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# *Pax4* regulatory elements mediate beta cell specific expression in the pancreas

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**Abstract**

*Pax4* is a member of the *Pax* (Pax, paired box) family of transcription factors with restricted expression and essential functions in the developing pancreas. *Pax4* deficient mice do not develop pancreatic beta and delta cells and thus die after birth due to diabetes. In this study using transgenic mouse technology we report the identification and characterization of a 0.9 kb DNA fragment in the 5′-region of the gene, which by itself is able to direct *Pax4* expression in the endocrine pancreas, recapitulating the beta-cell-specific in vivo expression pattern of *Pax4*. The 0.9 kb DNA fragment contains an area spanning 407 base pairs that is highly conserved between human and mouse showing 88% identity. This promoter region contains sequence motifs that have been shown to be involved in beta-cell-specific expression of insulin, *Pdx1* and islet amyloid polypeptide (IAPP). In addition, we determined a previously undescribed 5′intron. 

*Keywords*: *Pax4*; Pancreas; Beta-cell-specific expression

## 1. Introduction

The mammalian pancreas accomplishes numerous tasks concerning digestion and glucose homeostasis. Functionally it can be divided into two parts. The exocrine part synthesizes digestive enzymes and releases them to the duodenum. The blood sugar level is controlled by the endocrine part of the organ, whose smallest functional unit is the Islet of Langerhans. Within the islets four cell types can be found: alpha, beta, delta and PP cells that produce glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. These hormones are directly released into the blood stream. Malfunctions of the endocrine pancreas can seriously disturb the regulation of the blood sugar level carried out by these hormones and result in life threatening diseases such as diabetes.

The molecular mechanisms of pancreas development are not completely understood. However, several differentiation and transcription factors have been detected that are crucial for pancreatic organogenesis and differentiation of cell types (Slack, 1995; Edlund, 1998; Yamaoka and Itakura, 1999). These include *Activin, Shh, Pdx1, Isl1, ngn3, BETA2/NeuroD, Nkx2.2, Hes1, HNF6*, *Pax6* and *Pax4* (Hebrok et al, 1998; Furukawa et al., 1995; Apelqvist et al., 1997; Kim et al., 1997; Jonsson et al., 1994; Offield et al., 1996; Ahlgren et al., 1997; Gradwohl et al., 2000; Naya et al., 1997; Rudnick et al., 1994; Sussel et al., 1998; Jensen et al., 2000; Jacquemin et al., 2000; St. Onge et al., 1997; Sosa-Pineda et al., 1997). The *Pax* genes have been shown to have decisive influence on the maturation of all endocrine cell types (reviewed in Mansouri et al., 1999; Dohrmann et al., 2000). *Pax6* deficient mice lack alpha cells and the remaining endocrine cells do not form proper islets (St. Onge et al., 1997). *Pax4* turned out to be required for the differentiation of insulin-producing beta and somatostatin-producing delta cells. Even though islets can be found in *Pax4* deficient mice the number of glucagon-producing alpha cells within the islets appears to be increased and to replace the missing beta and delta cells (Sosa-Pineda et al., 1997).

Pancreas development in mice starts at about E 8.5 (E: embryonic days, days post coitum). Production of insulin and glucagon can be detected in the earliest precursor cells (Gittes and Rutter, 1992). Interestingly, even in the *Pax4* knock-out mice insulin-producing cells show up in the first pancreatic precursors (Dohrmann et al., 2000). At E 9.5, all insulin-containing cells coexpress glucagon, but the percentage of double labelled cells decreases after E 14.5, when the first somatostatin-expressing cells arise. Until E 18.5 all

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these cell types are not organized and can be found distributed throughout the exocrine tissue but at the end of gestation with the first appearing PP cells they gather to form mature islets (Yamaoka and Itakura, 1999).

Expression of *Pax4* during pancreas development is tightly regulated. *Pax4* mRNA is detected in few cells in the ventral spinal cord and pancreatic bud as early as E 9.5 in mice, but the *Pax4* expression is later restricted to pancreatic beta and delta cells and vanishes soon after birth (Sosa-Pineda et al., 1997). A maximum level of expression can be seen at E 15.5 (Dohrmann et al., 2000).

