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Direct and concentration-dependent regulation of the proneural gene *Neurogenin2* by Pax6

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SUMMARY

Expression of the proneural gene *Neurogenin2* is controlled by several enhancer elements, with the E1 element active in restricted progenitor domains in the embryonic spinal cord and telencephalon that express the homeodomain protein Pax6. We show that Pax6 function is both required and sufficient to activate this enhancer, and we identify one evolutionary conserved sequence in the E1 element with high similarity to a consensus Pax6 binding site. This conserved sequence binds Pax6 protein with low affinity both in vitro and in vivo, and its disruption results in a severe decrease in E1 activity in the spinal cord and in its abolition in the cerebral cortex. The regulation of *Neurogenin2* by Pax6 is thus direct.

Pax6 is expressed in concentration gradients in both spinal cord and telencephalon. We demonstrate that the E1 element is only activated by high concentrations of Pax6 protein, and that this requirement explains the restriction of E1 enhancer activity to domains of high Pax6 expression levels in the medioventral spinal cord and lateral cortex. By modifying the E1 enhancer sequence, we also show that the spatial pattern of enhancer activity is determined by the affinity of its binding site for Pax6. Together, these data demonstrate that direct transcriptional regulation accounts for the coordination between mechanisms of patterning and neurogenesis. They also provide evidence that Pax6 expression gradients are involved in establishing borders of gene expression domains in different regions of the nervous system.

Key words: Spinal cord, Cerebral cortex, Transcriptional enhancer, Chick, Electroporation

INTRODUCTION

The generation of a large variety of neuronal and glial cell types at defined positions is essential for the development of a functional nervous system. The establishment of neuronal and glial diversity is initiated by patterning of the neural tube along the anteroposterior and dorsoventral axes, in response to inductive signals produced by organizing centers. The secreted molecule Sonic Hedgehog (Shh) is the main ventral organizing signal, and is initially produced by the notochord and subsequently by the floor plate. Shh patterns the ventral neural tube by positively and negatively regulating different sets of homeodomain (HD) transcription factors, which in turn establish five discrete domains of progenitor cells in the ventricular zone through cross-repressive interactions (Jessell, 2000; Briscoe et al., 2000). The combinatorial action of these factors is thought to control the expression of a number of downstream genes encoding cell fate determinants, leading to the generation of specific neuronal types from each progenitor domain.

Pax6 is a HD protein involved in the establishment of progenitor domains in the ventral neural tube and in the specification of progenitors to particular cell fates. Pax6 expression is repressed by Shh signalling, resulting in a ventral^{low}-medial^{high} gradient of Pax6 protein in the spinal cord and its exclusion from ventral-most progenitors (Ericson et al., 1997). Subsequently, cross-regulatory interactions between *Pax6* and the HD gene *Nkx2.2* sharpen the boundary between the Nkx2.2-positive, V3 interneuron progenitor domain adjacent to the floor plate and the neighboring Pax6^{low} motor neuron progenitor domain (Briscoe et al., 2000). Analysis of mouse and rat embryos homozygous for the naturally occurring null mutation in the *Pax6* gene, *Small eye* (*sey*), has revealed that *Pax6* is required for the generation of the V1 and V2 subtypes of ventral interneurons and the correct specification of subsets of spinal and hindbrain motor neurons (Ericson et al., 1997; Takahashi and Osumi, 2002). However, no specific function has yet been ascribed to the distinct concentration gradient of Pax6 protein in the ventral spinal cord.

Strikingly similar regulatory interactions between HD genes are responsible for partitioning the telencephalic primordium into distinct territories (reviewed by Wilson and Rubenstein,

2000). Pax6 is also expressed in a graded manner in the dorsal telencephalon, reaching highest levels in a lateral and caudal domain of the cerebral cortex and gradually diminishing towards the medial-rostral cortex. Cross-regulatory interactions between *Pax6* and the HD gene *Gsh2* have been shown to establish the border between the cerebral cortex, dorsally, and the lateral ganglionic eminence, ventrally (Toresson et al., 2000; Yun et al., 2001). Pax6 has also been shown to control many properties of cortical cells, including the proliferation of cortical progenitors, their neuronal commitment, and the migration of newborn neurons (e.g. Stoykova et al., 2000; Muzio et al., 2002; Heins et al., 2002; Estivill-Torrus et al., 2002). Its graded expression has been implicated in the regionalization of the neocortex into distinct areas (Bishop et al., 2000).

