Transcriptional Regulation of the Human PAX6 Gene Promoter*

Zheng-Ping Xu and Grady F. Saunders‡

From the Department of Biochemistry and Molecular Biology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

PAX6, a member of the highly conserved paired-type homeobox gene family, is expressed in a spatially and temporally restricted pattern during early embryogenesis, and its mutation is responsible for human aniridia. Here we examined the transcriptional regulation of the PAX6 gene by transient transfection assays and identified multiple cis-regulatory elements that function differently in different cell lines. The transcriptional initiation site was identified by RNase protection and primer extension assay. Examination of the genomic DNA sequence indicated that the PAX6 promoter has a TATA like-box (ATATTTT) at –26 base pairs (bp), and two CCAAT boxes are positioned at –70 and –100 bp. A 38-bp poly(CA) sequence was located 992 bp upstream from the initiation site. Transient transfection assays in glioblastoma cells and leukemia cells indicate that a 92-bp region was required for basal level PAX6 promoter activity. A negative transcriptional element, silencer (bases –1518 to –1268), functioned differently in different cell lines. The activation of the promoter is positively correlated with the expression of PAX6 transcripts in all cells tested. These results indicate that a cis-regulatory element or elements is responsible for selective activation of the PAX6 promoter in cells that can express PAX6 mRNA.

The paired box (pax) gene family was originally identified in Drosophila as segmentation genes such as paired (prd), gooseberry-distal (gbd-d), and gooseberry-proximal (gbd-p) (Bopp et al., 1986) and was subsequently found in many other species (Burri et al., 1989; Ton et al., 1991; Chisholm et al., 1995). Based on their sequence homology to the Drosophila gbd-d paired box, nine pax genes have been found in mice (pax1–9) (Walther and Gruss, 1991) and humans (PAX1–9) (Gruss and Walther, 1992). All the pax genes contain a 128-amino acid N-terminal paired box that can be present alone (as in PAX1 and PAX9) or with a full-length (as in PAX3, PAX4, PAX6, and PAX7) or truncated (as in PAX2, PAX5, and PAX8) paired-type homeodomain. The PAX proteins make up a family of transcription factors involved in the regulation of cell morphogenesis and differentiation. The paired domain alone or in combination with the paired-type homeodomain, confers a novel DNA binding specificity (Czerny et al., 1993; Wilson et al., 1995).

PAX6 plays a key role in eye morphogenesis and has been implicated in the secondary inductive interaction of lens formation (Quiring et al., 1994; Li et al., 1994; Grindley et al., 1995). Ectopic expression studies indicate that pax6 is probably a master control gene for eye development (Halder et al., 1995). The expression pattern for PAX6 (PAX6) has been extensively characterized by northern and in situ hybridization analysis (Ton et al., 1991, 1992). PAX6 expression is restricted to defined regions of the forebrain, optic cup, hindbrain, and spinal cord, as well as the lens placode and nasal epithelium (Walther and Gruss, 1991; Puschel et al., 1992; Li et al., 1994). Heterozygous mutations in PAX6 result in eye abnormalities in human (aniridia) (Glaser et al., 1992; Jordan et al., 1992; Martha et al., 1994, 1995), mouse (small eye) (Hill et al., 1991; Hogan et al., 1988), and Drosophila melanogaster (eyeless) (Quiring et al., 1994). We know relatively little about the transcriptional regulation of PAX genes compared with those of the homeobox family (Sham et al., 1993) and Drosophila paired box genes (Gutjahr et al., 1993). Examining transcriptional regulation of PAX6 may reveal how it functions in different tissues during early embryogenesis.