For a better understanding of the mechanism of the temporally and spatially regulated expression pattern of *Pax4*, it is necessary to characterize the regulatory elements. Although the mouse *Pax4* cDNA has been cloned recently (Matsushita et al., 1998; Inoue et al., 1998; Kalousová et al., 1999) the genomic structure of the 5′ end of *Pax4* and the *Pax4* promoter has not been characterized in vivo yet. So far, 10 exons have been described. In this study we identify and characterize a 0.9 kb *Pax4* promoter fragment that mediates the described *Pax4* expression pattern using transgenic mice as a model system. Furthermore, we report the detection of a so far undescribed 5′intron.

## 2. Results

### 2.1. Identification of the functional Pax4 promoter element

A 19 kb genomic DNA clone (from liver DNA of BALB/c mice, see Walther et al., 1991 for further details) containing the mouse Pax4 locus was isolated and physically mapped (Fig. 1). Since most regulatory elements of genes are known to reside within the 5′-region of the genomic sequences (O'Shea-Greenfield and Smale, 1992), the most upstream 7.8 kb NotI/NheI DNA fragment was ligated in frame to the *lacZ* gene (Fig. 2a). We tested this construct for reporter gene activity in four transgenic mouse lines generated by microinjection. We analyzed E 10.5 mice and newborn pancreas for beta galactosidase activity by the x-gal reaction. Three of four transgenic lines showed reporter gene activity in the pancreas of E 10.5 mouse embryos (two of them shown in Fig. 3B,C) and in islets of newborn pancreas (Fig. 3E,F). As a control, we compared this expression pattern with that of heterozygous *Pax4* knockout mice generated by homologous recombination (Fig. 3A). In these mice, most of the *Pax4* coding sequences of one allel have been replaced by the *lacZ* gene (Sosa-Pineda et al., 1997). The expression mediated by the 7.8 kb fragment in all detectable cases showed pancreatic expression like the expression in the wild type situation (*Pax4* +/−). Occasionally, ectopic reporter gene activity most likely due to positional effects could be seen in the transgenic lines (Fig. 3B).

However, the expression in the pancreas in all cases was indistinguishable from that of heterozygous *Pax4* knock-out mice and like in the wild type *Pax4* expression dynamics vanished after birth. We therefore conclude that the 7.8 kb fragment harbors the *Pax4* promoter and is able to reproduce the in vivo expression pattern of *Pax4*. This fragment includes the 5′ end of the 19 kb genomic clone, the first exons and also part of the coding sequences of *Pax4* (Fig. 2a).

### 2.2. Delineation of the genomic structure of Pax4

The mouse *Pax4* full length cDNA sequence has already been published (Inoue et al., 1998) and the genomic structure of the gene has been described (Inoue et al., 1998; Kalousová et al., 1999). To delineate the genomic structure of the 5′region of *Pax4* concerning the functional *Pax4* promoter element we sequenced the 7.8 kb fragment (gene bank accession number AF304868) and compared it to the full length cDNA sequence of *Pax4*. This revealed the existence of a previously undescribed intron of 1948 bases. The transcription start is predicted to be 2211 nucleotides upstream of the translation start site (Fig. 1). These findings are consistent with the mouse transcription start site recently described by Smith et al. (2000). As a consequence, the ATG is located at position 95 of the second exon. The first exon spans 169 nucleotides of 5′untranslated DNA sequence so that the complete 5′-UTR (UTR, untranslated region) is predicted to be 264 base pairs (bp) in size.