The proneural genes that encode basic helix-loop-helix (bHLH) transcription factors, also play an important role in establishing the fates of neural progenitors (Kageyama and Nakanishi, 1997; Bertrand et al., 2002). Members of this gene family, which include Mash1, Math1 and the neurogenins, have the dual function of promoting the differentiation of individual progenitors, and of selecting the particular neuronal or glial lineage along which progenitors differentiate. In the spinal cord, the neurogenin gene *Ngn2* has been shown to promote cell cycle arrest and neuronal differentiation of neuroepithelial cells (Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001). *Ngn2* has also been shown to contribute to the specification of motor neuron progenitors, acting in conjunction with a major determinant of motor neuron fate, the bHLH protein Olig2 (Mizuguchi et al., 2001; Novitch et al., 2001). In the telencephalon, *Ngn*s have similar roles in neuronal commitment and specification of the identity of cortical progenitors (Fode et al., 2000; Nieto et al., 2001; Sun et al., 2001).

Proneural proteins are, like HD proteins, expressed in restricted progenitor domains, and cross-repressive interactions are similarly involved in establishing the sharp dorsoventral borders that separate these domains (Fode et al., 2000; Gowan et al., 2001) (reviewed in Bertrand et al., 2002). In the spinal cord, *Ngn3* is expressed in a ventral domain immediately adjacent to the floor plate, and *Ngn1* and *Ngn2* are expressed throughout most of the basal plate and in restricted domains of the alar plate, while *Mash1* is expressed in a large part of the alar plate and *Math1* is expressed in a dorsal domain immediately adjacent to the roof plate. In the telencephalon, *Mash1* is expressed at high levels in ventral progenitors and at reduced levels in a subset of dorsal progenitors, contrasting with the restricted expression of *Ngn*s in dorsal progenitors. In addition to this strict spatial regulation, there is recent evidence that the precise timing of proneural gene expression is important for the correct specification of progenitors. For example, it has been proposed that down-regulation of *Ngn2* expression in the motor neuron progenitor domain is involved in the transition from motor neuron to oligodendrocyte generation in this region of the spinal cord (Zhou et al., 2001a).

Despite the importance of these proneural expression patterns for the diversification of progenitor populations, little is known of how they are established. The restriction of proneural gene expression along the dorsoventral axis of the neural tube suggests an implication of inductive signals produced by dorsal and ventral organizing centers. Indeed, there is evidence that BMP signals simultaneously regulate the expression of proneural and HD proteins in the dorsal spinal cord (Timmer et al., 2002), and that Shh induces *Mash1* expression in the ventral telencephalon (Yung et al., 2002). In the ventral spinal cord, several factors, which are themselves regulated by Shh signalling, have been shown to control the expression of *Ngn* genes. *Nkx2.2* is required for the expression of *Ngn3* in a domain adjacent to the floor plate (Briscoe et al., 1999), and Olig2 regulates *Ngn2* expression in progenitors of motor neurons (Mizugushi et al., 2001; Novitch et al., 2001; Zhou and Anderson, 2002). In the cerebral cortex, *Ngn2* has been shown to be regulated by Pax6 (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Thus, regulatory interactions between patterning genes and proneural genes may be involved in coordinating the distinct genetic programs underlying the regional specification of progenitors and their lineage commitment.

