAGCCGAGTGCAGTTCATTCTCGTCTGAGTGAT
  Q     1 ---CCG-G---G--CG------G------A G----C-G------A-------------

  M    61 CTACAAATAGGGACGGAAAGGGTCGTTTTATCACGGTCCCGTTTGTCGTGACGATGCAAT
  Q    61 A-A-----GC-----------------------T-C-A-TA-------------------

  M   121 TTCCCGGAGCGGAGCACTGTCACAAAGTGACAAGGCTGCCACAAGCGCCCCGACTGATCT
  Q   121 --T-AAA---A---G-------T---------C-C----------T--T--A--------

  M   181 TTTCAATTAGCCTTCCATGCATGATCCGGAGCGACTTCCGCCTATTTCCAGAAATTAAGC
  Q   181 -----------------------------G------------------------------

  M   241 TCAAACTTGACGTGCAGCTAGTTTTATTTTAAAGACAAATGTCAGAGAGGCTCATCATA-
  Q   241 ---------------------------------------------C--------------T

  M   301 TTCCCCCCTCGTCTATATTTGGAGCTTATTTATTGCTAAGAAGCACAGGCTCCTGGAGTC
  Q   301 --T-------T-----------------------------G---CGG-----TC-C--C-

  M   361 AATTTATCAGGAGGCTCCAAGGAGAAGAGAGGAGAAGAGAGGAGACAAGAGAGGAGCTGA
  Q   361 C-----------------G-C----G----.-CGC--C---CGCGGAGCGGCGCTTCTCC

  M   421 GAACCACATTTCCTTGAGAGGGCTTCTGTC
  Q   421 AGGGATCGATCCTTCCCCCCGCCGCAGCCC
```

1


A
E9.0
Ec
B
E9.7
Pl
C
E10.5
LP
P
D
E9.0
Ec
E
E10.5
OCo
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F
E12.5
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L
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E13.5
LF
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LC
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13.5
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L
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E15.5
LGl
C
J
P1
K
E13.5
OB
L
E9.5
P
2
M
P1
N
P1

alternative splicing at the 5′UTR also takes place in the murine *Pax6* transcripts. By alignment of the murine genomic sequences with the corresponding areas of quail cDNA clones (Pax-QNR-I, Martin *et al.*, 1992; Pax-QNR-2 and QNR-B1, Dozier *et al.*, 1993) and a human cDNA clone (1HPx-2, Glaser *et al.*, 1992) we identified three sequences with high homology at the 5′UTR regions. The estimated nucleotide identity was of about 70% for exon 0 (transcript a), 93% for exon 1 (transcript b), and 86% for exon α (transcript c, Figs. 1a and 1b). The mouse exon 1 is located only 100 bp 5′ of exon 2, the alternative 5′UTR (exon 0) is found 6.2 kb further upstream of exon 1, while exon α is located between exon 4 and 5 (Fig. 1a). To determine whether the identified homologous regions are contained in authentic transcripts of the mouse *Pax6* gene and whether alternative splicing occurs at the 5′-end we performed RT-PCR experiments. The sequences of the RT-PCR products for transcript a and transcript b (Fig. 1b) in mouse matches with the 5′UTR regions of the quail transcripts, indicating a conservation of the transcription start sites in the two species. Similarly, high homology has been detected for the mouse and the quail exon α after genomic DNA sequencing.

Sequence analysis of the upstream promoter region of exon 0 in the mouse and the quail (Plaza *et al.*, 1993) revealed a conserved TATA-like sequence (ATATTAA) and a conserved CCAAT box as well as several putative transcriptional consensus sequences including a binding site for cAMP response elements and a binding site for c-*Myb*. Furthermore, the upstream promoter region of exon 1 in mouse contains consensus sequences for various basal promoter elements, such as a conserved TATA-like sequence (AATATTT), three CCAAT boxes, and consensus binding sites for Sp1 and Ap-2, which are also highly conserved in the *Pax6* gene of the quail (Plaza *et al.*, 1993) and human (Xu and Saunders, 1997). However, no conserved TATA-like sequences and CCAAT boxes were found in the 5′UTR of exon α.

Expression of Pax6 in Ectodermal Derivatives of the Developing Eye, in the Pancreas, and in the Olfactory Bulb Is Directed by a Regulatory Region Located 5′ from Exon 0

For the identification of regulatory elements which control the complex spatiotemporal expression of the mouse *Pax6* gene *in vivo*, we generated transgenic mice using *lacZ* as a reporter gene. The first fusion construct 406 (construct 1, Fig. 3) contains 3-kb sequences located upstream of exon 0. Injected embryos of generation 0 (F_0) were examined for the presence of the transgene by the expression of β-galactosidase (β-gal) from embryonic day (E) 10.5 to E12.5. Since the transgenic embryos showed either no or ectopic β-gal activity (Table 1), we elongated this construct with a further 5-kb upstream fragment (construct 2, 406/*Sal*I; Fig. 3). From the analyzed 25 transgenic embryos at E12.5, five showed restricted reporter β-gal staining in the lens, the cornea, and the pancreas. We concluded that the regulatory element localized on this additional 5-kb fragment 5′ to the first promoter P0 controls the *Pax6* expression in these tissues. Knowing the complex expression of *Pax6* in all tissues of the developing eye (Walther and Gruss, 1991), we were interested to examine whether the reporter gene expression would remain restricted during embryogenesis only to the ectodermal (lens and cornea) eye derivatives or whether it would extend to the retina and pigmental retinal layer—the neuroectodermal eye derivatives, where *Pax6* is also expressed strongly. Therefore, six stable transgenic lines were established with construct 2 and the expression of the transgene in two of them was examined in detail from E8.0 until adult stage (Fig. 2; Table 1).

Endogenous *Pax6* mRNA is initially detected (E8.0–8.5) in a broad region of the head surface ectoderm, including the region from which the lens placode will develop (Walther and Gruss, 1991; Li *et al.*, 1994; Grindley *et al.*, 1995). Later on, the expression is confined to the lens pit, the lens vesicle, differentiating lens, and also to the surface ectoderm forming the cornea. The expression of the reporter gene driven by construct 2 in the developing eye is illustrated in Fig. 2. The first expression of the transgene is detected at E9.0 in the surface ectoderm (Ec) over the presumptive eye region (Figs. 2A and 2D). At E9.5–E9.75 β-gal activity increases within the area of the presumptive lens placode (Fig. 2B and 2C) and at E10.5 the expression becomes confined to the forming lens pit (LP, Figs. 2C and 2E), presumptive corneal ectoderm (Fig. 2F) and a stream of cells that populates the anterior edge of the maxillary domain of the first branchial arch (arrowhead in Fig. 2C). Remarkably, no transgenic expression was found in the inner (Oci) and outer (Oco) layer of the invaginating optic cup (Figs. 2E–2G). Similarly to the endogenous *Pax6* expression (Macdonald and Wilson, 1996), a strong reporter *lacZ* signal is observed at stage E12.5 in proliferating cells of the lens (L, Fig.

FIG. 2. Developmental expression analysis of the *lacZ* reporter gene in a transgenic lines carrying the construct 2 (406/*Sal*). (A–C, H–K) and (D–G, L–N) Views of embryos after whole mount β-gal staining and after sectioning at the indicated stages, respectively. (A–K) Expression in the ectodermal derivatives of the developing eye. The arrowhead in C points to a stream of *lacZ*-positive cells that extend from the head mesenchyme over the anterior edge of the first branchial arch. The curved arrow in H points to the anlage of the duct of the lacrimal gland. Abbreviations used: C, cornea; Ec, surface head ectoderm; L, lens; LC, cavity of the lens vesicle; LF, lens fibres; LG1, lacrimal gland; LP, lens pit; Pl, lens placode; Oco- and Oci, outer and inner layer of the optic cup, respectively; OS, optic stalk; V, vessels. (C, L–M) Expression in the developing pancreas. (M) and (N) shows sections after β-gal whole mount staining and immunohistochemistry for detection of glucagon (M) or insulin (N). The arrows in M and N point to colocalization of the *lacZ* reporter expression with endocrine cells of the islands producing glucagon or insulin, respectively.

