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# Activation of the human *PAX6* gene through the exon 1 enhancer by transcription factors SEF and Sp1

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## ABSTRACT

**PAX6 is a transcription factor that plays a major role in ocular morphogenesis. PAX6 is expressed in the eye, central nervous system and pancreas. Two alternative promoters, P0 and P1, which are differentially regulated during development, drive *PAX6* transcription. We identified a 57 bp *cis*-regulatory element in exon 1 of the human *PAX6* gene exon 1 enhancer (EIE). EIE enhances P1-driven *PAX6* expression. Three regions in E1E (E1E-1, E1E-2 and E1E-3) have sequence similarities with binding sites of transcription factors ARP-1, Isl-1 and SEF, respectively. As shown by electrophoretic mobility shift assays, E1E-3, but not E1E-1 or E1E-2, bound to proteins in nuclear extracts of human glioma cells and transcription factor SEF bound to E1E-3. As shown by transient transfection experiments, deletion or site-specific mutations in E1E-3 dramatically decreased P1 promoter activity. Mutations in E1E-2, however, did not affect function of the P1 promoter. Co-transfection of SEF and *PAX6* promoter–reporter constructs showed that SEF up-regulates *PAX6* gene expression through the P1 promoter. Two Sp1 sites in the E1E region were also shown to be important by transient co-transfection assays. Data from immunoprecipitation and transient transfection assays demonstrated that SEF and Sp1 interacted *in vitro* and may act together *in vivo* to regulate *PAX6* expression.**

## INTRODUCTION

PAX6 is a transcription factor and the *PAX6* gene acts as a master control gene for eye development. Its expression is limited to the eye, central nervous system and pancreas. PAX6 is highly conserved throughout the animal kingdom and in man and mouse the PAX6 protein is identical. The level of PAX6 protein is critical since mutations that reduce PAX6 levels result in eye phenotypes of variable severity. A reduction in PAX6 level is caused by mutations in the *PAX6* gene and putative regultory regions controling *PAX6* gene expression.

*PAX6* transcription is controlled by two promoters, P0 and P1. In the mouse, P0-initiated transcripts predominate in the lens placode, cornea and conjuntival epithelia and in the developing pancreas (1,2). P1-initiated transcripts are expressed in lens placode, optic vesicle and central nervous system. The P0 promoter and several *cis*-acting elements have been well characterized in human, mouse, quail and pufferfish (1–4).

Since little is known about the molecular mechanisms that control expression of the *PAX6* gene, it is important to understand the regulation of *PAX6* through *cis*-regulatory elements (enhancers or silencers). It is known that *PAX6*, as a tissue-specific regulatory gene, is transcribed from two alternative promoters (P0 and P1) (3,4) that are differentially regulated in human brain development and in adult brain plasticity. Some distinct *cis* elements are involved in regulating the P0 and P1 promoters and in tissue-specific expression of *PAX6* in the eye, the central nervous system and pancreas (5–7) and several potential binding sites for transcription factors lie within the P0 and P1 promoters. The P0 promoter contains a TATA-like box, a CCAAT box, multiple consensus transcription factor binding sites for AP1, AP2, Sp1, c-Myb and Pit-1 and sites for the p53 transcriptional activator, NFκB and C/EBP protein. The P1 promoter contains a TATA-like box, two CCAAT boxes, a 38 bp polymorphic dinucleotide repeat sequence, $(AC)_m(AG)_n$, and potential transcription factor binding sites for AP1, AP2, AP4, Sp1, c-Myb, Pit-1, ARP-1, and Isl-1 (8–9; Fig. 1). Another region between –3.1 and –2.9 kb upstream of the P1 promoter contains a regulatory element that directs *PAX6* expression in the developing photoreceptor (10). Other putative *cis*-regulatory elements involved in human *PAX6* expression have been proposed to lie upstream or downstream of the *PAX6* transcription initiation site (10). In this paper we report our search for such elements and our identification of a novel enhancer element in exon 1 of the human *PAX6* gene, the exon 1 enhancer (EIE), which is activated by transcription factors SAA3 enhancer factor (SEF) and Sp1. We also demonstrate a physical association between SEF and Sp1 in regulating *PAX6* gene expression.

## MATERIALS AND METHODS

### Cell culture

Human glioma cell lines U251 and LN229 were derived from two different glioblastomas and both have mutations in the *p53* gene. The cell lines were grown in a Dulbecco's modified Eagle's medium and F-12 nutrient mixture (DMEM/F-12)

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**Table 1.** Oligonucleotides used for EMSA for mutant E1E binding sites

| Primer | Sequence |
|---|---|
| E1E-1 | 5′-ATGATGACAG**AGGTCA**GGCTTCGCTA-3′ |
| mE1E-1 | 5′-ATGATGACAG***ATCC*****CA**GGCTTCGCTA-3′ |
| E1E-2 | 5′-GAGGTCAGGCTTCG**CTAATG**GGCCAGTGAGGAG-3′ |
| mE1E-2 | 5′-GAGGTCAGGCTTCG***C GCC*****TG**GGCCAGTGAGGAG-3′ |
| E1E-3 | 5′-GGTCTACA**CCAGTGAGGAGCGGTG**AATT-3′ |
| m1E1E-3 | 5′-GGTCTACA**CCAG*CTC*GGAGCGGTG**AATT-3′ |
| m2E1E-3 | 5′-GGTCTACA***AAC*****GTGAGGAGCGGTG**AATT-3′ |
| m3E1E-3 | 5′-GGTCTACA**CCAGTGAGGA*AAA*GTG**AATT-3′ |

The binding site is in bold, the mutant position is italic.

supplemented with 10% fetal calf serum (Gibco BRL, Rockville, MD). The degree of tumorigenicity in nu/nu mice is higher for LN229 than for U251 (11).

### Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using nuclear extracts of cell cultures prepared following the method described by Schreiber *et al.* (12). Oligomers for use in the EMSAs were synthesized to be complementary, with one dG protruding at the 5′-end of each sequence for Klenow labeling with [α-$^{32}$P]dCTP. One strand of each oligomer sequence is shown in Table 1.

EMSAs were performed in a 20 µl reaction mixture that had a pH of 7.9 and contained 25 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 1% Nonidet P-40, 5% glycerol, 2 mg poly(dI–dC), nuclear protein extracts containing 7–10 mg of protein and $10^5$ c.p.m. (~3 fmol) of $^{32}$P-labeled oligomers. The reaction mixture was incubated at room temperature for 30 min. Samples were then loaded onto a 5% native polyacrylamide gel (19:1 crosslinking ratio). The gels were analyzed with a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). For supershift assays, the binding reaction mixture was incubated with antibody or unlabeled probe at room temperature for 30 min before being loaded onto the gel.

### Construction of *PAX6* promoter–luciferase constructs

A 346 bp sequence containing the *PAX6* P1 promoter and the downstream 157 bp exon 1 sequence were obtained by PCR amplification from the *PAX6* promoter–CAT construct pCSMNA-b (13) using primers PAX6p-1 (5′-TGAGCTCG-GGCTCGGGGGCCCTGC-3′) and PAX6p-2 (5′-GGCGCCC-GGCCTCGCCTCCA-3′). Primer PAX6p-1 was designed with a *Sac*I site at the 5′-end and after blunt-end treatment with Klenow DNA polymerase (Roche, Indianapolis, IN), the PCR product was digested with *Sac*I and cloned into a pGL3B luciferase vector (Promega, Madison, WI) between the *Sac*I and *Sma*I sites in the vector. The *PAX6* promoter–luciferase constructs (346pGL3B) were verified by sequencing.

### Construction of mutant *PAX6* promoter–luciferase constructs

PCR-based, site-directed mutagenesis was used to create mutations in the E1E region of the 346pGL3B construct. In brief, two partially overlapping primers, designated R and F, which contained mutations, were synthesized for each mutant construct (Table 2). Using PCR, two DNA fragments were amplified from 346pGL3B plasmid DNA in two separate reactions. The first reaction (first round PCR) contained primers R and RVP3, corresponding to the vector sequence upstream of the insert, or primers F and GLP2, corresponding to the vector sequence downstream of the insert. The two PCR products of the expected sizes were isolated from the first round PCR mixtures by electrophoresis on a 2% agarose gel and were cut out, mixed together and resuspended in 400–500 µl of water by heating for 5 min at 95°C. An aliquot of the suspension was used in a second PCR reaction (second round PCR) with primers RVP3 and GLP2.

The first round PCR conditions were 95°C for 1 min, 60°C for 1 min and 72°C for 1 min for one cycle, 94°C for 30 s, 58°C for 1 min and 72°C for 1 min for 30 cycles, and 72°C for 10 min for one cycle. The second round PCR conditions were 95°C for 1 min, 40°C for 1 min and 72°C for 1 min for one cycle, 94°C for 30 s, 58°C for 1 min and 72°C for 1 min for 30 cycles, and 72°C for 10 min for one cycle. The PCR product of the second round PCR was purified with a Gel Extraction Kit (Qiagen, Hilden, Germany). After digestion with *Sac*I and *Bgl*II (the

**Table 2.** Oligonucleotides used in PCR-based site-directed mutagenesis

| Construct | Primer | Sequence |
|---|---|---|
| 346pGL3B-mE1E-1 | R primer | 5′-CAGA**ATC**CAGGCTTCGCTAATGGGPCR-3′ |
| | F primer | 5′-GAAGCCTG**GAT**TCTGACATCATCCTCCAG-3′ |
| 346pGL3B-mE1E-2 | R primer | 5′-CTTCGC**GCC**TGGGCCAGTGAGGAGCG-3′ |
| | F primer | 5′-CCCA**GGC**GCGAAGCCTGACCTCTG-3′ |
| 346pGL3B-m2E1E-3 | R primer | 5′-ATGGG**AAC**GTGAGGAGCGGTGGAGGC-3′ |
| | F primer | 5′-CCTCAC**GTT**CCCATTAGCGAAGCCTGAC-3′ |
| 346pGL3B-m3E1E-3 | R primer | 5′-TGAGGA**AAA**GTGGAGGCAGAGCCGGG-3′ |
| | F primer | 5′-CTCCAC**TTT**TCCTCACTGGCCCATTAGCG-3′ |
| 346pGL3B-mSp1 | 3′-Sp1Xho primer | 5′-ACTCGAGCACCGCTCCTCACTGGCC-3′ |

Mutant sites are in bold.

same sites as on the pGL3B vector), the mutated DNA fragment was removed from the 346pGL3B vector and inserted into the pGL3B vector between the corresponding digestion sites. Positive clones were selected by PCR screening with primers RVP3 and GLP2.

The 346pGL3B-mSp1 construct, 346pGL3B with the Sp1 site deleted from its 3′-end, was generated by amplifying the *PAX6* P1 promoter region with primers RVP3 and 3′-Sp1Xho (Table 2) and inserting the DNA between the *Sac*I and *Xho*I sites of the pGL3B vector. The PCR conditions were the same as the first round PCR conditions mentioned above. The PCR product was isolated by digestion at the *Sac*I and *Xho*I sites. The mutated DNA fragment was then inserted into the pGL3B vector between its *Sac*I and *Xho*I sites.