To further elucidate the mechanisms controlling the spatial and temporal expression of *Ngn2*, we have initiated a study of the regulatory sequences of this gene and identified four distinct enhancer elements (Scardigli et al., 2001). These enhancers drive gene expression in subsets of the *Ngn2* expression domain, and together cover most of this domain. Interestingly, analysis of *Ngn2* enhancers in small eye mice revealed that the activity in the ventral spinal cord of one of the enhancers, named E1, requires *Pax6* function, probably explaining the role of Pax6 in regulating *Ngn2* expression in this domain (Scardigli et al., 2001). In contrast, Pax6 only has a minor role in the regulation of other *Ngn2* enhancers, thus explaining that much of *Ngn2* expression in the spinal cord is unaffected in *Pax6* mutants. In this work, we have further characterized the regulation of the E1 element by Pax6. We have specifically asked whether this interaction is direct, and whether Pax6 controls the spatial domain of activity of this *Ngn2* enhancer.

MATERIALS AND METHODS

EMSA and in vitro mutagenesis

EMSAs were carried out as previously described (Marquardt et al., 2001). Disruption of the Pax6 binding site in E1 was performed by in vitro mutagenesis using a QuickChangeTM Kit (Stratagene), as recommended by the manufacturer. Briefly, two primers that were complementary to the sequence of interest, carried the desired mutation and introduced a new *Spe*I restriction site, were used to PCR amplify the *E1hsplacZ* vector (Scardigli et al., 2001). The template was then eliminated by *Dpn*I digestion, the PCR-derived plasmid was transformed into *E. coli* and the presence of the mutation was identified by *Spe*I digestion. Replacement of low affinity Pax6 binding sequences by a consensus binding site (consE1.1 and consE1.2) was achieved by two rounds of PCR using internal oligonucleotides with the appropriate optimizing mutations in the E1.1 and E1.2 sequences and external oligonucleotides corresponding to the 5′ and 3′ end of the E1 element. A *Not*I restriction site was added at the end of the 5′ oligonucleotide and a *Spe*I site at the end of the 3′ oligonucleotide to allow cloning of the resulting PCR fragment into the *βglobinlacZ* vector (Yee and Rigby, 1993). The sequences of the oligonucleotides used in EMSA and for the in vitro mutagenesis experiments, are outlined below, with consensus positions in the Pax6 binding site (Epstein et al., 1994; Czerny and Busslinger, 1995) underlined and mutated positions in bold.

E1.1 (581+) 5′-TCATTCACGCCTAGAAGCAG –3′
mtE1.1 5′-TCA**CT**A**GTAA**C**G**AGAAGCAG-3′
consE1.1 5′-ACGC**A**T**GA**A**T**GCA**C**AGCCGGGTGGAGAAGG-3′
E1.2 (1066-) 5′-CTTTTTACGCTTTACTCCTG-3′
mtE1.2 5′-CT**AC**T**AGTAA**T**G**TACTCCTG-3′
consE1.2 5′-TAAAACAG**T**TTTTTTACGCTT**G**ACT**T**CT**C**GG-3′
E3.2 (824+) 5′-TGCTTCATGCATTATTTATC-3′

To generate the constructs *4×E1.1βglobinlacZ* and *4×E3.2βglobinlacZ*, 26 bp-long oligonucleotides corresponding to the E1.1 and E3.2 binding sites, with a *Bam*HI restriction site and a *Bgl*II site at either end, were hybridized, oligomerized, and cloned into the pKSBluescript vector (Stratagene). Inserts containing 4 copies of the oligonucleotide were selected and cloned as *Not*1-*Spe*1 fragments into the *βglobinlacZ* (BG*Z*A) vector (Yee and Rigby, 1993).

Generation, genotyping and analysis of transgenic and mutant mice

Transgenic mice were generated by standard procedures using fertilized eggs from FVBN mice, and founder animals were genotyped for the *lacZ* sequence by PCR as previously described (Scardigli et al., 2001). *PAX6YAC* transgenic mice [(Schedl et al., 1996) kindly provided by A. Schedl] were bred with *E1hsplacZ* transgenic mice. *PAX6YAC* transgenic embryos were identified by their eye phenotype, and *E1hsplacZ* embryos by X-gal staining**.** Embryos were dissected from the uterus in cold PBS and fixed at room temperature in 4% paraformaldehyde for 30 minutes to 1 hour depending on the stage. Whole-mount X-gal staining was performed as described (Beddington et al., 1989). After staining, some embryos were embedded in 1-2% agarose and vibratome-sectioned at 100 μm.