TABLE 1
Transgene Expression in Transient and Founder Embryos

Construct*	Embryo age (days)	No. of β-gal-positive embryos/transgenes	Transgene expression patterns		
			Cornea lens	Pancreas	Ectopic
1 (406)	10.5–12.5	0/4	−	−	4
2 (406/*Sal*)	10.5–13.5	5/5	+	+	—
	8.5–adult	6/9*	+	+	1
3 (406/*Spe*)	9.5–13.5	5/5	+	+	—
4 (TK-1)	10.5–12.5	0/21	−	−	—
5 (406/*Hin*cII)	10.5–13.5	6/19	+	−	1
6 (406/H)	10.5–13.5	4/15	+	−	—
7 (406/e)	10.5–13.5	0/23	−	−	6
8 (406/A)	10.5–13.5	11/12	+	−	6
9 (406/b)	10.5–13.5	3/6	+	−	—
10 (406/*Xba*)	10.5–13.5	0/10	−	−	6
11 (406/BN)	10.5–13.5	6/11	+	−	1
			Cornea/lens/ pancreas	Telencephalon	Ectopic
12 (Fugu)	12.5	1/3	+	+	—
13 (2218/14P)	10.5–14.5	5/9	−	CNS	4
14 (ENN1)	10.5–14.5	4/11	−	+,HB,SC	2
			Retina		
15 (PGNA)	10.5–13.5	2/5	+		1
16 ((TK-2)	10.5–13.5	0/5	−		1
17 (406/8)	10.5–13.5	13/14	+		2
	9.5–19.5	2/3*	+		—
18 (406/Fugu)	10.5–13.5	2/3	+		—
19 (406/BX)	10.5–13.5	3/4	+		—
20 (406/DX)	10.5–13.5	0/5	−		—

2F). One day later, when the differentiation of the lens fibers (LF, Fig. 2G) starts, the transgene activity starts to decline in these areas. At stage E13.5 the β-gal staining in the developing cornea (C, Figs. 2F–2I) becomes more prominent. A further domain of transgenic activity was detected within the temporal orbita in a duct that will later form the lacrimal gland (LG1, Fig. 2I) and also has ectodermal origin. The transgenic expression in the lacrimal gland (Fig. 2J) and in the cornea (data not shown) was maintained 1 day after birth (P1). Consistent with the endogenous expression of *Pax6* in conjunctiva (Koroma *et al.*, 1997), the conjunctival epithelium of the adult eye was also β-gal positive (data not shown).

Construct 2 (406/*Sal*I) is also able to direct the *Pax6* reporter gene expression in the pancreas (Figs. 2L–2M). As previously reported endogenous *Pax6* is expressed in the developing pancreas (Walther and Gruss, 1991) and *Pax6* transcripts are detected in all four cell types (α,β, γ,δ) of pancreatic islet cells, but not in the exocrine cell lines (Turque *et al.*, 1994). At stage E9.5 β-gal staining appears in all transgenic lines in a subset of fore- and midgut cells (the pancreatic bud, P, Fig. 2L), similar to the endogenous *Pax6* (Sander *et al.*, 1997) and at E10.5 the expression is clearly seen in the pancreas (P, Fig. 2C). At later stages and 1 day after birth (P1), the *Pax6* reporter transgene is expressed throughout the entire endocrine pancreas (Figs. 2M and 2N). Double histostaining for *lacZ* and immunostaining for insulin (Fig. 2M) or glucagon (Fig. 2N) confirms the colocalization of the *Pax6* promoter/*lacZ* expression with insulin-producing (β) and glucagon-producing (α) cells of the pancreatic islets.

It is noteworthy that apart from the expression in the developing lens and pancreas, two of six transgenic lines that were analyzed in detail showed at stage E12.5 and E13.5 a very restricted *lacZ* expression within the anlage of the olfactory bulb (Fig. 2K), a region where *Pax6* is also specifically expressed (Stoykova and Gruss, 1994).

Distinct Regulatory Elements Are Necessary for the Expression of Pax6 in Eye Ectodermal Tissues and in the Pancreas

To further delineate the *cis*-acting regulatory elements that specifically control the expression of *Pax6* either in eye ectodermal tissues or in the pancreas we performed a

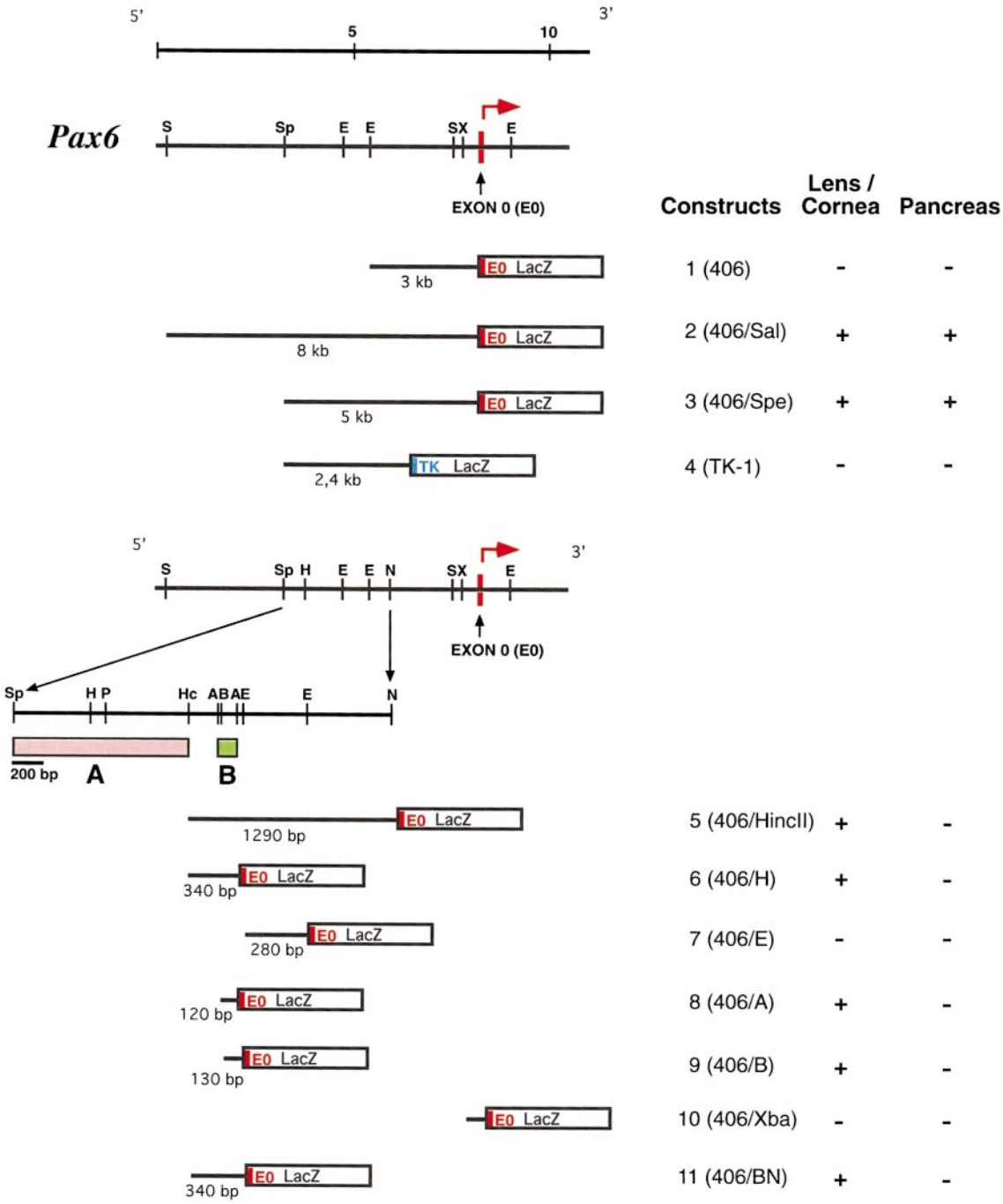


FIG. 3. Identification of the mouse *cis*-control elements for the transgene expression in the lens, the cornea, and the pancreas. The restriction map of the mouse *Pax6* genomic locus surveyed in this study is shown once on the top and further in the middle as a magnification. The arrow indicates the transcriptional start site in exon 0 (E0, marked in red). The reporter constructs carry a transcriptional start point either from the *Pax6* gene (red box, B) or from the TK gene (blue). The identified 1100-nt *cis*-element for the pancreas is indicated by an apricot box (A) while the 120-nt element for the lens and cornea element is shown by a green box. Abbreviations: A, *Acc*I; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; N, *Nsi;* P, *Pst*I; S, *Sal*I; Sp, *Spe;* X, *Xba*I.