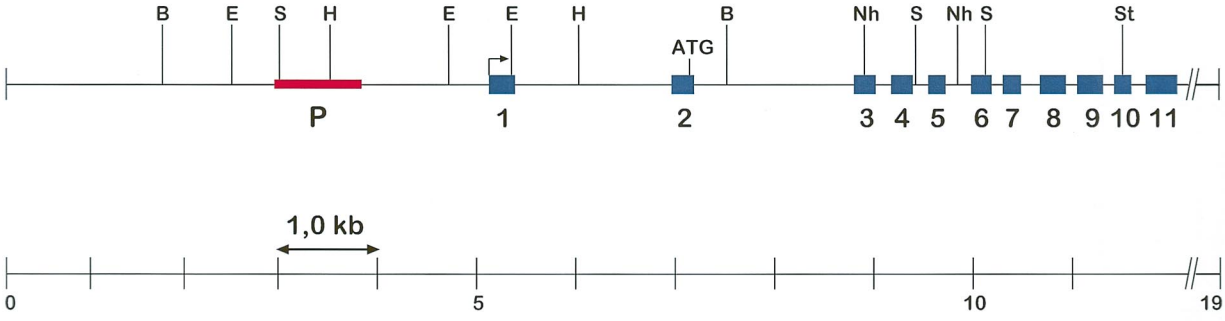


Fig. 1. Physical and genomic map of the *Pax4* locus showing the existence of an additional intron between exons 1 and 2 and the location of the described 0.9 kb promoter fragment (P). Exons 1–11 are in blue, the putative transcription start is marked with an arrow. The 0.9 kb promoter region is given in red. Cutting sites for restriction enzymes: B, BamHI; E, EcoRI; H HindIII; Nh, NheI; S, SmaI; St, StuI.

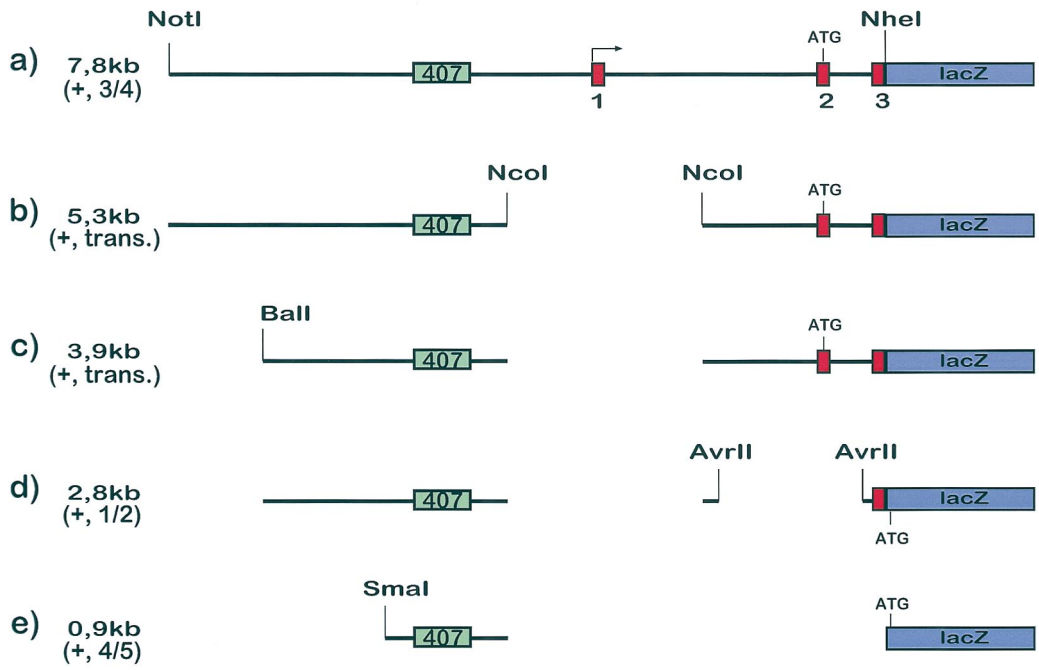


Fig. 2. Constructs that were tested in transgenic mice. All of them were tested in transient transgenic mice and gave positive results (+). For the constructs that have been used to establish mouse lines the ratio of reporter gene expressing lines to the total number of generated transgenic lines is given on the left. In (b) and (c) 'trans' is meaning only transient transgenic mice have been analyzed. Starting from the 19 kb genomic clone (Fig. 1), the constructs were created in a row from (a) to (e). The enzymes that have been used for creating deletions within the previous construct are shown, respectively. The 407 bp cassette with high homology to the human sequence is shown as a green box, the putative transcription start is marked with an arrow, exons are in red, the *lacZ* reporter gene is outlined in blue. (a) A NotI/NheI fragment representing the mostly 5′ 7.8 kb of the 19 kb genomic clone (Fig. 1) was ligated in frame to the *lacZ* reporter gene. It contains the native transcription start and the first three exons. (b–d) Deletions using the enzymes NcoI (b), BalI (c) and AvrII (d) lead to the given constructs. (e) A 0.9 kb SmaI/NcoI fragment was cloned in front of *lacZ*.