In this paper, we describe analyses of the human PAX6 promoter and upstream regulatory region and report the presence of multiple repressors and activators within the regulatory region. One of the repressive elements was a strong silencer (SX250) that could specifically repress PAX6 promoter activity in glioblastoma and HeLa cells.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa (a human cervical carcinoma cell line), U87 (a human glioblastoma cell line), and 293 (a human embryonic kidney cell line) were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, 50 μg/ml glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. NIH 3T3 fibroblasts, K562 (a human chronic myelogenous leukemia cell line), and a normal human lymphoblastoid cell line were grown in RPMI 1640 medium containing 10% fetal calf serum, 50 μg/ml glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. HBL100 (a human breast cell line) was grown in McCoy’s 5a medium containing 10% fetal calf serum, 50 μg/ml glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

RNA Preparation and Northern Analysis—Total RNA was prepared from cultured cells by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987), and mRNA was isolated with the poly(A) tract system (Promega) and analyzed by Northern blotting. The probe used for hybridization of the Northern blots was a random primed 1.6-kilobase (kb) human PAX6 cDNA clone (ph-12) (Ton et al., 1991); the membranes were then stripped and rehybridized with a human β-actin cDNA probe.

Isolation of Genomic DNA Clones Containing the Human PAX6 Promoter—The PAX6 genomic clone ch1–7 (Ton et al., 1991) was isolated from a pWE15 cosmids library by screening with a 1.6-kb PAX6 cDNA probe (ph-12). Restriction endonuclease mapping revealed that ch1–7 con-
FIG. 1. Genomic structure of the human PAX6 gene. The two overlapping cosmids ch1–7 and ch1–2 were characterized by restriction mapping and Southern analysis. The region not covered by these two cosmids is adapted from Glaser et al. (1992). The alternative exon 5a is indicated, and the untranslated 5’ and 3’ regions are depicted by open boxes. The restriction map of the 26 kb of the 5’ region, including the 5-kb promoter region, was determined. B, BamHI; E, EcoRI; H, HindIII; N, NotI; S, SalI; X, XbaI.

tained a 36-bp insert. A 300-base pair (bp) probe derived from the 5’ end (from the HindIII site to the StuI site) of a human PAX6 cDNA clone (ph-12) was used to localize the most 5’ genomic sequence.

DNA Sequencing—A 6.5-kb EcoRI-XbaI genomic DNA fragment, which hybridized to the 300-bp ph-12 probe was cloned into pBluescript II KS (Stratagene). The insert was then further dissected to generate small overlapping subclones. The sequence of the 2.9 kb between the II KS (Stratagene). The insert was then further dissected to generate small overlapping subclones. The sequence of the 2.9 kb between the 5’-HindIII site and the 3’ end of exon 2 was determined by the dideoxy chain termination method. The sequence was analyzed for candidate transcription factor binding sites by using the Genetics Computer Group (Madison, WI) program.

RNase Protection Assay—Total cellular RNA (30 μg) was hybridized with gel-purified labeled antisense transcripts (10^6 cpm). The probe was transcribed with T7 polymerase using [γ-32P]CTP from a genomic fragment corresponding to the 5’ region of the PAX6 cDNA (−180 to +166 bp) cloned into the vector pBluescript SK+. The hybridization was carried out at 42°C overnight. Hybrids were digested for 30 min at 30°C with RNase A and T1 (ribonuclease protection assay kit, Ambion). The sizes of the protected fragments were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel using a DNA sequencing ladder as size marker.

Primer Extension—A 33-mer antisense primer corresponding to bases 133 to 161 of the human PAX6 cDNA sequence was end-labeled with T4 polynucleotide kinase using [γ-32P]ATP. Total cellular RNA (15 μg) was hybridized with 105 cpm of the 32P-labeled oligonucleotide by heating at 90°C for 5 min in 20 μl of hybridization buffer (50 mM Tris-Cl, pH 8.3, 150 mM KCl, 1 mM EDTA) and then immediately incubated at 42°C overnight. The DNA-RNA hybrid was then collected by ethanol precipitation and dissolved in 20 μl of reverse transcription buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM dNTP). The primer was extended by SuperScript II RNaseH− Reverse Transcriptase (Life Technologies, Inc.) at 42°C for 1 h. After completion of the reaction, samples were extracted with phenol-chloroform, precipitated with ethanol, and analyzed on 6% polyacrylamide gel. The sequencing ladder was done with the same primer used for primer extension, and the DNA template is a genomic fragment corresponding to the 5’ region of the human PAX6 gene.