### Luciferase and β-galactosidase assays

Cells were plated in 6-well tissue culture dishes at a density of 2–3 × $10^5$ cells/well 24 h before transfection. At 60–80% confluence, cells were transfected with 0.5 µg of luciferase constructs using 3 µl FuGene6 (Roche) transfection reagent per well. Cells were co-transfected with 5 ng of SV40-pSV2 β-galactosidase expression vector as an internal control. Forty-eight hours after transfection, cell lysates were extracted and luciferase activity was estimated using a Luciferase Assay Kit (Tropix, Bedford, MA) according to the manufacturer's protocol. Luciferase activity was measured with a luminometer (Lumat LB 9507; EG & G Berthold, Woldbad, Germany).

β-Galactosidase activity was detected using a Galacto-Light Plus chemiluminescent assay kit (Tropix) according to the manufacturer's protocol. Because SEF activates the SV40 promoter, luciferase activities were normalized to the molar amount of protein in the SEF/Sp1 co-transfection experiments.

### Immunoprecipitation and immunoblotting

For co-immunoprecipitation assay of nuclear extracts ~300–400 µg of nuclear protein from LN229 cells was diluted with binding buffer (150 mM NaCl, 12.5 mM HEPES, pH 7.9, 5 mM KCl, 0.05% Nonidet P-40, 100 mg BSA, 0.1 mM EDTA) and then supplemented with 1× Complete protease inhibitor cocktail (Roche), 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM $Na_3VO_4$ to a total volume of 300 µl. The diluted nuclear extracts were then preabsorbed to protein G–Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 h and then centrifuged at the highest speed for 3 min. The supernatant was incubated with a polyclonal anti-SEF antibody (14) at 4°C overnight. The immunocomplexes were then absorbed onto protein G–Sepharose at 4°C for 1 h and washed three times with 200 µl of phosphate-buffered saline. Elution of associated proteins in SDS sample buffer was performed by boiling for 5 min; the proteins were then resolved by 6% SDS–PAGE, transferred to a polyvinylidene difluoride (PVDF) immunoblot membrane (Qiagen), probed with anti-Sp1 antibody (Santa Cruz Biotechnology) and detected using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia). The SEF antibody was prepared according to Bing *et al*. (14) and its specificity was demonstrated by them. Anti-Sp1 antibody was purchased from Santa Cruz Biotechnology.

For immunoprecipitation of *in vitro* proteins the SEF and Sp1 proteins were synthesized from SEF/pCS2+MT (14) and Sp1/pCDNA (15), respectively, using the TNT Coupled Rabbit Reticulocyte Lysate system (Promega). In brief, 15 µl of SEF protein and 15 µl of Sp1 protein were mixed in 170 µl of binding buffer, incubated at 4°C for 2 h, supplemented with anti-SEF antibody and then absorbed onto protein G–Sepharose for 1 h at 4°C. The proteins were then denatured in SDS sample buffer and resolved by SDS–PAGE.

## RESULTS

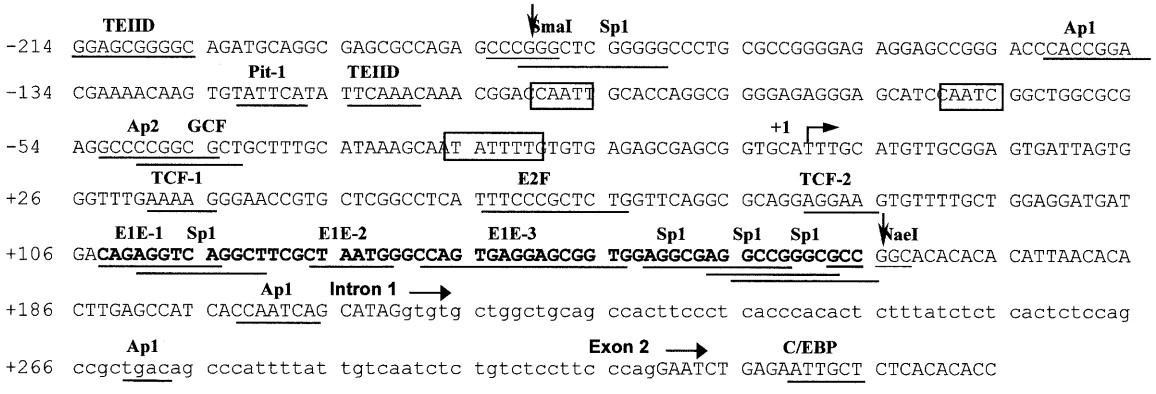
### Identification of the EIE in the human *PAX6* P1 promoter

Previously (13) we identified a 57 bp region in exon 1 (from position +109 to position +166) (Fig. 1) that increased *PAX6* promoter activity in U87 and K562 cells. Sequence analyses of E1E revealed several potential regulatory factor binding sites, including an E1E-1 site (CAGAGGTCA), an E1E-2 site (CTAATG), an E1E-3 site (CCAGTGAGGAGCGG) and four Sp1 sites (Fig. 1). The sequences of the E1E-1, E1E-2 and E1E-3 binding regions are similar to the binding sequence of the transcription factor apolipoprotein AI regulatory protein-1 (ARP-1), Islet-1 (Isl-1) and *Drosophila* serendipity-β (D-Sry-β), respectively.

### Characterization of the *PAX6* P1 promoter enhancer E1E by EMSA

To characterize the specificity of the DNA–protein binding affinity of each potential transcription factor binding site in E1E, double-stranded oligonucleotides corresponding to each binding site were synthesized and end-labeled with $^{32}P$ for use as probes. Nuclear extracts were prepared from U251 cells. As shown by EMSA, no DNA–protein complexes formed with the E1E-1 oligonucleotide (Fig. 2, lane 1) and a weak complex was formed with the E1E-2 oligonucleotide (Fig. 2, lane 2). In contrast, a strong complex was formed with the E1E-3 oligonucleotide and nuclear extracts from U251 cells (Fig. 2, lane 4), indicating the presence of proteins binding both the E1E-2 and E1E-3 regions in *PAX6* exon 1.