In ovo chick electroporation

In ovo electroporation of chick embryos was performed as described previously (Funahashi et al., 1999) using a BTX electroporator (Electro Square Porator, ECM 830), with the following parameters: 3 times 25 V square pulses of 30 mseconds. DNA was purified using a Maxiprep EndoFree kit (Qiagen) and injected into the neural tube of HH stage 10-12 (E1.5) embryos (Hamburger and Hamilton, 1992). 1-1.5 μl of reporter construct at a concentration of 2 μg/μl was injected, together with 0.2 μg of *CMVeGFP* plasmid (Clontech) as tracer, and in some experiments the same amount of *CMVPax6* construct (Marquardt et al., 2001). Either 6 hours or 48 hours after electroporation, GFP-positive embryos, identified with UV light under a dissection microscope (Leica MZFL3), were collected and analyzed by immunocytochemistry. At least four electroporated embryos were analysed in each experiment.

Immunohistochemistry

Mouse and chicken embryos were fixed in 4% paraformaldehyde at room temperature for 30 minutes to 2 hours depending on the stage, impregnated with 20% sucrose overnight, embedded in OCT compound (Tissue Tek), and cryosectioned at 10 μm. Double immunofluorescence experiments were performed as previously described (Scardigli et al., 2001) by simultaneous incubation with two primary antibodies. The following antibodies were used: mouse monoclonal anti-β-galactosidase (Promega), rabbit polyclonal anti-β-galactosidase (5 prime-3 prime, Inc.), rabbit polyclonal anti-GFP (Molecular Probes), rabbit polyclonal anti-Pax6 (Babco), mouse monoclonal anti-Pax6 (Developmental Studies Hybridoma Bank), rabbit polyclonal anti-cNgn2 [(Zhou et al., 2001) kindly provided by D. Anderson], rabbit polyclonal anti-Nkx6.1 and guinea pig polyclonal anti-Nkx6.2 [(Vallstedt et al., 2002) kindly provided by J. Ericson]. Alexa 488- and Alexa 594-coupled secondary antibodies were purchased from Molecular Probes. Whole-mount immunocytochemistry was performed on HH stage 13-15 chicken embryos collected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde at room temperature for 30 minutes, washed in PBS, and incubated overnight at 4°C with the appropriate antibody diluted in 0.1% Triton X-100, 3% bovine serum albumin and 10% fetal calf serum in PBS. Embryos were then extensively washed in PBS plus 0.1% Tween 20 and incubated overnight at 4°C with a secondary antibody. Embryos were then washed and flat mounted in AquaPolymount (Polysciences Inc.). Sections and whole-mount samples were analysed using a confocal microscope (Leica Sp1). 3 stacks of pictures were merged to generate images of sections and 10 stacks of pictures (representing 25 μm in thickness) were merged to generate images of whole-mount embryos.

RESULTS

The *Ngn2* enhancer, E1, is active in regions of the spinal cord and telencephalon that express high levels of Pax6

To examine the mechanism by which Pax6 regulates *Ngn2*, we focused on the enhancer element E1, whose activity in both the ventral spinal cord and the lateral telencephalon is entirely dependent on Pax6 function (Scardigli et al., 2001). The activity of E1 was revealed by the expression of the β-galactosidase (β-gal) protein in embryos carrying an *E1hsplacZ* transgene, in which E1 was inserted upstream of the *hsp68* minimal promoter driving the *lacZ* gene (Scardigli et al., 2001). The domain of E1 activity was compared with the expression of Pax6, which is distributed in gradients along the dorsoventral axis of the embryonic spinal cord and cerebral cortex (Ericson et al., 1997; Stoykova et al., 2000; Bishop et al., 2000). In double-labeled transgenic embryos at E10.5, βgal-positive cells were found exclusively within Pax6 expression domains, in both spinal cord and cerebral cortex (Fig. 1). Within these domains, βgal-positive cells are present in regions that express highest levels of Pax6 (Fig. 1C,F).