PAX6 Promoter Constructs—DNA fragment (6.5-kb EcoRI-XbaI) derived from the 5’-flanking region clone described above was inserted immediately upstream from the chloraaminophenyl acetyltransferase (CAT) reporter gene in the promoterless and enhancerless CAT expression vector pCAT-basic (Promega). We then made a series of CAT expression constructs that contained various lengths of the 5’-upstream sequence of the PAX6 gene and had the same 3’ end, the NotI site in exon 1. DNA fragments for minimal promoter analyses were prepared by PCR amplification with the following oligonucleotides: 5’-CCCA-AGGTTCCGGTCGGCGAGGCCC-3’ (bases −66 to −50) for the 5’ ends of the PAX6 inserts and 5’-GCTCTAGAGTCATCATCCCTCCAGCA-3’ (bases +83 to +100), 5’-GCTCTAGACCCATTTAATCCACCGG-3’ (bases +13 to +29) for the 3’ end of the PAX6 inserts. The amplified products were digested with HindIII and XbaI and inserted immediately upstream of the CAT reporter gene in the vector’s multiple cloning site.

PAX6 Silencer Constructs— A 1147-bp HindIII-PstI (bases −2404 to −1256) fragment was amplified by PCR and inserted downstream of the CAT gene, which was driven by a 346-bp Smal-NotI PAX6 promoter fragment. A series of deletions from the 5’ and 3’ ends were generated by PCR with the 5’ primers and 3’ primers. The PCR fragments were digested with BamHI and PstI and inserted into the BamHI site of the PAX6 promoter construct pCSMA. 5’ primers: 5’-CGGGAATTCCTCGGGAGAAGGACGAGC-3’, 5’-CGGGAATTCCTCTGTGAAATTACGGCC-3’, 5’-CGGGAATTCCTACAGGAGCTCG-3’, and 5’-CGGGAATTCAGAAAATCTCTTCTTCC-3’. and 3’ primers: 5’-CCCGATCCAGGATTCGCTGCTG-3’, and 5’-GAAGATCGCTTGACCGGCGCA-3’.

Transfection and CAT Assay—CAT expression constructs were transfected into cells growing in monolayers or suspension by electroporation. 20 μg of plasmid DNA and 5 μg of internal control (pSV-β-gal) were electroporated normally into 5 × 10^6 cells in 0.3 ml of culture medium with a Bio-Rad gene pulse at 260 V and 960 microfarads. The transfected cells were harvested after 48 h, and cell lysates were prepared by three cycles of freezing and thawing. The β-galactosidase activity was determined by the method of Rosenthal (1987), and the protein concentration was determined with a Bio-Rad protein assay kit. Aliquots of cell extracts containing 30–50 μg of protein were used for the CAT assay. The acetylated chloraaminophenol was separated from the unacetylated form by thin-layer chromatography in chloroform:methanol (97:3, v/v). The acetylated [14C]chloraaminophenol was quantitated with a PhosphorImager (Molecular Dynamics). The CAT activity of each construct was determined with at least three independent transfection assays and normalized to the β-galactosidase activity.

Preparation of Nuclear Extracts and Western Blot—Nuclear extracts were prepared following the method described by Schreiber et al. (1989). The nuclei were isolated following the disruption of cells by the addition of 0.4% Nonidet P-40 after being swollen on ice in the lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 μg/ml leupeptin, 2.0 μM aprotinin, and 0.5 μg/ml benzamidine). The nuclear pellet was resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithio-
threitol, 0.5 mM phenylmethylsulfonylfluoride, 2.0 mM leupeptin, 2.0 mM aprotinin, and 0.5 mg/ml benzamidine) and centrifuged for 5 min at 4°C, and the supernatant (nuclear extracts) was stored at −70°C.