To determine whether the 6 bp E1E-2 and the 14 bp E1E-3 sequences were critical for the formation of DNA–protein complexes, mutant E1E-2 and E1E-3 oligonucleotides (mE1E-2 and mE1E3, respectively) were synthesized (Table 1) and used as probes in EMSA. In contrast to the wild-type oligonucleotide, mE1E-2 did not form DNA–protein complexes with proteins in the nuclear extracts (Fig. 2, lane 3). Since the sequence of E1E-2 is similar to that of the binding site of transcription factor Isl-1, Isl-1 antibody (Santa Cruz Biotechnology) was added to the EMSA reaction to determine if the E1E-2 binding protein was Isl-1. Surprisingly, no supershift or decrease in the amount of DNA–protein complexes was demonstrated (data not shown), indicating that the protein in the E1E-2 complex was not Isl-1. Gel shift competition experiments were used to examine nuclear protein binding to E1E-3. The E1E-3 probe bound well with nuclear proteins (Fig. 2, lane 4) and excess unlabeled oligonucleotide effectively competed labeled oligonucleotide binding (Fig. 3A, lane 2). Mutation of the three central nucleotides in E1E-3 (m1E1E-3) reduced binding (Fig. 2, lane 5). Mutation of three nucleotides on either side of the E1E-3 binding site completely abolished binding (Fig. 2, lanes 6 and 7). Thus, proteins in the glioma cell nuclear extract bind to both the E1E-2 and E1E-3 regions in a sequence-specific manner and the nucleotides adjacent to the




**E1E-1: ARP-1 (apolipoprotein AI regulatory protein-1)**
**E1E-2: Isl-1 (islet-1)**
**E1E-3: *D*-Sry-β (*Drosophila* serendipity-beta)**

**Figure 1.** Nucleotide sequence of the P1 promoter of the human *PAX6* gene. The transcriptional start site (+1) is marked with the bent arrow. The *cis* element E1E is indicated in bold. The TATA-like box, CCAAT boxes and potential *cis*-regulatory elements in the proximal promoter region mapped by Xu and Saunders (13) and Okladnova *et al*. (20) are either in boxes or underlined and labeled. Essential restriction sites used in subsequent subcloning are underlined and labeled above the sequence. Intron 1 is in lower case and labeled. The exon 2 region is labeled.

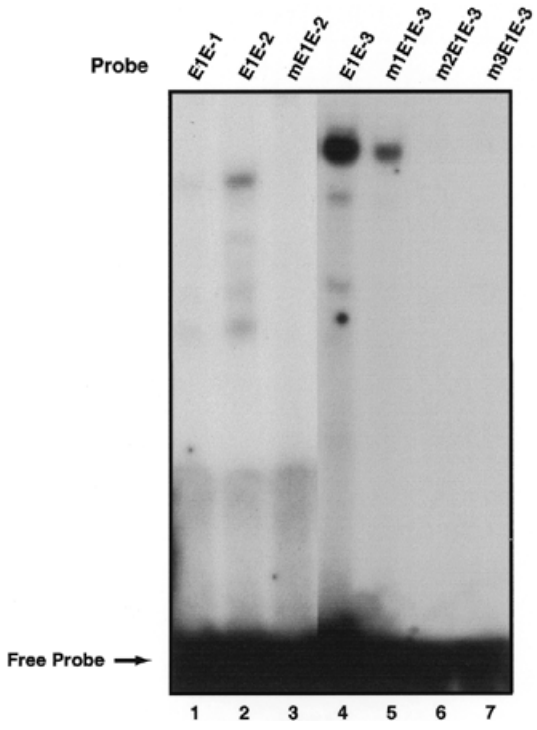



**Figure 2.** EMSA of potential protein binding sites in E1E. $^{32}$P-labeled probes for E1E-1, E1E-2, mE1E2, E1E-3, m1E1E3, m2E1E3 and m3E1E3 (Table 2) sites were incubated with equal amounts of protein in nuclear extracts from U251 cells.

E1E-3 region appear to be more critical for binding than those in the middle. Comparison of the E1E-3 region (5′-CCAGTGAGGAGCGGT-3′) with the consensus binding site 5′-CNRGN$_{5-6}$CNRG-3′ (14,16) showed matches at 13 of 14 positions.

## SEF binding to E1E-3

Because of the high sequence similarity of the EIE-3 and SEF binding sequences, we investigated whether SEF could be the transcription factor that binds the E1E-3 site. SEF is a transcription factor encoding the human serum amyloid A3 (*SAA3*) gene enhancer (14,15). SEF has also been called LBP-1c/CP2/LSF (17–19). To determine whether SEF binds to the E1E-3 region in *PAX6*, the oligonucleotides corresponding to E1E-3 and the binding site of SEF in the *SAA3* promoter were labeled and used as probes in EMSA with nuclear extracts from LN229 human cells. As shown in Figure 3A, the binding activity of E1E-3 oligonucleotide was competed by itself (lane 2) and by wild-type SEFB (lane 4), but not by the mutant forms of each oligonucleotide (Fig. 3A, lanes 3 and 5). The binding activity of SEF was competed by itself and by wild-type E1E-3, but not by the mutant forms of each oligonucleotide (Fig. 3A, lanes 8 and 10). These results indicate that SEF protein binds to the E1E-3 region. To determine if SEF binds to the E1E-3 region, supershift experiments were performed with anti-SEF antibody and preimmune rabbit serum as a control (Fig. 3B). Both SEF and E1E-3 DNA–protein complexes were supershifted by anti-SEF antibody (Fig. 3B, lanes 3–5 and 8–10). Thus, the protein complexes formed with E1E-3 and the SEF probe involved the same SEF protein. The conclusion that SEF protein binds to the E1E-3 binding region of the *PAX6* P1 promoter was confirmed by EMSA using SEF protein synthesized *in vitro*. As shown in Figure 4, both EIE-3 and SEF oligonucleotides bind to SEF protein and form protein–DNA complexes (Fig. 4, lanes 1 and 11). The binding activities of both EIE-3 and the SEF probe are competed by themselves but not by their mutant forms (Fig. 4, lanes 2–6 and 7–10). In order