Pax6 is both necessary and sufficient to regulate *Ngn2* expression and activate the E1 element in the neural tube

We have previously shown that activity of the E1 enhancer in the spinal cord is restricted to the p1 and p2 progenitor domains and that it is almost completely abolished in *Sey* mutant embryos, which lack *Pax6* function (Scardigli et al., 2001). To determine if the expression of endogenous *Ngn2* is similarly dependent on *Pax6* activity in p1 and p2, as expected if E1 is the main regulatory element for *Ngn2* in these domains, we examined in detail β-gal expression in mice carrying a *lacZ* knockin allele of *Ngn2* (*Ngn2*^{KIlacZ}) (Scardigli et al., 2001). In wild-type embryos carrying the *Ngn2*^{KIlacZ} allele, β-gal expression was detected in cells in the p1 domain, marked by expression of Nkx6.2, and in the p2 domain, corresponding to the dorsal part of the Nkx6.1 expression domain (Vallstedt et al., 2001) (Fig. 2A,C), thus confirming that E1 activity in these domains reflects the expression of the endogenous *Ngn2* gene. In *Sey* mutant embryos carrying the same *Ngn2*^{KIlacZ} allele, βgal expression was not detected in the Nkx6.2-positive domains or in the dorsal part of the Nkx6.1-positive domain (Fig. 2B,D). Thus *Pax6* function is required for endogenous *Ngn2* expression in the p1 and p2 domains, suggesting that regulation of *Ngn2* in these domains relies mostly or exclusively on the E1 element.

To determine if Pax6 is not only necessary but also sufficient to activate E1, we performed gain-of-function experiments by co-electroporating Pax6 expression and E1 reporter constructs

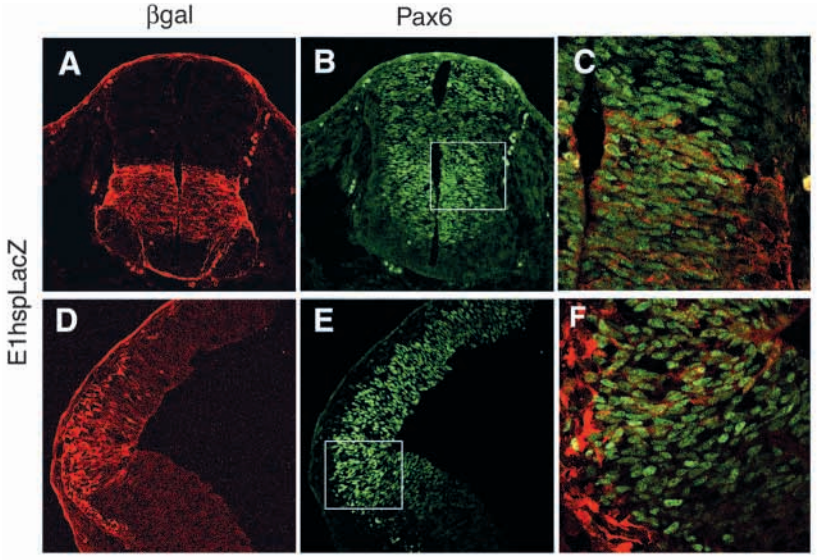

Fig. 1. Activity of the *Ngn2* enhancer, E1, is restricted to domains of the embryonic spinal cord and telencephalon expressing high levels of Pax6 protein. Double immunocytochemistry with an α-β-galactosidase antibody (red) and an α-Pax6 antibody (green), on transverse sections of spinal cord (A-C) and frontal sections of telencephalon (D-F) from an E10.5 mouse embryo transgenic for the *E1hsplacZ* construct. C and F show high magnifications of areas boxed in B and E, respectively, with merged α-β-gal and α-Pax6 staining. Activity of the E1 element is restricted to a ventromedial domain in the spinal cord and a lateral domain in the cerebral cortex.