The in vitro translated protein and nuclear extract were resolved on 10% SDS-polyacrylamide gels, electrotransferred to nitrocellulose, probed with a rabbit polyclonal antibody against the full-length PAX6 protein, and detected by chemiluminescence (ECL, Amersham Corp.).

Nucleotide Sequence Accession Number—The nucleotide sequence of the human PAX6 promoter has been deposited in GenBank (accession number U63833).

RESULTS

Cloning and Sequencing of the Human PAX6 Promoter Region—A human genomic library constructed in the cosmid vector pWE15 was screened with a 1.6-kb human PAX6 cDNA probe. Cosmid cH1–7 was selected and found to contain the first seven exons of the PAX6 gene. cH1–7 spans 36 kb, including 24 kb of the untranscribed 5′ sequence.

In order to identify the PAX6 promoter region, it was necessary to first identify the transcriptional start site. For this purpose we used both primer extension and RNase protection analysis. A 5′-labeled 33-mer antisense primer (corresponding to position +133 to +101) was used for primer extension and hybridized to total RNA isolated from PAX6-expressing cells and non-PAX6-expressing cells. A single 134-bp primer extension product was generated in three PAX6-expressing cells (U87, K562, and HeLa cells) but not in the Y79 cells that do not contain endogenous PAX6 mRNA (Fig. 3A). By comparison with a DNA ladder generated on a genomic plasmid by the same oligonucleotide primer, the transcriptional initiation site was located 108 bp upstream from the beginning of the reported longest cDNA sequence (Glaser et al., 1992). This result was confirmed by the RNase protection assay (Fig. 3B). A 32P-labeled antisense RNA probe corresponding to a 346-bp SmaI-NaeI fragment containing the 66-bp published 5′-terminal sequence was used to hybridize with 15 μg of total RNA isolated from PAX6-expressing cells and non-expressing cells. A single protected fragment of approximately 166 bp was observed in PAX6-expressing cells but not in non-PAX6-expressing cells. In addition to primer extension and RNase protection analyses, we also carried out a 5′ RACE assay of the isolated total RNA from PAX6-expressing cells and non-expressing cells. A single protected fragment of approximately 166 bp was observed in PAX6-expressing cells but not in non-PAX6-expressing cells. In addition to primer extension and RNase protection analyses, we also carried out a 5′ RACE assay of the isolated total RNA from PAX6-expressing cells and non-expressing cells. A single protected fragment of approximately 166 bp was observed in PAX6-expressing cells but not in non-PAX6-expressing cells.
cDNA and numbered base +1.

Sequence analyses of the 2.9-kb upstream promoter region (Fig. 2) from the HindIII site to the end of exon 2 revealed a TATA-like sequence (ATATTTTT) located 26 bp from the initiation site, and two CCAAT boxes located −70 and −100 bp, respectively. The position of TATA-like box and CCAAT boxes are consistent with those found in other eukaryotes. A third consensus sequence (PyAPyPyPy), a pyrimidine-rich initiator element, is also found at the PAX6 transcriptional start site (Breathnach et al. 1981; Burley et al. 1996).

Several putative transcriptional regulatory consensus sequences (Faiast and Meyer, 1992) were identified within the NotI-NaeI proximal promoter region (at bases −452 to +166), including single binding sites for Sp1 protein (at bases −395 to −387), Myc protein (at bases −379 to −373), PuF protein (at bases −410 to −405), CCCT binding factor (at bases −334 to −329), and TCF-2 (at bases +81 to +86). In addition, we found two consensus TCF-1 binding sites (at bases +33 to +37 and −348 to −344), three potential binding sites for activator protein-2 (AP2) (at bases −51 to −44, −145 to −136, and −281 to −274), and four potential candidate binding sites for GCF (at bases −450 to −444, −392 to −386, −236 to −230, and −49 to −43). A 38-bp poly(CA) sequence was located at bases −992 to −955.