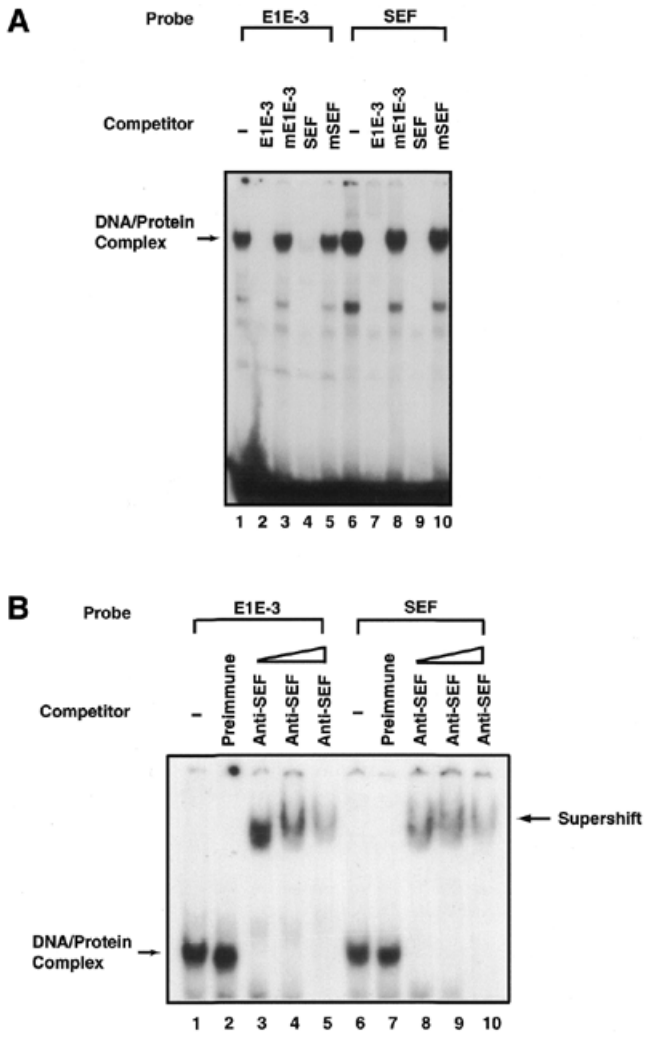



**Figure 3.** DNA binding specificity of SEF to E1E-3. (**A**) EMSA with nuclear extracts. Double-stranded oligonucleotides of E1E-3 in the *PAX6* P1 promoter and the SEF binding site (SEF) from the serum amyloid A3 promoter were $^{32}$P-labeled and incubated with nuclear extracts from LN229 cells. DNA–protein complexes were competed with wild-type or mutant E1E-3 or SEF oligonucleotides (lanes 2–5 and 7–10). (**B**) The reaction was incubated with preimmune or anti-SEF antibodies to supershift the DNA–protein complex (lanes 1–5 and 6–10) and the amounts of anti-SEF antibodies were increased to supershift the DNA–protein complex.

to confirm that the SEF protein synthesized *in vitro* was the oligonucleotide binding component, SEF protein was mixed with SEF antibody before addition of labeled EIE-3 or SEF oligonucleotide and complex formation was assayed by EMSA. In the absence of SEF antibody appropriate complexes were formed. Increasing amounts of SEF antibody reduced the amount of SEF protein driven into protein–DNA complexes with the oligonucleotide probe (Fig. 4, lanes 13–16 and 18–20). These results indicate that SEF protein binds to the EIE-3 region of the *PAX6* promoter.

### Functional analysis of the putative transcription factor binding sites in E1E

The function of the three putative transcription factor binding sites in the exon 1 region of *PAX6* (E1E-1, E1E-2 and E1E-3) and the Sp1 binding site (Fig. 1) were examined. Constructs with mutations in the binding sites were generated by PCR-based site-directed mutagenesis of 346pGL3B (Table 2). The mutations were the same as those in the mutated oligonucleotides shown in Table 1. The luciferase constructs were examined for promoter activity by transient transfection into U251 and LN229 cells. For comparison, the cells were also transfected with the luciferase construct without the promoter and enhancer (pGL3B). The structures of the mutant constructs and their promoter activities determined by transient expression assays are shown in Figure 5. For comparison, the luciferase activities were normalized to the activity of 346pGL3B.

Although the E1E-1 region did not bind to nuclear extracts of human glioblastoma cell lines (Fig. 2), mutations in this region resulted in a 70–90% reduction in transactivation. However, a search of the database of transcription factor binding sites indicated that an Sp1 binding site overlaps the E1E-1 region (Fig. 1). Thus, this decrease in promoter activity may have been caused by a mutation in the Sp1 site rather than in E1E-1, and it is possible that this Sp1 binding site is important in *PAX6* promoter function.