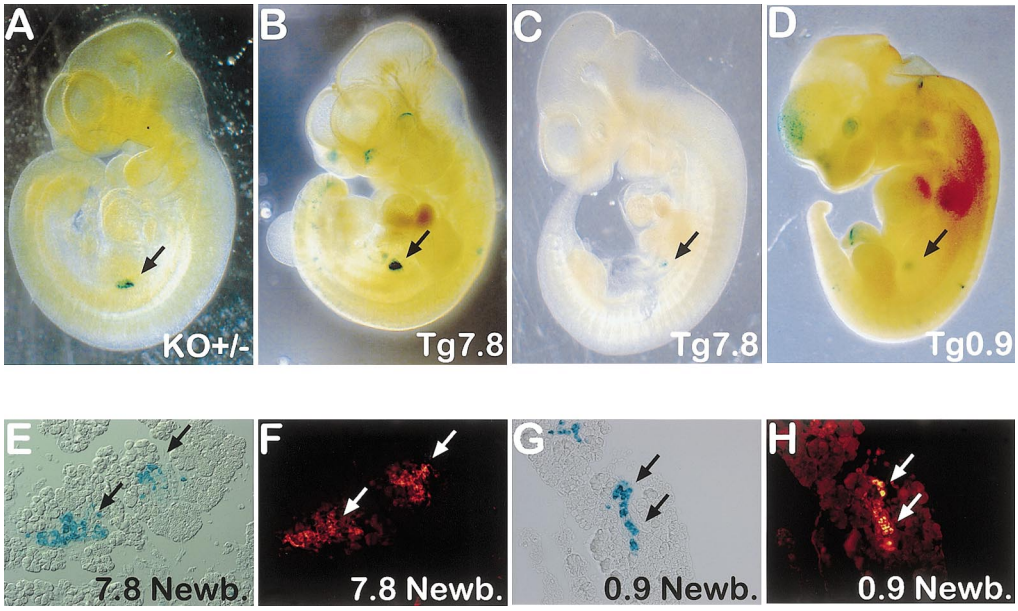


Fig. 3. Reporter gene activity in transgenic mouse lines. (A) Control: heterozygous E 10.5 knockout mouse (KO + /−) showing *lacZ* expression in the pancreas (black arrow). (B,C) Mouse lines transgenic for the 7.8 kb construct (Tg7.8, Fig. 2a). (B) E 10.5 mouse showing reporter gene activity in the pancreas (black arrow) but also ectopic signals in eye, nose and tail. (C) E 10.5 mouse showing no ectopic signals, but weak pancreatic expression (black arrow). (D) Mouse line transgenic for the 0.9 kb construct (Tg0.9, Fig. 2e). An E 10.5 mouse showing background staining in the umbilical cord, ectopic expression and expression in the pancreas (black arrow). Newborn pancreas of mouse lines transgenic for the 7.8 kb element (7.8 Newb., E,F) and the 0.9 kb element (0.9 Newb., G,H) is shown in the bottom row. Reporter gene expression in islets of newborn pancreas (E,G, black arrows) co-localized with beta cells visualized by detecting insulin expression using fluorescent immunohistochemistry (F,H, white arrows).

Absence of the proximal TATA or CAAT boxes or a Sp1 motif indicates a deviation from the classical promoter structure.