Deletion Analysis of the Human PAX6 Promoter Region—To examine the PAX6 promoter activity and identify important regulatory domains within the 5′ region, a series of fragments with 5′ and 3′ deletions in the 5′ region of the PAX6 gene were ligated upstream of the bacterial CAT gene in the promoterless and enhancerless CAT expression vector pCAT-basic (Fig. 4A). These CAT constructs were tested for promoter activity by transfection into U87, K562, and NIH 3T3 cells. For comparison, the cells were also transfected with two control CAT constructs, one with a simian virus 40 promoter and enhancer (pCAT-control) and one without promoter and enhancer (pCAT-basic). The β-galactosidase-expressing vector p8SV-β-gal was used as an internal control for transfection efficiency, and CAT activity was normalized to β-galactosidase activity. Fig. 4 shows the structures of the deletion constructs and their promoter activity determined by transient expression assays. The longest construct, pCENA-b (bases −4900 to +166), had high promoter activity in both U87 and K562 cells (Fig. 4B). The 1.4-kb PstI-NaeI construct (pCPNA, bases −1254 to +166) had a very high level of CAT activity in K562 and U87 cells. It is interesting that the 346-bp SmaI-NaeI fragment (pCSMNA, bases −180 to +166) had the highest CAT activity in U87 and K562 cells. It is interesting that the 346-bp SmaI-NaeI fragment (pCSMNA, bases −180 to +166) had the highest CAT activity in U87 and K562 cells. It is interesting that the 346-bp SmaI-NaeI fragment (pCSMNA, bases −180 to +166) had the highest CAT activity in U87 and K562 cells. It is interesting that the 346-bp SmaI-NaeI fragment (pCSMNA, bases −180 to +166) had the highest CAT activity in U87 and K562 cells. It is interesting that the 346-bp SmaI-NaeI fragment (pCSMNA, bases −180 to +166) had the highest CAT activity in U87 and K562 cells. 

Deletion of a 2.4-kb EcoRI-HindIII fragment from pCENA to form pCHNA caused a 9-fold decrease in CAT activity in U87 cells but not in K562 cells. Deletion of a 1.1-kb HindIII-PstI fragment from pCHNA to form pCPNA caused a 16-fold decrease in CAT activity in U87 cells but only a 2-fold increase in K562 cells. This suggested that there was a strong repressor...
PAX6 protein; different cell lines with rabbit anti-human PAX6 serum. A 46-kDa band and human analysis. An approximately 2.7-kb transcript was detected with a 1.7-kb repressor between the HindIII and PstI sites. The multiple activators and repressors between the EcoRI and HindIII sites was only active in U87 cells, and the strong repressor between the HindIII and PstI sites functioned differently in U87 and K562 cells. The multiple activators and repressors in the 4.9-kb upstream promoter region may function differently in combination and in different cells.

To determine the tissue specificity of the PAX6 promoter, murine and human cell lines of different origins were tested for promoter activity by using the constructs pCHNA and pCPNA. PAX6 promoter activity was detected in cell lines that expressed PAX6 mRNA (as revealed by Northern blotting, Fig. 5), including U87 glioblastoma cells, K562 erythroleukemia cells, 293 kidney cells, and HeLa cells (data not shown). No CAT activity was observed in cell lines that did not express pax6 mRNA, including NIH 3T3 murine fibroblast cells and HeLa cells but not in NIH 3T3 cells. The rabbit anti-PAX6 serum (Tang et al., in press) can specifically recognize the PAX6 protein, because no cross-interaction was observed using an in vitro translated PAX8 protein. The size of the in vitro translated PAX6 protein is consistent with the predicted molecular weight of 46,500 for the 422-amino acid human PAX6 protein. The human PAX6 transcripts are well correlated with the protein level, indicating that PAX6 expression is mainly controlled at the transcriptional level, at least in the cell lines tested.