detailed functional analysis of the positive 8-kb region contained in the construct 2. Reporter transgenes containing various subfragments from the 8-kb fragment, the *Pax6* promoter P0, *lacZ*, and the SV40 poly(A) sequences were used to generate transgenic embryos (see Fig. 3). Truncation at the 5′ end of construct 2 (406/*Sal*I) resulted in construct

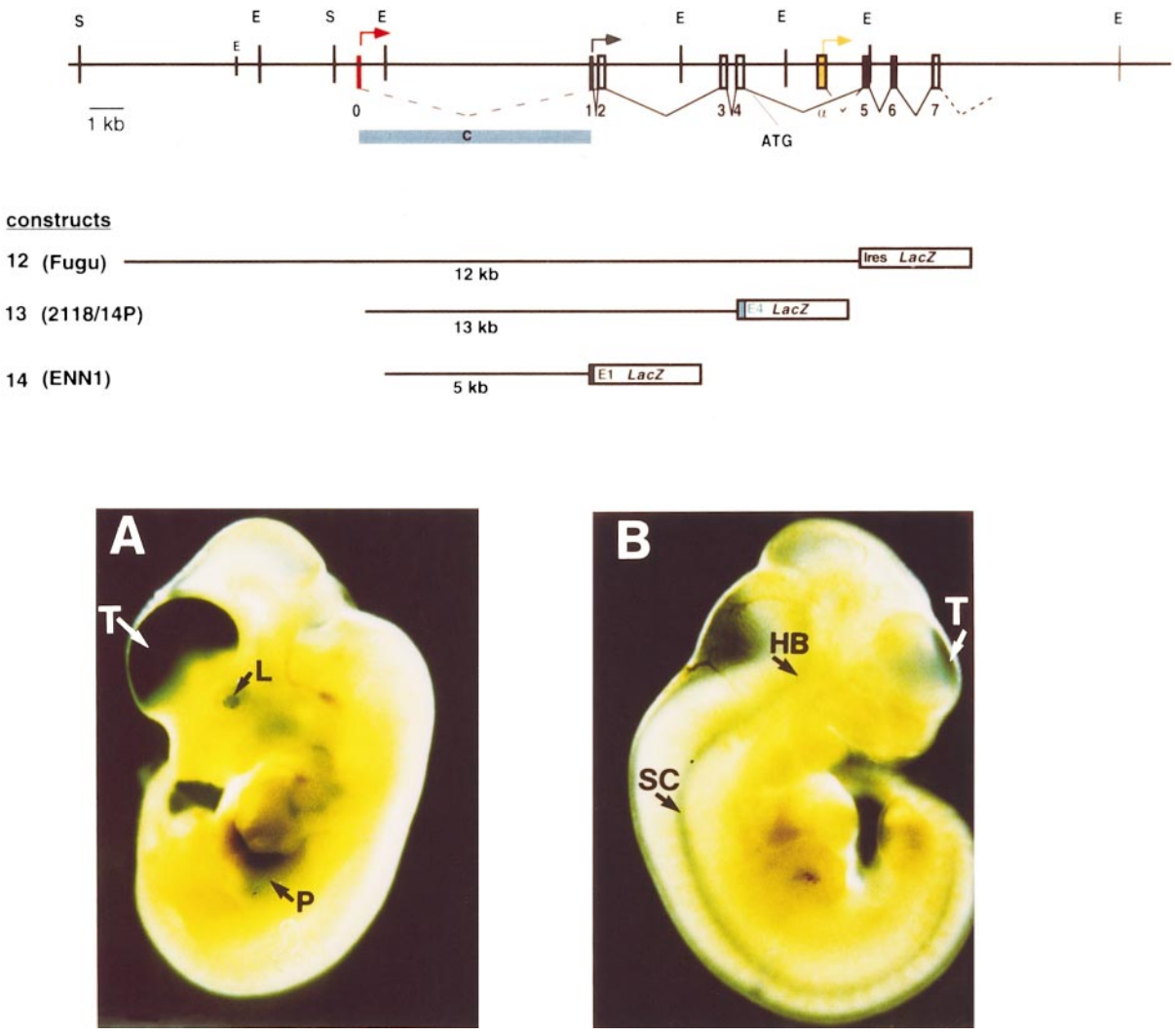


FIG. 4. (A) Transgene *lacZ* expression driven by the construct 12 (containing Fugu control sequences), in mouse telencephalon (T), lens (L) and pancreas (P) at stage E 12.5. (B) Transgene *lacZ* expression driven by construct 14 (containing mouse control sequences) in dorsal telencephalon (T), hindbrain (Hb), and spinal cord (SC) at stage E11.5.

3 (406/*Spe*), which was still able to drive the transgenic *lacZ* expression in the lens, the cornea, and the pancreas. As already mentioned, transgenic mice carrying the 3-kb *Pax6* promoter/*lacZ* fusion construct 1 lack any β-gal activity, indicating that the regulatory regions for the lens, cornea, and pancreas are located within a 2.4-kb fragment (Fig. 3). However, insertion of this 2.4-kb fragment upstream to the *lacZ* driven by the minimal TK promoter failed to support any *lacZ* expression, indicating most probably that these regulatory elements are nonfunctional with the minimal TK promoter. Therefore, various overlapping fragments of the 2.4-kb regulatory region (construct 5–9, Fig. 3, Table 1) were placed upstream of the *Pax6* promoter P0. The negative control, construct 10 (406/*Xba*I), which contains only the minimal *Pax6* promoter P0 provides no specific transgene activity. Interestingly, while the construct 3 (406/*Spe*) was still sufficient to direct the reporter gene expression in both the surface ectoderm derivatives and the pancreas, the 1.29-kb fragment in construct 5 (406/*Hin*cII) directs the *lacZ* expression only in the lens/cornea. These results indicate that the pancreas specific regulatory element (box A in apricot, Fig. 3) is located on a 1100-bp *Spe*/*Hin*cII fragment 4.6-kb upstream of exon 0. Furthermore, the sequence comparison performed among corresponding mouse, human, and fugu DNAs revealed a 124-bp sequence of 74% homology (Fig. 6b) in the *Pax6* regulatory region which might be responsible for controlling the expression of the gene in the pancreas.