### 2.3. Homology to human sequences and further delineation of the Pax4 promoter

We then compared the mouse *Pax4* promoter sequence to the human cosmid clone AC000359 sequence known to contain the human *Pax4* coding region to find evolutionary conserved sequences. Starting at 2003 nucleotides upstream of the murine transcription start we identified a 407 bp DNA cassette showing 88% sequence identity to the homologous human region (Fig. 4a, red label). To further delineate the *Pax4* promoter and assuming an evolutionary conservation of important regulatory sequences, we deleted areas surrounding the conserved region starting from the previously tested 7.8 kb construct. This was accomplished by taking advantage of suitable restriction enzymes (Fig. 2). First, by excising a 2.5 kb NcoI fragment the first exon and surrounding sequences were deleted (Fig. 2b). Since we removed the transcription start we expected the reporter signal to be silenced. Surprisingly, the resulting 5.3 kb fragment of genomic sequence also mediated a *Pax4*-like expression pattern in transient transgenic mice (not shown) revealing the presence of a second putative transcription initiation site. Further deletion constructs were then created starting from this 5.3 kb construct as shown in Fig. 2c,d. As occurred with the 7.8 kb fragment, the transgenic mice generated using these constructs showed a reporter gene activity that recapitulated the in vivo expression pattern of *Pax4* in the pancreas. We verified this for both, 3.9 and 2.8 kb constructs analyzing the expression patterns of the reporter in E 10.5 embryos and newborn pancreas of transient transgenic mice (3.9 and 2.8) and a mouse line (2.8, not shown). Finally, we placed the *lacZ* gene under the control of a 0.9 kb SmaI/NcoI genomic fragment containing the highly conserved region (Fig. 2e). The sequence identity of these 0.9 kb to the homologous human sequences is 81%. No heterologous minimal promoter element was added to this construct. Using this 0.9 kb promoter construct five transgenic mouse lines were generated. In four cases expression in the pancreas of E 10.5 mice (Fig. 3D) and in islets of newborn embryos (Fig. 3E,F) was detected. As in the wild type situation no expression was detectable soon after birth (not shown). These data indicate that the 0.9 kb genomic fragment contains all relevant regulatory elements that are sufficient for driving the *Pax4* expression in the pancreas in vivo.

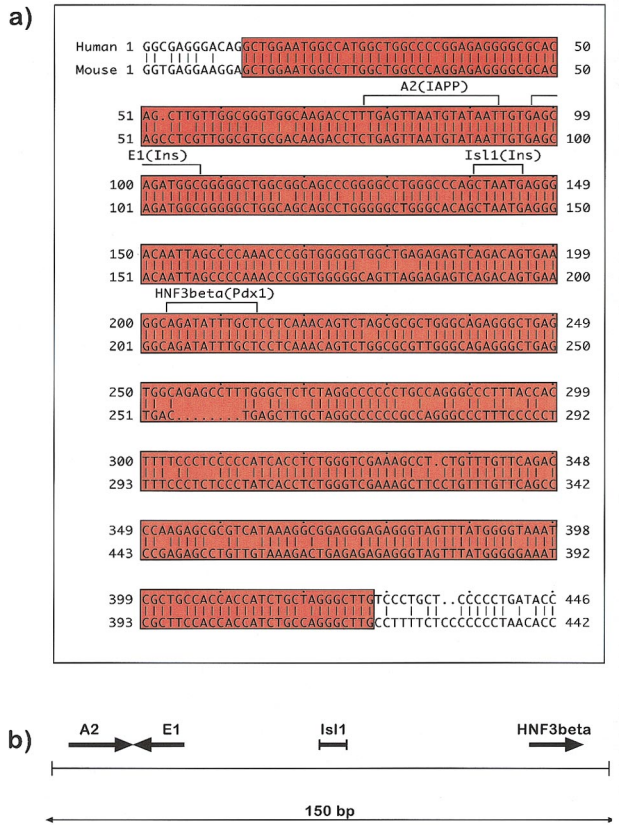


Fig. 4. Homology between human and mouse DNA sequence within the *Pax4* promoter and location of targets for transcription factors. (a) The human sequence is outlined in the upper, the mouse sequence is given in the bottom row. The 407 nucleotides spanning cassette showing 88% identity between the two species is marked in red. The sequence motifs, that have been shown to be recognized by transcription factors expressed in beta and delta cells are marked with frames: *A2(IAPP)* = A2 element known from the *IAPP* promoter, recognized by *Pdx1;* E1(Ins) = inverted E1 element known from the insulin promoter, recognized by a BETA2/NeuroD containing protein complex; *Isl1* (Ins) = core sequence of motifs recognized by *Isl1* on the insulin promoter; *HNF3beta(Pdx1)* = sequence motif similar to a *HNF3beta*-recognized element of the *Pdx1* promoter. Details and references in the text. (b) Overview of the position and orientation of the given motifs.

### 2.4. Sequence motifs present in the Pax4 promoter

Within the highly conserved 407 base pair cassette we found sequence motifs that are identical or similar to promoter elements known to mediate beta cell or delta cell specific expression of genes (Fig. 4, details in Section 3). One of them is a perfectly fitting inverted mouse insulin control element (ICE, 5′-GCCATCTGCT-3′, Fig. 4a, nt. 98–107; which had later been renamed E1 element in accordance with the nomenclature proposed by German et al., 1995). Another motif (5′-CTAATG-3′) has been shown to be recognized by the *Isl1* protein in the insulin promoter (Karlsson et al., 1990) and can be found 43 bp downstream of the inverted E1 element in the *Pax4* promoter (Fig. 4a, nt. 141–146).