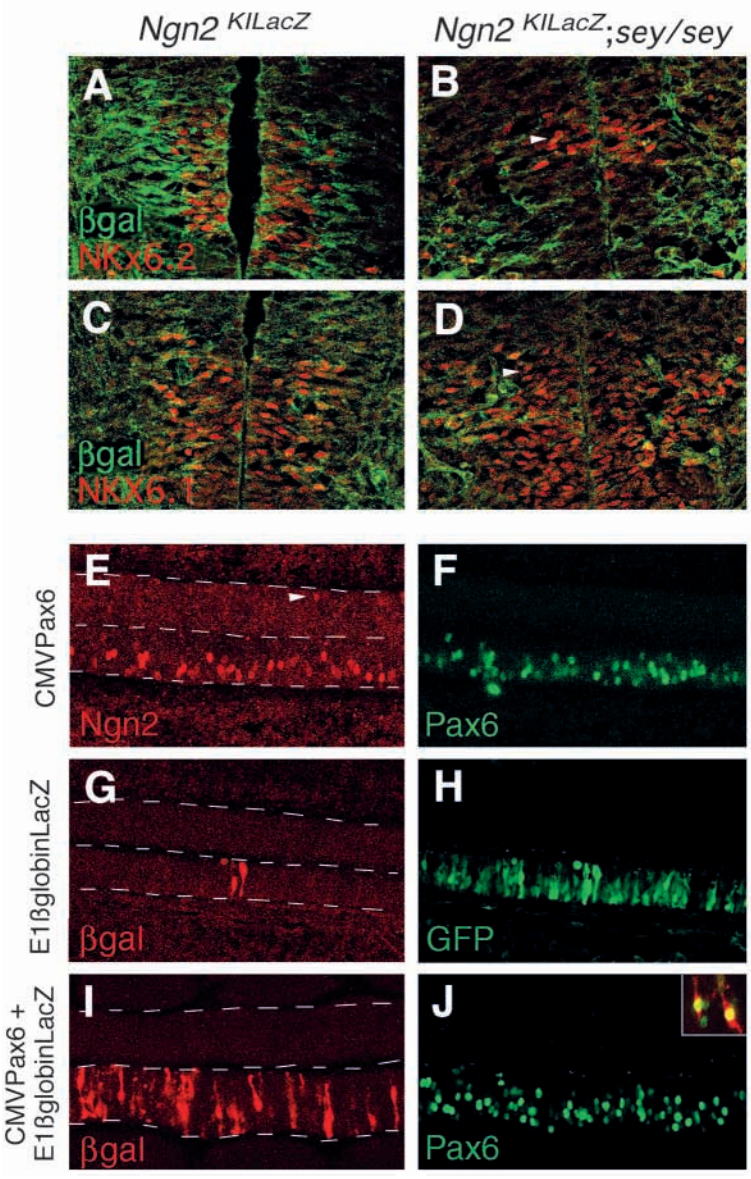

Fig. 2. Pax6 is both necessary and sufficient to regulate endogenous Ngn2 expression and activate the E1 enhancer. (A-D) Double immunocytochemistry with an α-β-galactosidase antibody (green) and an α-Nkx6.2 antibody (red, A,B), or an α-Nkx6.1 antibody (red, C,D), on transverse sections of spinal cord from E10.5 embryos, heterozygous for the *Ngn2*^{KIlacZ} allele, and either wild-type (A,C) or homozygous *Sey* mutants (B,D) at the *Pax6* locus. β-gal expression is down-regulated in Nkx6.2-expressing cells (p1 domain, arrowhead in B) and dorsal Nkx6.1-expressing cells (p2 domain, arrowhead in D). (E-J) Dorsal views of whole-mount chick neural tubes labelled for Ngn2 (E), Pax6 (F,J), β-gal (G,I) and GFP (H). Embryos were harvested 6 hours after being electroporated with a *CMVPax6* vector (E,F,I,J), an *E1βglobinlacZ* vector (G-J) or a *CMVGFP* vector (H). The electroporated side of neural tubes is at the bottom of the panels. Only a few Ngn2-positive and β-gal-positive cells are detectable at this stage (arrowhead in the unelectroporated side of the neural tube in E, and electroporated side in G, respectively), where endogenous levels of Pax6 are low (top in F). In the presence of high exogenous levels of Pax6 protein (bottom in F and J), the number of cells expressing endogenous Ngn2 (E) and activating the E1 element (I) is strongly increased. The inset in J shows two cells co-expressing β-gal and high levels of Pax6. Dashed lines in left panels outline the neural tube.