Mutation in the E1E-2 region (the same mutation that abolished binding shown in Fig. 2) did not substantially affect promoter activity, suggesting that the E1E-2 region is not necessary in *PAX6* P1 promoter function. Two mutations in E1E-3 abolished binding activity and resulted in a 70–90% reduction in promoter activity. Deletion of the Sp1 binding region in E1E led to an 80–95% reduction in promoter activity. Together, these data suggest that the E1E-3 binding site in *PAX6* and the adjacent Sp1 binding site are both important for *PAX6* P1 promoter activity.

### *In vitro* interaction between SEF and Sp1

Sp1 is a ubiquitous transcription factor whose binding site, a GC box, is widely distributed in promoter regions of many genes. Because the SEF binding site (E1E-3) in E1E is adjacent to Sp1 binding sites, we hypothesized that the two transcription factors SEF and Sp1 may cooperate in the regulation of *PAX6* gene expression. LN229 nuclear extracts and SEF and Sp1 protein mixtures were immunoprecipitated with polyclonal anti-SEF antibody in separate experiments. Then the proteins in immunocomplexes were detected with anti-Sp1 antibody. As a positive control, immunoprecipitation by Sp1 antibody was done (Fig. 6, lanes 3, 9 and 11). Negative controls showed that the Sp1 antibody was unable to detect *in vitro* synthesized SEF protein (Fig. 6, lanes 7 and 12) and ruled out a cross-reaction between Sp1 antibody and SEF protein. As shown in Figure 6, Sp1 protein was detected in the SEF immunocomplexes from nuclear extracts and from the mixture of *in vitro* synthesized SEF and Sp1 proteins (Fig. 6, lanes 2 and 6). In contrast, an unrelated antibody (anti-PAX6 antibody) failed to precipitate Sp1 protein (Fig. 6, lane 4). These results indicate that SEF can interact directly with Sp1 *in vitro*.

### Involvement of SEF and Sp1 in *PAX6* regulation

To determine whether SEF and Sp1 were involved in the regulation of *PAX6* gene expression, LN229 cells were co-transfected with the human *PAX6* promoter–luciferase construct 346pGL3B, an Sp1 expression construct, Sp1/pCDNA3.1, or an SEF expression construct, SEF/pCS2+MT. Because SEF can bind and activate the SV40 major late

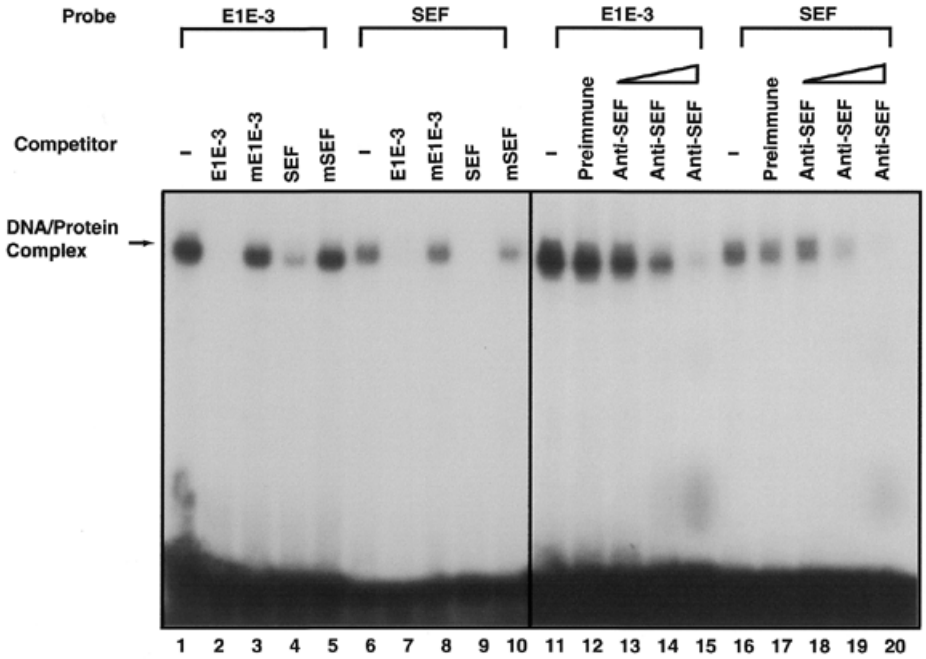



**Figure 4.** DNA binding properties of SEF protein to E1E-3. In an experiment similar to that in Figure 3, $^{32}$P-labeled E1E-3 or SEF oligonucleotides were incubated with SEF protein. The competitors used were the wild-type or mutant oligonucleotides, E1E-3 or SEF (lanes 2–5 and 7–10) or preimmune serum or anti-SEF antibodies to abolish the DNA–protein complex (lanes 12–15 and 17–20). In lanes 13–15 and 18–20 the amounts of anti-SEF antibodies were increased to abolish the DNA–protein complex.