## 3. Discussion

The molecular dynamics of the developing pancreas requires a complex interaction between transcription and growth factors during maturation of pancreatic cells and structures. An understanding of the mechanisms of differentiation and proliferation during pancreas development

will be important to devise novel therapies for pancreatic disorders. Since *Pax4* is a main regulator of the development of insulin-producing beta cells, knowledge of its promoter regions would be essential to our understanding of pancreas development and diseases. In this study we have been able to isolate a genomic DNA fragment of 0.9 kb that contains cis-regulatory elements sufficient to mediate *Pax4* expression in mice. Within this fragment we detected a region of 407 bp showing 88% identity to the homologous human region. Inside this highly conserved area previously described target sequences of four transcription factors were discovered. All of them have already been shown to participate in beta or delta cell specific expression of genes.

### 3.1. The E1 element of the Pax4 promoter

The expression of genes responsible for endocrine pancreas development undergoes remarkable changes during gestation. Earliest endocrine precursor cells coexpress numerous genes like *Pax4, Pax6, Pdx1, Nkx2.2,* insulin and glucagon which later in development become selectively restricted to distinct cell types of the Islets of Langerhans (Yamaoka and Itakura, 1999). *Pax4* expression during later development is restricted to beta cells, showing a maximum expression level at E 15.5, and is switched off at birth (Dohrmann et al., 2000). In contrast, other genes like *Pdx1, IAPP* and insulin remain or become selectively active in beta cells. The E1 element of the insulin promoter has been shown to be responsible for beta-cell-specific insulin expression (Sharma et al., 1997). According to our sequence analysis it can also be found within the *Pax4* promoter in an inverted orientation. The protein complex that regulates the E1 motif of the insulin promoter is composed of tissue-specific (*BETA2/NeuroD*), and generally distributed (*E2A* and *HEB* encoded) gene products of the basic helix-loop-helix (*bHLH*) family (Sharma et al., 1997). The mechanisms of E1 activation mediated by the *BETA2/NeuroD* containing complex have been shown to act selectively in beta cells. However, a similar type of promoter regulation can be found as tissue specific activators of the *bHLH* class, the best characterized of which are the myogenic *bHLH* activators (i.e. *MyoD*, *myogenin*, *myf-5*, and *MRF-4*; Bengal et al., 1992; Li et al., 1992). Therefore, the E1 element of the *Pax4* promoter has the potential to act as a multifunctional regulator element on *Pax4* expression.

### 3.2. The A2 element

A 17 bp motif contacting the E1 element at the 5′ end contains 94% homology to the A2 element known from the human islet amyloid polypeptide (IAPP) gene promoter (Carty et al., 1997; Fig. 4a, nt 78–93). IAPP is exclusively expressed in beta and delta cells (Carty et al., 1997) and has been proposed to play an important role in regulating plasma glucose levels in mammals (Carty et al., 1997). The A2 element of the IAPP promoter, known to be an activator of IAPP expression, has been shown to be a target of *Pdx1* protein (Carty et al., 1997), Pdx1 is ubiquitously expressed in the early developing pancreas and later becomes restricted to beta cells. *Pdx1* was shown to be a main regulator of early pancreas development and is involved in the glucose dependent insulin response of the mature beta cells (Yamaoka and Itakura, 1999). Since the A2 element regulates gene expression in differentiated beta and delta cells these findings suggest that *Pdx1* could be also a candidate to influence *Pax4* expression in mature beta cells.

### 3.3. HNF3beta and Isl1

An inverted consensus sequence that regulates *Pdx1* expression by binding *HNF3beta* (Wu et al., 1997) shows up further downstream (Fig. 4a, nt. 204–214). *HNF3beta* is expressed in pancreatic beta cells and duct cells. The functional consensus sequence recognized by *HNF3beta* shows 91% identity to the sequence motif present in the *Pax4* locus. Hence *HNF3beta* represents another promising candidate for *Pax4* regulation.