To determine the regulatory region that is sufficient to control the expression of reporter gene in the lens and the cornea we created transgenic mice carrying construct 6 and construct 7 (see Fig. 3). In transient assays, the 340-bp

fragment of construct 6 (406/H) directed expression only in the developing lens and the cornea, while construct 7 (406/E) containing a 280-bp fragment gave no β-gal staining. Further trimming of construct 6 to a 120-bp fragment (construct 8, 406/A) resulted in *lacZ* expression in the lens and additional ectopic patchy staining in retina (in 6 of 11 transient assays, Table 1). We then created transgenic mice carrying the construct 9 (406/B) that contains an 130-bp *Bgl*II/*Eco*RI fragment overlapping with construct 8, but in 3′–5′ orientation. The reporter lacZ expression was detected in the lens and the cornea, indicating that this regulatory element can act as an independent enhancer. However, similar to construct 8, this construct also gave in addition to the correct lens and cornea specificity, additional ectopic expression in the retina, suggesting that a negative regulatory element might be missing on these two constructs. By comparing constructs 8 and 9, we therefore assume that an overlapping sequence of 107 bp (*Bgl*II–*Acc*I, Fig. 6a) located 3.6 kb upstream of exon 0 (Fig. 3) is the minimal sequence sufficient to direct the *lacZ* activity in the ectodermal derivatives of the developing eye, lens, and cornea, while a sequence beyond this element appears necessary to strictly limit this expression. Interestingly, a high homology was also found for the 107-bp regulatory element in mouse, human, and fugu (see below and Fig. 6a).

Taken together, these results demonstrate that two different regulatory elements are located within a 4.6-kb region 5′ of exon 0, a 107-bp fragment which is sufficient to drive the reporter *lacZ* expression in the lens, cornea, and lacrimal gland and a 1100-bp fragment which is responsible for *lacZ* expression in the pancreas.

Identification of Fugu Pax6 Regulatory Elements Directing lacZ Expression in the Mouse Telencephalon, Lens, and Pancreas

The tetraodontoid fish, *F. rubripes,* has a compact genome of approximately 400 Mb, which is nine times smaller than the mouse genome (Brenner *et al.,* 1993), thus making the analysis of regulatory sequences less time consuming. As *Pax6* is strongly conserved both structurally and functionally through evolution, the availability of information on the *Pax6 cis*-regulatory element in the fish would facilitate the identification of further regulatory elements within the large mouse *Pax6* locus. The identification of enhancer regions using cross-species comparison has already been successfully applied (Marshall *et al.,* 1994; Aparicio *et al.,* 1995; Kimura *et al.,* 1997).

We isolated a 12-kb fugu genomic phage clone from the *Pax6* locus containing the paired box and the 5′ untranslated region (including exons 1, 2, 3, 4, and α; see Fig. 4). Comparison of the intron/exon structure revealed that the fugu *Pax6* locus is one-third smaller than the corresponding human (Glaser *et al.,* 1992) and mouse sequences. Noteworthy, the presence of exon 0 could not be detected on the fugu genomic clone either by DNA hybridization or by sequence comparison.

We further tested the functional activity of this sequence using the mouse *in vivo* reporter assay. In transgenic mice, the 12-kb fugu genomic sequence (construct 12, Fig. 4), directs the *lacZ* expression in the lens and in the pancreas, thus demonstrating functional conservation of *cis*-regulatory elements between the fish and the mouse. In addition, a very intensive β-gal staining was detected in the dorsolateral domain of the telencephalon (Fig. 4A) that is one of the most prominent characteristics of the endogenous *Pax6* expression. Unfortunately, of the analyzed 79 embryos (F_0) three were transgenic and only one expressed the *lacZ* (Table 1).

To identify corresponding mouse DNA sequences that may regulate the *Pax6* expression in the telencephalon, a 13-kb fragment encompassing the region from exon 0 to exon 4 (thus lacking the lens, cornea, and pancreas elements) was used to make construct 13 (2118/14P, Fig. 4). The transgenic embryos exhibited a β-gal staining similar to the expression of the endogenous *Pax6* gene thus including the regions of dorsal telencephalon, diencephalon, pretectum, hindbrain, spinal cord, and nasal epithelium (data not shown). Additional ectopic expression in the vertebrae and the kidney was also seen. In several other embryos in addition to the activity in telencephalon and spinal cord an ectopic expression was evident in the mesencephalic roof. However, a further truncation of this large fragment to a 5-kb fragment located upstream of exon 1 (construct 14, ENN1, Fig. 4) showed a more restricted expression. Until now, in 4 of 11 transgenic mice carrying construct 14, the reporter *lacZ* expression was detected within the dorsal telencephalic cortex, hindbrain, and in the spinal cord (Fig. 4B). However, some ectopic expression in midbrain was also detected in two of four *LacZ*-positive embryos.

To sum up, a 5-kb fragment located between exon 0 and exon 1 in the mouse Pax6 locus appears to contain regulatory sequences for directing the *Pax6* expression in the telencephalon, hindbrain, and spinal cord. Further extensive analysis is needed to precisely dissect this regulatory region and in order to delineate the functional capacity of the regulatory sequences.

Localization of Conserved Mouse and Fugu Regulatory Elements Directing Transgenic Expression in the Neural Retina

Results from *in vitro* experiments with the quail *Pax6* gene revealed a region 7.5 kb downstream of the quail P0 promoter acting as an enhancer in neural retina cells (Plaza *et al.,* 1995a, 1995b). To identify regulatory elements specific for *Pax6* expression in the mouse neural retina we used several constructs carrying different regions between exon 1 and exon 5 for generating transgenic mice (see Fig. 5). Construct 17 (406/8, Fig. 5) contains a 1.8-kb *Acc*I fragment upstream of the minimal promoter P0. Of the 14 transgenic embryos carrying this construct, 13 exhibited β-gal staining only in the retina. In order to analyze in detail the spatiotemporal expression controlled by this regulatory element we established a stable transgenic line. The initial transgenic expression was detected at E9.0 in the nasal and temporal region of the developing

neural retina (Fig. 5A). At midgestation stage (Fig. 5B), intensive β-gal staining still appears confined mostly to the nasal and temporal domain missing the dorsal aspect known from the endogenous expression of *Pax6* (Walther and Gruss, 1991; Grindley *et al.*, 1995). It should be mentioned, however, that in several transgenic embryos the size of the β-gal-negative domain within the dorsal retina is smaller and in a few cases even a thin layer of *lacZ*-positive cells connected the two strongly positive retinal domains (curved arrow in Fig. 5D). As illustrated in Fig. 5E the transgenic β-gal activity is very strong in the inner (arrowhead) and in the pigmental layer of retina (PL). On sections of E18 d.p.c. embryonic eyes the staining was observed in the ganglionic and amacrine cells (data not shown). As at early developmental stages, a regionalized *lacZ* expression in the retina (at intermediate level) and in the iris (at very high level) was detected after birth (Fig. 5C).

Construct 15 (Fig. 5) harboring a 1.2-kb *Eco*RI/*Xba*I fragment and the *lacZ*–poly(A) gene as an insertion into exon α, directed similar expression pattern in the neural retina of transgenic embryos, indicating the location of the regulatory sequences in a 900-bp region (Fig. 5). Trimming the fragment to 530 bp included in construct 19 (406/BX, Fig. 5) resulted in a similar *lacZ* activity in retina, while a further smaller 290-bp fragment in construct 20 (406/DX) failed to show a transgenic expression (Fig. 5). No β-gal staining could be detected when using a heterologous minimal TK promoter, indicating that this specific minimal promoter is not sufficient to activate the *Pax6* regulatory sequences (construct 16, Fig. 5).