**Fig. 6. Deletion analysis of PAX6 proximal promoter region.** The 346-bp SmaI-NaeI proximal promoter fragment was cloned into pCAT-basic. The deletion of the 5’ 118 bp of pCSMNA-b produced pCBSNA-b. Construct pCHX-176 was synthesized by PCR amplification of a 171-bp fragment (bases –62 to +109). Construct pCHX-95 was synthesized by PCR amplification of a 92-bp fragment (bases –62 to +30). All PCR-generated fragments were sequenced. The CAT activity was normalized to the activity of pCSMNA-b.

between the HindIII and PstI sites that could regulate cell type-specific expression of the PAX6 gene. Further deletion of the 0.7-kb PstI-SacII fragment from pCPNA to form pCSANA caused 3-fold and 2.5-fold decreases in CAT activity in K562 and U87 cells, respectively. The deletion of a 363-bp SacII-SmaI fragment from pCSANA to form pCSMNA produced 3.5-fold increases in CAT activity in K562 and U87 cells. The deletion of a 500-bp sequence (bases –62 to +166) pCBSNA) had very high CAT activity (Fig. 4B), especially in U87 cells. Four potential transcriptional factor binding sites in this region were identified in the nucleotide sequence, including AP2, GCF, TCF-1, and TCF-2 (Fig. 2). The relative activity of the 3’ deletion constructs created by PCR amplification was tested in U87 and K562 cells (Fig. 6). The 3’ deletion of bases +109 to +166 (pCHX-176) resulted in a nearly 50% decrease in promoter activity in both U87 and K562 cells, indicating that the 57-bp sequence (bases +109 to +166) contained a positive regulatory element or elements for PAX6 promoter function. Further deletion of the 79-bp 3’ fragment (bases +30 to +109, pCHX-95) still retained appreciable promoter activity. These observations demonstrated that sequences both upstream and downstream of the transcriptional start sites are required for PAX6 promoter activity. From the deletion analysis, we can conclude that the 92-bp sequence flanking the TATA-like box and the initiation start site is important for promoter activity. The TATA-like sequence (ATATTTT) identified in human PAX6 promoter is similar to that found in the quail pax6 promoter (ATATTAA) (Plaza et al., 1993).

**Functional Analysis of PAX6 5’ Silencer—**Analysis of the 5’ deletion constructs shown in Fig. 4 suggested the presence of a repressor between the HindIII and PstI sites (–2404 to –1256). This repressor repressed the PAX6 promoter in U87 cells but not in K562 cells. We then tested the repressive activity of this 1.1-kb fragment when it was inserted downstream of the PAX6 promoter. The fragment acted on the PAX6 promoter and a SV40 as a strong repressor in a position- and orientation-independent manner in U87 cells but not in K562 cells (data not shown).

Next, we dissected the 1.1-kb repressor to more precisely locate the sequence responsible for its repressive activity. After deletion of the 5’ 406-bp sequence (bases –2404 to –1998) from the 1.1-kb fragment, the remaining 730 bp (SX730) showed repressor activity both in U87 and HeLa cells but not in K562 cells (Fig. 7, A and B). Further dissection analysis of the 730-bp fragment showed that the 5’ 481-bp region (bases –1998 to –1519, SX480) had no repressor activity in all the cell lines tested, but the 3’ 250 bp (bases –1518 to –1268, SX250) caused
a 2.5-fold decrease of CAT activity in both U87 and HeLa cells. The repressor activity was absent when the 250-bp sequence was split into two overlapping 5' and 3' fragments. Because the 5' SX45 and 3' SX54 (Fig. 7A) have a 31-bp overlap sequence, it is likely that cis-elements upstream and downstream of the 31-bp region are required for the repressor activity. The 250-bp repressor sequence functions equally well in either orientation (Fig. 7B). These results demonstrate that more than 31 bp overlap sequence is required for its repressor activity, and it functions as a cell type-specific silencer. Sequence analysis of the 250-bp silencer showed several transcription factor binding sites, but no potential binding site within 31 bp overlapped sequence (Fig. 7C).