Mice deficient of *Isl1* show severe defects in the development of the pancreas and the surrounding mesenchyme (Ahlgren et al., 1997). Therefore the *Isl1* recognition site detected in the *Pax4* promoter region might be a hint for a more general regulation of *Pax4* carried out by *Isl1*.

### 3.4. Additional factors present in the developing endocrine pancreas

The presence of binding motifs for *Pdx1, BETA2/NeuroD, Isl1* and *HNF3beta* protein suggests that these genes might be involved in the regulation of the *Pax4* gene.

However, all of the described regulations carried out by these transcription factors are positive (Sharma et al., 1997; Carty et al., 1997; Wu et al., 1997; Ahlgren et al., 1997). To explain how the silencing of *Pax4* expression at birth is accomplished, additional genes that are expressed during endocrine pancreas development and might act as repressors need to be examined for their influence on *Pax4* expression.

The functional promoter element contains several TAAT motifs (Fig. 4a), that might be targets for different homeobox genes and there might be competition of several protein complexes for binding to the E1 motif. *Neurogenin* (*ngn3*) regulates endocrine pancreas development (Gradwohl et al., 2000) and is an early developmental regulator of E-box motifs (Huang et al., 2000). It might be involved in the control of *Pax4* expression. Other genes which might play such a role are *HNF6, Hes1, Notch* genes and *Nkx6.1*.They have recently been shown to participate in endocrine pancreas development or being expressed in the developing tissue (Jacquemin et al., 2000; Jensen et al., 2000; Lammert et al., 2000; Jensen et al., 1996). Their influence on *Pax4* expression will be the subject of our future experiments. In this regard it is worthwhile to mention that *Pax4* itself has been described as a transcriptional repressor (Smith et al., 1999). After submission of this manuscript an article has

appeared in which an autoregulatory loop during *Pax4* expression is suggested (Smith et al., 2000).

### 3.5. Alternative transcription initiation site

The putative *Pax4* transcription start site predicted by sequence comparison lacks the classical promoter elements. However, the analyzed 0.9 kb promoter construct mediates reporter gene activity without a classical minimal promoter or the sequences surrounding the native transcription start. Our results suggest the presence of an alternative transcription start site within the remaining sequences of all smaller constructs.

Taken together, our results concerning the *Pax4* promoter provide a first insight into possible mechanisms of regulation of this gene. Because of their expression pattern and the presence of target sequences on the *Pax4* promoter, *Pdx1, BETA2/NeuroD, HNF3beta* and *Isl1* appear as promising candidates for the regulation of *Pax4*. Further experiments are needed to analyze each module of the promoter for its role in regulating *Pax4* expression in vivo. It is also necessary to prove if the same cis-regulatory elements, that mediate an activation of expression in a particular promoter (E1, A2) might act as repressors on a different promoter within the same cell.

## 4. Experimental procedures

### 4.1. DNA constructs

For the genomic clone a 19 kb genomic mouse DNA fragment containing the *Pax4* locus was cloned into the NotI site of the p-bluescript cloning vector provided by Stratagene.

For the 7.8 kb construct a 11.2 kb NheI/SpeI fragment was replaced by a 3.7 kb HindIII/BamHI-fragment out of the Pharmacia pCH110 vector which contains the *lacZ* gene. A blunt end ligation placed the *lacZ* gene to the third exon in frame of the native *Pax4* ATG in the second exon. The orientation was checked by sequencing. Further constructs have been created out of this one by cutting out NcoI (2.5 kb), SacII/BalI (1.2 kb) and AvrII (1.0 kb) fragments and relegation.

For the 0.9 kb construct a SmaI/NcoI fragment was cloned blunt into the SmaI site of the pbeta-Gal-basic vector provided by Clontech. The orientation was checked by HindIII digestion.

### 4.2. 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 BamHI for southern blot analysis, using a labelled *lacZ* probe amplified by PCR out of pCH110 or pbeta-Gal-basic to confirm the integration of the transgene.

### 4.3. lacZ staining

Beta galactosidase activity of E 10.5 embryos and newborn pancreas was determined as described in St. Onge et al. (1997). For sections the tissue was embedded in paraffin after staining.

### 4.4. Immunohistochemistry

Primary antibodies mouse anti-insulin (Sigma) were applied on paraffin sections after beta-galactosidase staining as previously described (Sosa-Pineda et al., 1997) and detected with a red-fluorescent secondary antibody (alexa 594).

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