Sequence comparison revealed that this mouse genomic area is highly conserved (87% in 403 bp, Fig. 6c) with the identified neuroretina-specific enhancer element of the quail *Pax6* gene (Plaza *et al.*, 1995b). Additionally, the same genomic area exhibits a high sequence identity (81% in 414 bp) to the *Pax6* gene of the pufferfish. A 600-bp genomic fragment of *Fugu* carrying this conserved region was inserted upstream to the mouse promoter P0 and the *lacZ* gene (construct 18, 406/Fugu, Fig. 5). Interestingly, this construct (carrying the *Fugu*-conserved sequence) was able to reproduce the restricted expression pattern in the nasal and temporal part of the retina seen in the transgenic embryos (compare Figs. 5A and 5B to 5F). These results demonstrate the functional conservation of *cis*-regulatory sequences in the *Pax6* gene during eye evolution.

Conservation of Putative Regulatory Regions in the Pufferfish Pax6 Locus

The nucleotide sequences of the identified regulatory elements reveal several DNA-binding motifs of transcription factors which are highly conserved among mouse, human, and fugu, suggesting that they may act as upstream regulators of the *Pax6* gene. The 340-bp *Hinc*II/*Eco*RI murine fragment (construct 6) responsible for the surface ectoderm expression shows a high sequence homology within 245 bp of human and *Fugu* genomic *Pax6* sequences (Fig. 6a). The 245-bp sequences contain two conserved TAAT-core motifs, critical components of many homeodomain DNA-binding sites. Motif A, CTTAATG, is located in position nt 56–nt 62, while motif B, GCTAATGTCT, is located in position nt 210–nt 220.

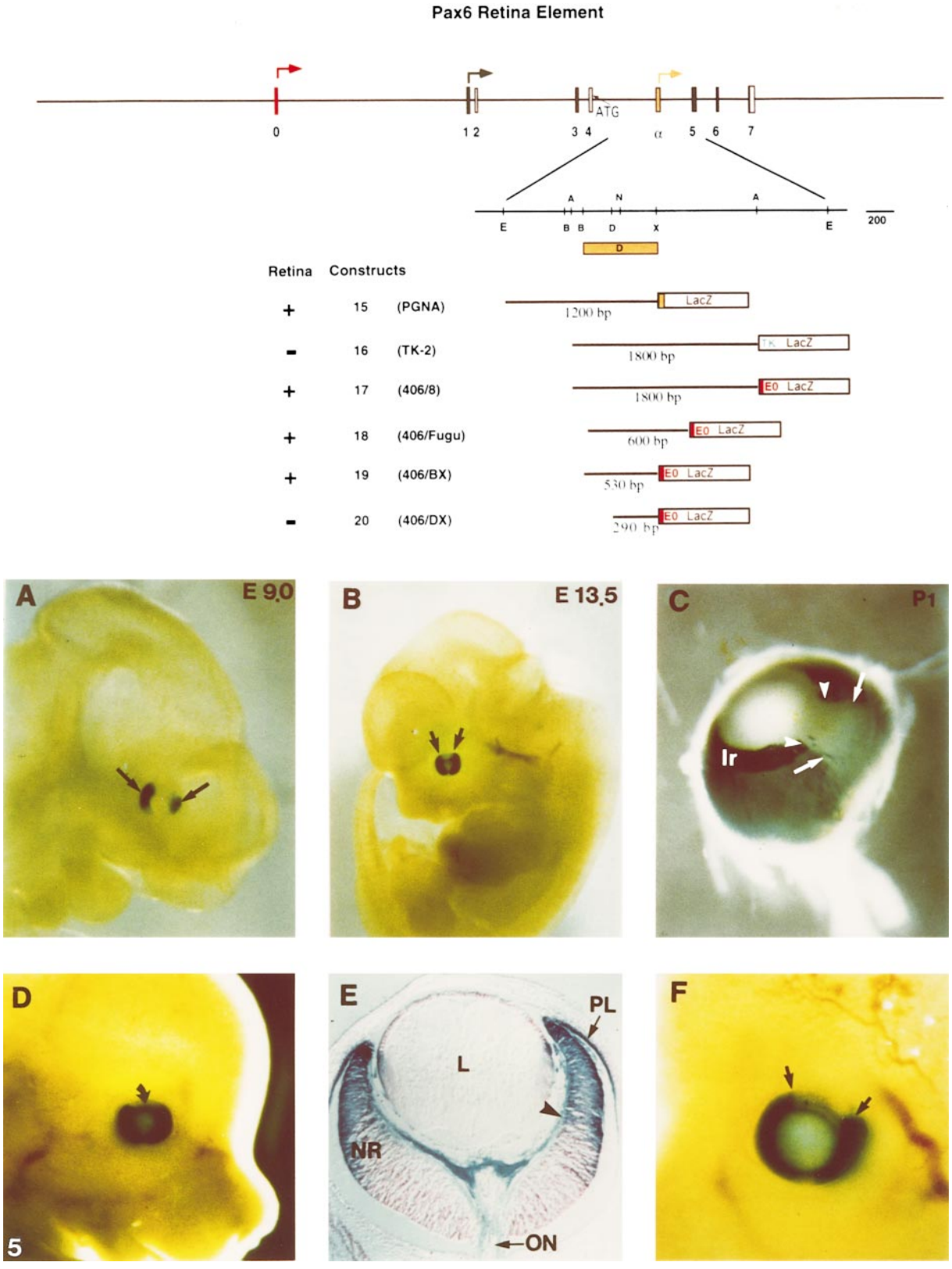
The 1100-bp fragment for the pancreas-specific element revealed a sequence of 120 nt with high sequence identity to human and *Fugu* genomic *Pax6* DNA, containing two motifs for homeodomain DNA-binding sites: motif C, CATTATTGT, in position nt 60–nt 68; and motif D, TTTAATCCAATTATA in position nt 156–nt 170, (Fig. 6b). Furthermore, a *PBX-1* consensus binding site AATCAATCA is located in position nt 97 (Lu *et al.*, 1995) which may regulate *Pax6* expression by direct binding.

Additionally, the sequence of the retina-specific fragment shows a high conservation among mouse, human, *Fugu*, and quail (Fig. 6c). Position nt 185 reveals a homeodomain-binding site for the transcription factor *MSX-1* CAATTAG (Catron *et al.*, 1993). Two further putative homeodomain-binding sites AAATTAAG and GTTTTATT are located at positions nt 233 and nt 262, respectively. The sequence at nt 199 reveals a binding motif for the transcription factor *Pax2* (Czerny *et al.*, 1993; Epstein *et al.*, 1994).

DISCUSSION

The transcription factor *Pax6* plays an essential role in eye and pancreatic morphogenesis and in regionalization and differentiation of telencephalon and spinal cord. Therefore, deciphering the molecular mechanisms for initiation and maintenance of the *Pax6* gene expression in a tissue-specific manner will help to elucidate important aspects of the mechanisms involved in the morphogenesis of these organs. Using a

FIG. 5. (Top) Identification of the *Pax6 cis*-elements responsible for the transgene expression in neural retina. The arrow indicates the transcriptional start site in exon 0, exon 1, and exon α. The reporter constructs carry the transcriptional start point from the *Pax6* gene of exon α (yellow box, construct 15) or of exon 0 (red box, constructs 17, 18, 19, 20) or from the TK gene (blue box, construct 16). The identified 530-nt *cis*-element controlling the transgene expression in the retina is indicated by a yellow box. Abbreviations used: A, *Acc*I; B, *Bgl*II; E, *Eco*RI; D, *Dra*III; X, *Xba*I. (Bottom) Transgene *lacZ* expression in developing neural retina. (A–E and F) are views after whole mount lacZ staining in transgenic mice, carrying construct 17 (406/8) or construct 18 (406/Fugu), respectively. The arrows in (A–C) and (F) point to a region within the dorsal neural retina that appears negative, observed in mice carrying the mouse or *Fugu* regulatory sequence, respectively. In (C) note the strong *Pax6*/*lacZ* expression in the iris (Ir) of the eye at postnatal stage (P1) and the lack of signal in the dorsal domain (arrowheads). The curved arrow in D (stage E13.5) points to a thin layer of *lacZ*-positive cells, connecting the strongly positive nasal and temporal retinal domains, observed in few transgenic embryos. The arrowhead in (E) points to a strong β-gal staining in the inner nuclear layer of the neural retina. Abbreviations used: L, lens; NR, neural retina; PL, pigmental layer of retina; ON, optic nerve.