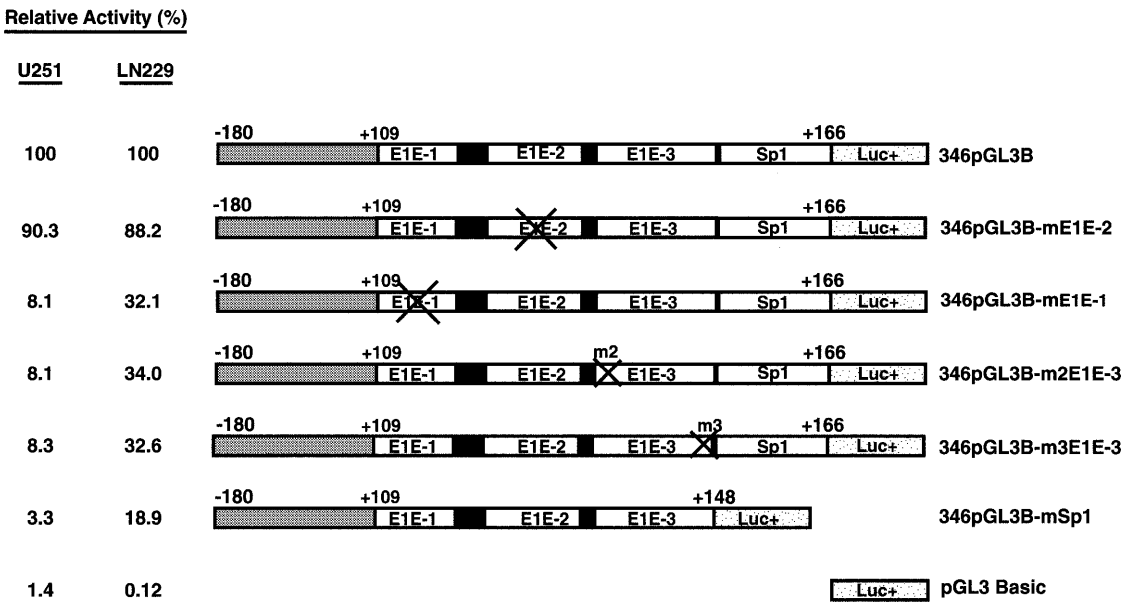



**Figure 5.** Luciferase activity of the *PAX6* P1 promoter with various mutations. The 346pGL3B construct was prepared by inserting the 346 bp *Sma*I–*Nae*I region of the human *PAX6* P1 promoter into a luciferase basic vector (pGL3B). Each binding site was mutated (as described in Materials and Methods). U251 and LN229 cells were transfected with 0.5 µg of the constructs shown. The results were normalized to the activity of the control pGL3 basic transfected cells, to which a value of 100 was assigned.

promoter, the β-galactosidase-expressing vector pSV40-pSV2 cannot be used as an internal control in these transfection experiments. Instead, luciferase activity was normalized to the amount of protein in the cell lysate. The same amounts of 346pGL3B were used in each transfection experiment and different amounts of vector pCS2+MT were used to maintain the same total amount of DNA. The amount of SEF expression construct SEF/pCS2+MT was increased from 0 to 10 ng (Fig. 7A)

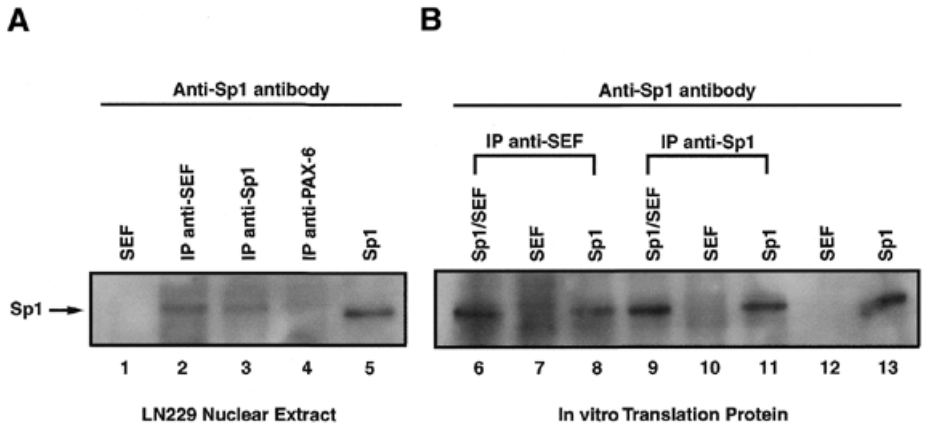



**Figure 6.** Co-immunoprecipitation analysis of the association of SEF and Sp1. (**A**) Nuclear extract from LN229 cells was subjected to immunoprecipitation with anti-SEF antibody, anti-Sp1 antibody or anti-PAX6 antibody (as a control). *In vitro* synthesized Sp1 protein was used to indicate the position of the Sp1 band (lane *5*) and a sample of *in vitro* synthesized SEF protein was used as a negative control for immunoblotting (lane 1). (**B**) *In vitro* synthesized SEF and Sp1 were immunoprecipitated together with anti-SEF antibody or anti-Sp1 antibody (lanes 6 and 9) or immuniprecipitated separately with anti-SEF antibody or anti-Sp1 antibody (lanes 7, 8, 10 and 11). Anti-Sp1 antibody was used to probe the membrane.

and the amount of Sp1 expression construct SP1/pCDNA3.1 was increased from 0 to 150 ng (Fig. 7B). These results showed that SEF could increase *PAX6* gene promoter activity up to 4-fold (Fig. 7A) and Sp1 could increase the promoter activity up to 6-fold (Fig. 7B). Although there seems to be a physical interaction between SEF and SP1 this interaction alone does not result in synergistic activation of the *PAX6* promoter. It seems likely that other factors are necessary for high level transactivation.

## DISCUSSION

Transcriptional regulatory regions of *PAX6* have been identified in mouse, quail, pufferfish and human. These regions are generally very highly conserved. The two upstream promoters P0 and P1 are remarkable in both their nucleotide sequence arrangement and spatio-temporal expression during evolution. The P0 promoter is activated at the onset of neuronal differentiation and P0 transcripts are found in the lens placode, corneal and conjuntival epithelia and pancreas (1,2). *cis*-regulatory elements for P0 have been found within 6 kb upstream of the P0 promoter (1–3). The P1 promoter control region is less well characterized. P1-initiated transcripts are found in all eye structures expressing *PAX6* and several *cis*-acting elements have been described (1–4,13), but no specific transcriptional activators have been identified.