DISCUSSION

In this study, we report the isolation and characterization of the human PAX6 promoter. A 92-bp basal promoter (bases −62 to +30) was able to drive transcription of a CAT reporter gene in PAX6-expressing glioblastoma and erythroleukemia cell lines. The promoter activity was well correlated with the ex-
pression level of endogenous PAX6, and high CAT activity was found in cell lines with high levels of PAX6 transcripts. Deletion analysis of the 346-bp fragment (−180 to +166, pC5SMA) indicates that multiple cis-elements located upstream and downstream of the initiation start site are responsible for high level basal promoter activity and cell specificity.

The 346-bp PAX6 minimal promoter region contains a single consensus binding site for the transcription factors TCF-1, TCF-2, and GCF and two binding sites for the AP2 protein. The PAX6 promoter has a typical initiator element, a TATA-like sequence and two CCAAT boxes at positions −70 and −100. This structure is similar to that of many tissue-specific genes that have a typical TATA box and initiator sequences (Arcioni et al., 1992; Hu et al., 1993). Because the quaii pax6 promoter has been well characterized, we did sequence comparison of the 2.9-kb human PAX6 promoter and the quaii 1.5-kb pax6 promoter (Plaza et al., 1993). Like many TATA box containing gene, they both have a TATA-like box and CCAAT box(es) in the corresponding position.

One of the most intriguing results of the functional analysis of the PAX6 upstream region was the discovery of multiple repressors and activators. A series of 5’ deletions of these cis-regulatory elements altered promoter activity (Fig. 4). The various cis-regulatory elements had various levels of activity in different cell lines. No single cis-element was shown to be essential for PAX6 transcriptional activation. The tissue-specific and temporal expression of the PAX6 gene may be controlled by a combination of different cis-regulatory elements upstream and downstream of the initiation site. The cis-regulatory regions of the Drosophila paired box genes prd, gsb, and gshb (Li and Noll, 1994) control the temporal and spatial expression of functionally equivalent genes. The three paired box genes play different roles during early embryogenesis and are controlled by cis-regulatory elements. All pax genes contain a very conserved paired domain and bind to a consensus recognition sequence (Czerny et al., 1993; Epstein et al., 1994). It is possible that several pax genes also have some redundant functions as do the homeobox genes. Therefore, some cis-regulatory elements may be responsible for the functional diversity of the pax genes. This hypothesis can be confirmed by mutation analysis of candidate cis-regulatory elements in aniridia patients. These elements can be positive or negative, because either haploinsufficiency or overdosage of PAX6 protein can cause eye defects (Schedl et al., 1996). So far, less than half of the aniridia patients examined have been found to have mutations in the coding region of the PAX6 gene. It is likely that some of the unidentified mutations are present in regulatory elements. This is the case for the Drosophila pax6 (eyeless) gene, in which two eyeless phenotypes were found to be caused by mutation in a 200-bp cis-regulatory element (Quiring et al., 1994).

Transient transfection analysis of the PAX6 promoter constructs showed that there is a transcriptional (silencer) in the upstream region. This silencer (bases −1518 to −1268) had different activities in different cell lines (U87, HeLa, and K562 cells). Sequence analysis showed multiple potential transcription factor binding sites in the silencer region (Fig. 7C). One binding site for GCF, a negative regulator of the epithelial growth factor receptor gene (Kageyama and Pastan, 1989), was identified in the silencer region. Notochord transplantation experiments indicated that signals produced by the notochord can repress the region-specific expression of pax6 in the developing spinal cord (Goulding et al., 1993). It was suggested that one of the signal molecules, activin A, is involved in the dorsal and ventral down-regulation of pax6 gene expression (Pituello et al., 1995). Another signal molecule, sonic hedgehog, appears to either directly or indirectly inhibit the expression of PAX6 (MacDonald et al., 1995). The cell-specific silencer in combination with other positive and negative elements may contribute to tissue-specific and temporal expression of the PAX6 gene.

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