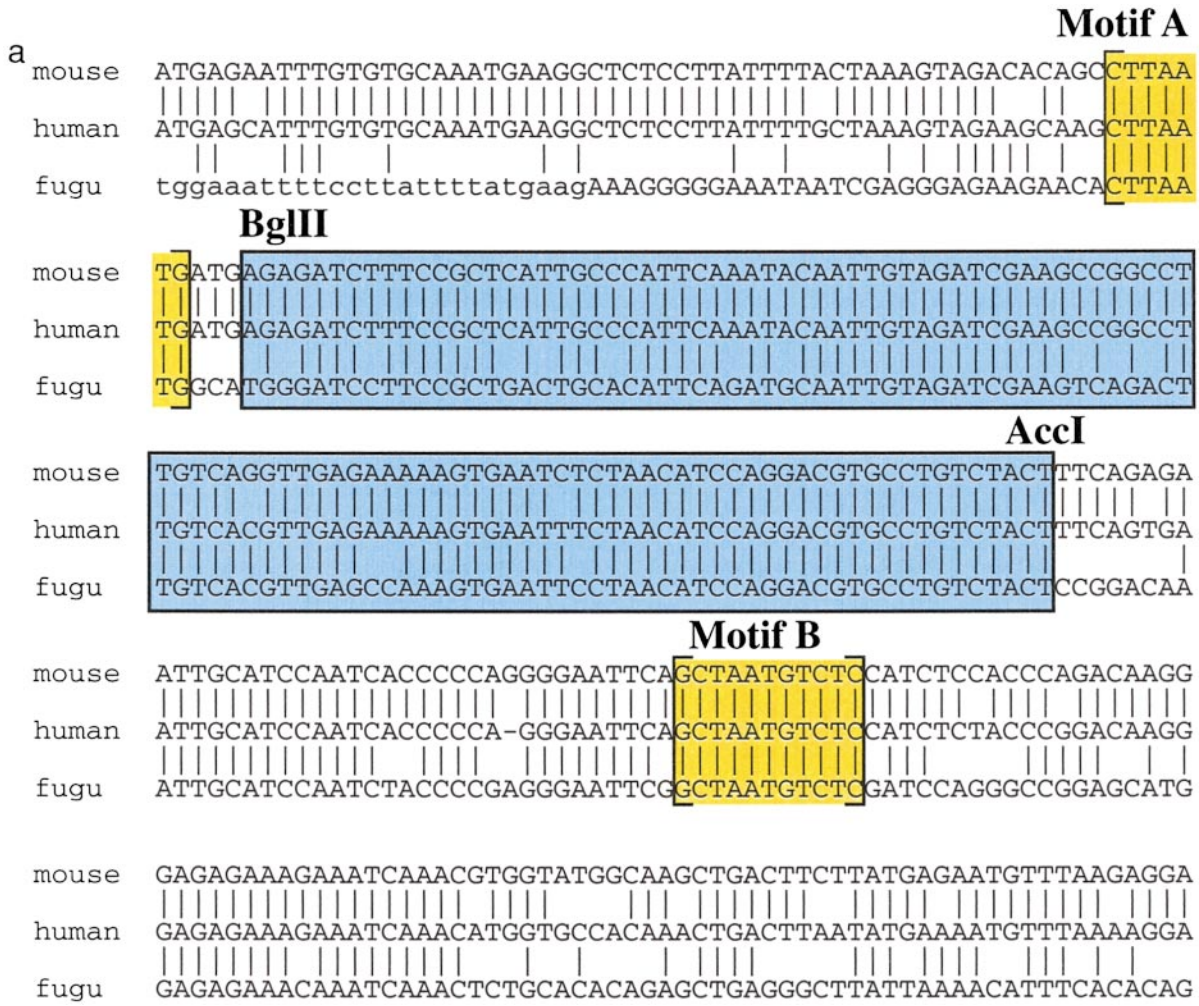


FIG. 6. (a) Sequence comparison of the conserved elements in mouse, human, and fugu *Pax6*/genomic DNA that control the expression in the eye tissues of head surface ectodermal origin. The minimal element which is necessary for driving the *lacZ* expression of the reporter gene into the lens and cornea is a 107-nt *Bgl*II/*Acc*I fragment (boxed). Two potential homeobox-binding sites are shown as DNA motifs A and B. (b) Sequence comparison of the mouse element controlling the expression in the pancreas with the human and the Fugu *Pax6* genomic DNA reveals a fragment of 124 nt with high percentage identity. This sequence contains potential binding sites for *Pbx1*. Two possible motifs (C and D) for homeobox binding sites are boxed. (c) Sequence comparison of the retina element between mouse, human, and Fugu genomic region. This sequence contains potential binding sites for the *Msx1* and *Pax2* and possible motifs for homeobox binding sites (motif E and motif I).

transgenic mouse approach we describe in this study the promoter region of the *Pax6* gene and the localization of distinct tissue-specific elements. We present further functional data for the evolutionary conservation of these *Pax6* control elements in fugu (F. *rubripes*), mouse, and human.

Structure of the 5′-Untranslated Region of the Pax6 Gene

Recent data indicated that two different promoters (P0 and *P1*) of the quail *Pax6* gene (*Pax6*-QNR) generate two transcripts which have distinct expression patterns during embryogenesis (Dozier *et al.*, 1993; Plaza *et al.*, 1995a). As described in this work, we identified three 5′-untranslated regions in the mouse *Pax6* transcripts: Exon 0 (located approximately 6.2 kb upstream of exon 1), exon 1, and exon α (found between exon 4 and exon 5). Alignment of the untranslated mouse genomic sequence (this study) with the respective cDNA sequences of quail (Martin *et al.*, 1992; Dozier *et al.*, 1993) and human cDNA clones (Glaser *et al.*, 1992) revealed a high sequence homology for exon 0, exon 1, and exon α. The RT-PCR products obtained for exon 0 and exon 1 confirmed that the mouse transcript a and transcript b (Fig. 1a) are real transcripts as in quail (Plaza *et al.*, 1995a), thus indicating that also in the mouse alternative splicing occurs within the 5′-untranslated region of the *Pax6* gene.