Xu and Saunders (13) previously cloned and characterized the human *PAX6* P1 promoter and identified the transcription initiation site and the 92 bp *PAX6* minimal promoter (bp–62 to +30). They found by deletion analysis of the 346 bp fragment (–180 to +166, pCSMNA) that multiple *cis* elements located upstream and downstream of the *PAX6* transcription initiation site are responsible for high level basal promoter activity and cell specificity. Using deletion mutants of the *PAX6* P1 promoter, Xu and Saunders (13) performed transient transfection experiments in different cell lines and predicted that there may be a 57 bp enhancer in the P1 promoter region. In this paper we have identified and characterized this 57 bp region (E1E), which is important for expression of the human *PAX6* gene. This *cis*-regulatory element is located in the 5′-untranslated region of the *PAX6* gene, 109 bp downstream of the transcription start site and 370 bp before the start of translation in *PAX6* mRNA. We have determined that the transcription factors SEF and Sp1 are essential transcription factors for E1E and that they up-regulate *PAX6* transcription.

By mutation of the putative TATA box in the *PAX6* P1 promoter and transient transfection experiments, we confirmed that the putative TATA box in the *PAX6* P1 minimal promoter is functional for the *PAX6* P1 promoter (data not shown).

Okladnova *et al.* (20) identified an Isl-1 binding motif within the promoter P1. This was of particular interest because *PAX6* is a key regulator for normal pancreatic islet cell development and because Isl-1 is a LIM homeodomain transcription factor that is required for motor neuron and islet cell development (8,21–23). However, our supershift experiments showed no binding of E1E-2 (the putative Isl-1 binding motif indicated by Okladnova *et al.*; 20) to anti-Isl-1 antibody and our transfection experiments showed that mutation of the E1E-2 region had no effect on *PAX6* promoter activity. These data indicate that Isl-1 was not the factor bound to E1E-2. Although we found weak sequence-specific binding to the E1E-2 sequence *in vitro*, our transient transfection data strongly implied that the protein(s) that bound to this region was not involved in the regulation of *PAX6* gene expression.

In EMSA no E1E-1 DNA–protein complexes were formed. However, mutation of this region dramatically decreased *PAX6* P1 promoter activity. One possible explanation is that, because there is also a Sp1 binding site overlapping the E1E-1 region, the mutation in the E1E-1 region may affect the Sp1 site, thus affecting transcription regulation by Sp1. Alternatively, under our *in vitro* conditions we were unable to detect the transcription factor binding E1E-1.

Both our *in vitro* and *in vivo* results show that the E1E-3 region is critical for *PAX6* gene expression. When we searched the TESS transcription factor database (www.cbil.upenn.edu/tess/) the sequence of E1E-3 matched the sequence of the zinc finger protein D-Sry-β in *Drosophila*, which is involved in the onset of zygotic transcription (24) but has no known

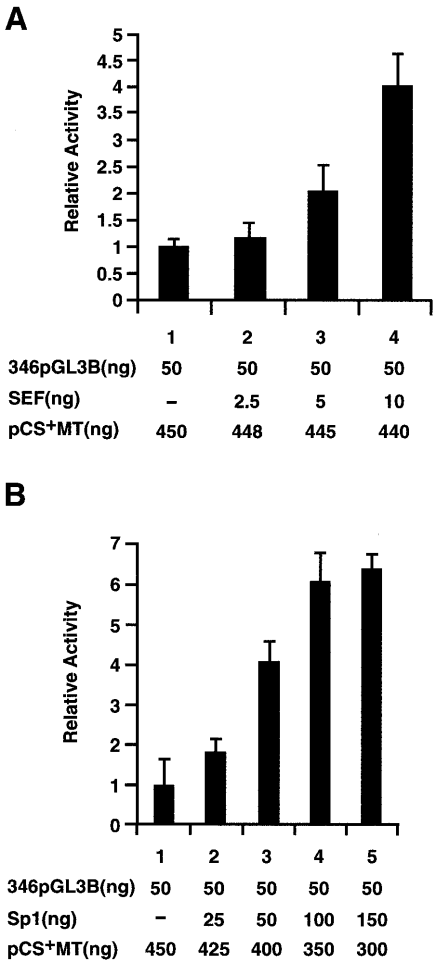



**Figure 7.** Transactivation of the *PAX6* promoter by SEF and Sp1 expression constructs. (**A**) LN229 cells were co-transfected with 50 ng of 346pGL3B and increasing amounts of SEF expression plasmid DNA. The luciferase activities were normalized to the activity of 346pGL3B with co-transfection of the empty vector, to which a value of 1.0 was assigned (column 1). (**B**) LN229 cells were co-transfected with 50 ng of 346pGL3B and increasing amounts of the Sp1 expression plasmid DNA. The data were calculated from nine independent experiments repeated three or four times.

human homolog. However, part of the E1E-3 region (5′-CCAGTGAGGAGCGG) matched the consensus binding site of SEF (5′-CNRGN$_{5-6}$CNRGN). As our EMSA and transient transfection experiments showed, SEF is an activator for *PAX6* transcription activation. Also, we have demonstrated an interaction between SEF and Sp1 *in vitro*. As our data show, the activity drops with increasing amounts of overexpressed Sp1 or SEF. Since co-transfection of SEF and Sp1 activates transcription slightly more than additively (data not shown), it is possible that additional factors besides SEF and Sp1 are involved in *PAX6* gene expression *in vivo*. Studies of how SEF and Sp1 regulate *PAX6* gene expression will provide a better understanding of the temporal and spatial regulation of *PAX6* gene expression.

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