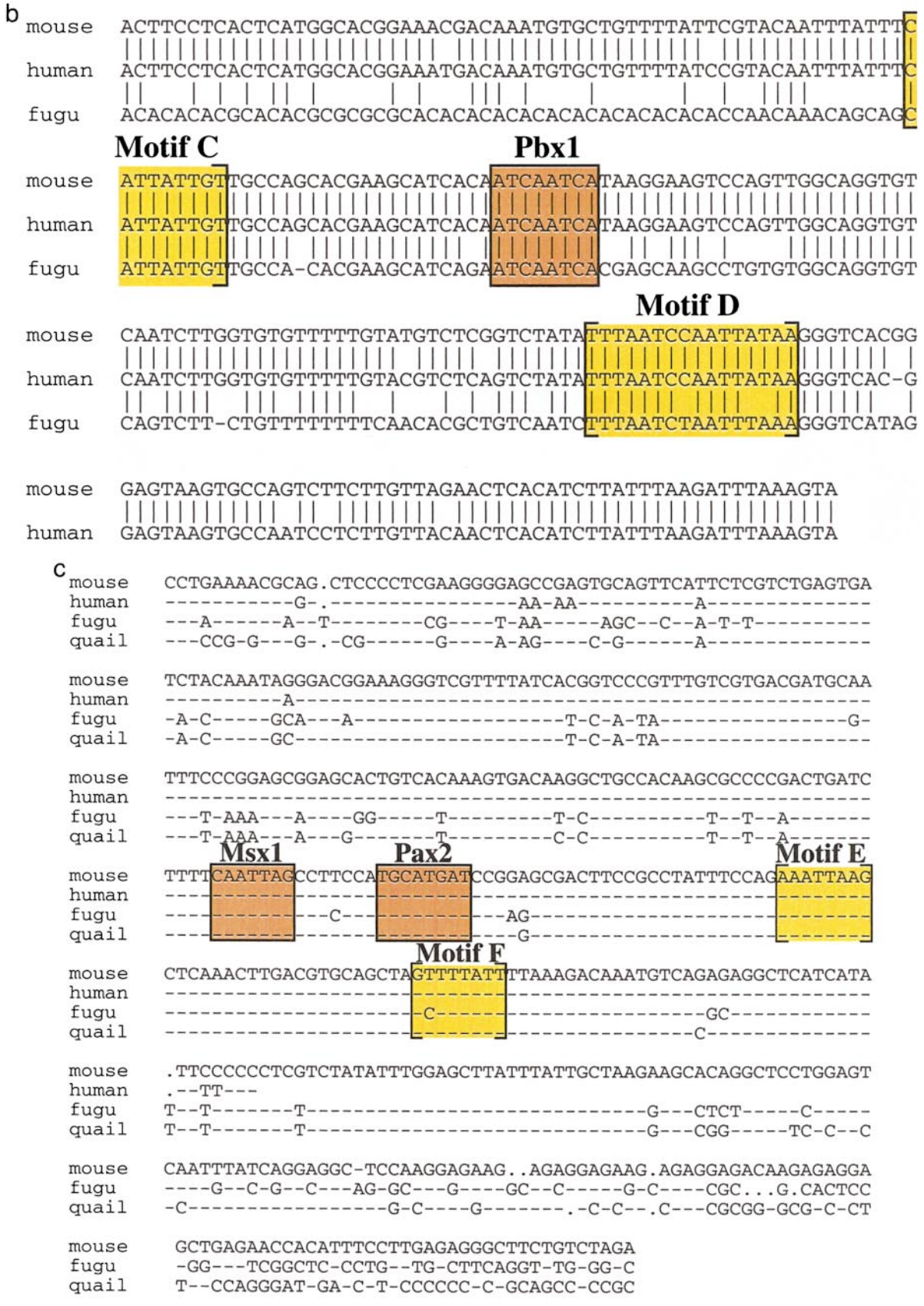


FIG. 6—*Continued*

Eye Control Elements

Pax6 regulatory elements that control expression in tissues of surface ectoderm origin. In the embryonic vertebrate eye *Pax6* is expressed in the surface ectoderm (lens, cornea, conjunctiva) and in the neural ectoderm (neural retina, iris, pigmental layer) derivatives (Walther and Gruss, 1991; Li *et al.*, 1994; Grindley *et al.*, 1995; Koroma *et al.*, 1997). We describe in this study that distinct regulatory elements control the expression of *Pax6* in these two type of tissues, located in a distance from each other, upstream to exon 0 and between exon 4 and exon 5, respectively.

Transgenic mice generated with construct 2, containing an 8-kb fragment upstream from exon 0, faithfully reproduced the expression of *Pax6* in the ectodermal derivatives of the developing eye. The earliest detectable expression occurs around E9.0 in a broad area of the lateral head surface ectoderm thus being only slightly delayed compared with the commencement of the endogenous *Pax6* expression around E8.0–E8.5 (Grindley *et al.*, 1995). In accordance with the reported expression of the endogenous *Pax6*, the expression of the transgene from E9.5 onward is progressively restricted to the lens pit, lens vesicle, lens, and the cornea, but also to the developing lacrimal gland, shown thus far only by a *lacZ* reporter gene expression in the *Pax6*−/− targeted mutated mice (St-Onge *et al.*, 1997). In addition to the transgene expression throughout the cornea, a strong β-gal signal was detectable in the prenatal stage in the entire eye conjunctiva, which has an ectodermal origin and expresses *Pax6*, as recently reported (Koroma *et al.*, 1997).

By deletion analysis of the 5′ and 3′ sequence from this 8-kb fragment we identified a 340-bp element that is sufficient to determine the spatiotemporal activation of the reporter gene in the eye ectodermal tissues. During the preparation of the manuscript a paper was published reporting the identification of a 341-bp fragment of the mouse *Pax6* gene that controls the expression of a reporter construct in eye components to nonectodermal origins (Williams *et al.*, 1998). This sequence is identical to the sequence we describe here. We show in this work, however, that an even shorter fragment of 120 bp orientated in both the 5′–3′ and the 3′–5′ directions (constructs 8) mimics the correct *lacZ* expression in ectodermal derivatives of developing eye. We found that this element is highly conserved within the corresponding sequences in pufferfish and human. Constructs 8 and 9 containing an overlapping 107-bp sequence directed the *lacZ* expression into the lens and cornea. We therefore suggest that the conserved 107-bp sequence in the *Pax6* locus can act as an lens+ cornea-specific enhancer. It should be noted that in some of the transient assays with these two constructs (constructs 8 and 9) ectopic patchy *lacZ* expression was detected in the retina. One reason for this ectopic expression might be that a regulatory repressor sequence exists outside of the 107-bp enhancer element. This is supported by our sequence analysis indicating that exactly this region harbors two potential homeobox-binding sites. Based on the overlapping expression domain with *Pax6* in the eye ectodermal tissues and comparable ocular phenotypes in mutant mice, several homeobox-containing transcription factors are good candidates for a regulatory interplay with the *Pax6* gene. Similar to *Pax6*, the expression of *Sox-1* and *Sox-2* genes that belong to the HMG-box gene family of transcription factors is initially detected in the head surface ectoderm and later in the developing lens placode and the lens (Collignon *et al.*, 1996). Furthermore, SOX proteins are involved in the lens-specific activation of crystallin genes (Kamachi *et al.*, 1995) as also shown for the *Pax6* gene (Cvekl and Piatigorsky, 1996). A further possible regulator, the homeobox gene *Lhx2*, may be responsible for the maintenance of *Pax6* expression in the lens placode (Porter *et al.*, 1997).

Pax6 regulatory elements that control expression in tissues of neuroectodermal origin. Our analysis revealed that the specific expression of *Pax6* in the eye tissue of neuroectodermal origin (neural retina and pigmental layer of retina) is directed by a distinct 530-bp element, located downstream of the translational start site. We failed to obtain a *lacZ* expression in transgenic mice carrying this regulatory element driven by a heterologous minimal TK promoter, indicating that a combination of specific promoter and other *cis*-elements is necessary to provide the specificity of *Pax6* expression. However, it should be mentioned that we have not tried any other heterologous promoter. The *lacZ* expression in the neuroectodermal eye derivatives was detected only by a *lacZ*-fusion constructs either with the exon 0 or with the exon α. Similar results have been reported for the promoter element controlling the *Pax6* expression in the quail neural retina (Plaza *et al.*, 1995b), supporting the view that individual sequence modules are responsible for the *Pax6* gene regulation.

Sequence alignment revealed that the mouse genomic area containing the *Pax6* retina control element is highly conserved in quail (Plaza *et al.*, 1995a) and in pufferfish *Pax6* (this study). Interestingly, when using the mouse neural retina control elements, we observed that the reporter transgene shows expression predominantly confined to the nasal and the temporal part of the retina. Homologous pufferfish sequences (construct 18, Fig. 5F) also directed a similar expression in a transient transgenic mouse assay. These results strongly suggest that additional control elements in the *Pax6* locus might contribute to the full expression pattern of the gene throughout the entire retina as demonstrated in *in situ* hybridization experiments (Walther and Gruss, 1991; Grindley *et al.*, 1995). Several genes, including the transcription factors BF1 and BF2, are topographically expressed along the nasotemporal axis of the developing retina (Hatani *et al.*, 1994; Yuasa *et al.*, 1996). The availability of transgenic lines with the constructs described here will facilitate the study of a possible involvement of *Pax6* in the establishment of correct retinotectal projections.

Our comparative sequence analysis between the mouse retinal control element and homologous regions from the human, quail, and pufferfish genome revealed the presence of highly conserved consensus binding sites for several

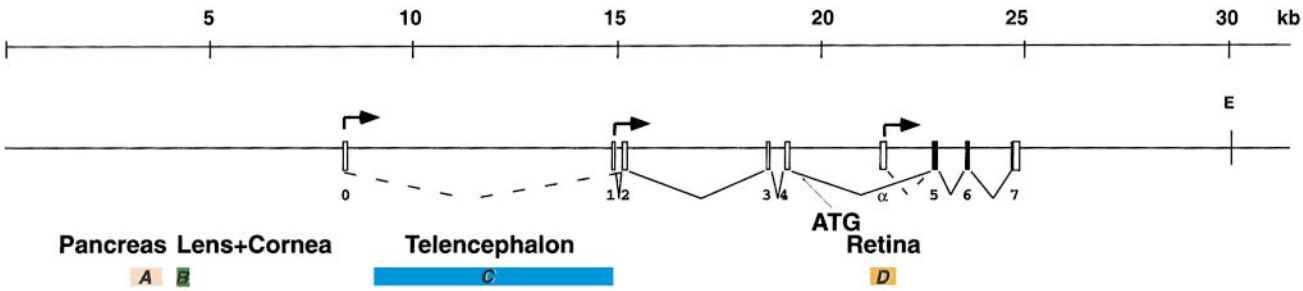


FIG. 7. Scheme illustrating the localization of the identified control elements in the mouse *Pax6* locus that control the expression of the gene in the pancreas (box A, apricot); lens, cornea, lacrimal gland, and conjunctiva (box B, green); telencephalon, spinal cord, and hindbrain (box C, blue); and neural retina (box D, yellow).

transcription factors, e.g., *Pax2* (Czerney *et al.*, 1993; Epstein *et al.*, 1994) and *Msx1* (Catron *et al.*, 1993) as well as a TAAT homeobox-binding site. The presence of a conserved binding site for the transcription factor *Pax2* within the *Pax6* promoter regulatory sequence controlling expression in the developing retina is of special interest since the two genes seem to play important roles in the early regionalization of developing optic cup (Grindley *et al.*, 1995; Macdonald and Wilson, 1996). Analysis of the cyclops phenotype in zebrafish (Macdonald *et al.*, 1995) and homozygous *Shh*−/− mice (Chiang *et al.*, 1996) indicates that the product of the *Shh* gene might be a negative regulator of the expression of *Pax6* by promoting the *Pax2* gene expression. Furthermore, mice lacking *Pax2* by targeted deletion of the gene have abnormally extended neural and pigmental layers of retina (Torres *et al.*, 1996). In electrophoretic mobility shift assay Pax2 binds to the mouse 530-bp retina regulatory region and this binding is abolished by deletion of the putative Pax2-binding site within the *Pax6* regulatory sequence (B. Kammandel, unpublished results). Taken together, these results strongly suggest a regulatory link between the *Pax2* and *Pax6* genes in eye morphogenesis.

Based on the lack of the normal *Msx1* expression in the nasal region of *Small eye* embryos, a similar link between the transcription factors *Msx1* and *Pax6* has been suggested (Grindley *et al.*, 1995).

Control elements for Pax6 expression in the pancreas. In this work we report the identification of a 1.1-kb (*Spe*I/*Hin*eII) *Pax6* element located 4.6 kb upstream of exon 0 that controls the specific expression of the *Pax6* in the developing pancreas. Consistent with the endogenous expression of *Pax6* in this organ (Walther and Gruss, 1991; Turque *et al.*, 1994; St-Onge *et al.*, 1997) the *lacZ* staining in transgenic mice generated with this construct was initially (E9.5) detected in a few cells of the pancreatic bud and later on in all cells of the pancreatic islets. Furthermore, we show here that the 1.1-kb regulatory element contains a 126-bp sequence that is highly conserved in the mouse, human, and *Fugu* genome. Interestingly, this regulatory elements contains consensus binding sequences for the transcription factor *Pbx1* (Lu *et al.*, 1995), involved in the control of gene expression in the pancreas (Peers *et al.*, 1995), and, additionally, two homeobox protein-binding motifs. It will be therefore interesting to study a possible regulation of the *Pax6* expression in the pancreas by these homeobox-containing genes.

Control elements for Pax6 expression in the telencephalon, hindbrain, and the spinal cord. In the developing brain *Pax6* transcripts are initially detected within the proliferative neuroepithelium of the entire CNS (Walther and Gruss, 1991). At midgestation embryonic stage the *Pax6* expression becomes restricted to specific nuclei of the ventral thalamus and hypothalamus, in structures of mesencephalic tegmentum, in the developing olfactory bulb and cerebellum (Stoykova *et al.*, 1996), and in the radial glia precursor cells of telencephalic cortex (Götz *et al.*, 1998). Most of these structures continue to express *Pax6* also in the mature brain (Stoykova and Gruss, 1994) and the same structures fail to form in *Pax6* loss-of-function mutant brain. This very complex expression of *Pax6* during brain development implicates a complex regulation. Until now our efforts have resulted in the identification of a 5-kb mouse genomic fragment, upstream from exon 1 in the *Pax6* locus, that drives the expression of the reporter gene in transgenic mice to a dorsal domain of the telencephalic cortex and hindbrain and in spinal cord. Although with a very low frequency, a larger fragment including further upstream and downstream sequences was able to mimic the entire expression of the endogenous gene in the developing CNS, including the telencephalic cortex, diencephalon, hindbrain, and the spinal cord. Further experiments are in progress to dissect and characterize in detail the functional capacity of this intriguing regulatory domain of the *Pax6* locus.

To conclude, our analysis of the murine *Pax6* regulatory region revealed a modular structure of distinct promoter and/or enhancer elements responsible for the restricted endogenous expression of *Pax6* in the developing (1) lens, cornea, and conjunctiva; (2) neural and pigmental layer of retina and iris; and (3) pancreas. Additionally a 5-kb fragment between promoter 1 and promoter 0 appears to control the expression of *Pax6* in the telencephalon, hind-

brain, and the spinal cord. However, further work is needed to localize the respective individual elements. We also show a striking structural and functional conservation of the *Pax6* locus in pufferfish and mouse—not only for the regulatory sequences mediating the spatiotemporal *Pax6* expression, but also for putative transcription factors that might be acting upstream of the *Pax6* gene during organogenesis. Evidence has been provided that not only a reduced (Glaser *et al.*, 1994), but also an increased, level of Pax6 results in dramatic ocular abnormalities (Schedl *et al.*, 1996), indicating a strong gene dosage effect at least for the eye development. It appears, therefore, that a complex evolutionarily conserved regulation of the *Pax6* ensures the fine tuning of distinct levels of Pax6 protein in different tissues. The availability of transgenic lines, faithfully reproducing various aspects of the complex spatio/temporal expression pattern of the *Pax6* gene, will be a valuable tool in performing tissue-specific knockouts to obtain further insights into the multiple roles of the transcription factor *Pax6* in the eye and pancreas and CNS morphogenesis.

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