into the neural tube of chick embryos (Funahashi et al., 1999). Experiments were performed in 1.5 day-old embryos [E1.5; Hamburger and Hamilton (HH) stage 10-12 (Hamburger and Hamilton, 1992)], a stage when Pax6 is expressed at very low levels throughout the neural tube (Fig. 2F, top part of the neural tube corresponding to the non-electroporated side). At this stage, Ngn2, as revealed by immunocytochemistry, is only expressed in a few cells (Fig. 2E, arrowhead). To assess the activity of E1, HH stage 10-12 embryos were electroporated with a construct in which the E1 element was cloned in front of the basal βglobin promoter driving expression of *lacZ* (*E1βglobinlacZ*) (Scardigli et al., 2001). Six hours later (corresponding to HH stage 13-15 embryos), β-gal expression was detected in only a few scattered cells, indicating that E1 has little activity in the early neural tube (Fig. 2G; for this and all subsequent electroporation experiments, $n>4$). To determine if this is due to low level of Pax6 expression at this stage, a *CMVPax6* expression vector was co-electroporated with the *E1βglobinlacZ* construct. Six hours later, the number of β-gal-positive cells was clearly increased, as compared to electroporation of *E1βglobinlacZ* alone (compare Fig. 2I with 2G). Thus, ectopic expression of Pax6 is sufficient to activate the E1 element.

To determine if the regulation of E1 by exogenous Pax6 reflects a similar regulation of the endogenous *Ngn2* gene, the *CMVPax6* construct was co-electroporated with a GFP expression construct into the neural tube of HH stage 10-12 embryos, and Ngn2 expression was examined 6 hours later by immunocytochemistry. Increased level of Pax6 protein in electroporated cells correlated with a strong expression of Ngn2, which was not observed in the non-electroporated side (Fig. 2A,B), thus indicating that exogenous Pax6 protein is able to induce endogenous Ngn2 expression. Pax6 is thus a limiting factor for the activity of E1 as well as for the expression of endogenous Ngn2 in the early neural tube. Altogether, loss-of-function experiments (Fig. 2A-D) (Scardigli et al., 2001) and gain-of-function experiments (Fig. 2E-J) demonstrate that *Pax6* is both necessary and sufficient to activate the E1 enhancer and induce *Ngn2* expression in the embryonic neural tube. We next examined the molecular mechanisms underlying the regulation of E1 by Pax6.

A low affinity Pax6 binding site is present in the E1 enhancer

To determine if the regulation of *Ngn2* expression by Pax6 is direct or indirect, we searched for the presence of putative Pax6 binding sites. A sequence with high similarity to published consensus binding sequences was found in the E1 element. This sequence, designated E1.1, contains 11 nucleotides of the 16-nucleotide consensus binding sequence for the paired box of Pax6 (Epstein et al., 1994; Czerny and Busslinger, 1995). Putative Pax6 binding sites were also found in other *Ngn2* enhancers (Fig. 3A and data not shown). *Ngn2* enhancer elements contain blocks of sequence that are highly conserved between the mouse and human *Ngn2* genes (Scardigli et al., 2001). The E1 element has one block of 544 bp, situated between residues 63 and 607, that is 94% identical between the two species (Fig. 3A). The E1.1 sequence is located between residues 583 and 600, at the 3′ end of this block of homology, and 14/16 bp are conserved in the human sequence (Fig. 3A).

To determine if Pax6 protein binds to the different consensus sites found in *Ngn2* enhancer elements, we performed electromobility shift assays (EMSA) using 25 bp-long oligonucleotides with sequences corresponding to the putative binding sites and surrounding sequences (Marquardt et al., 2001) (see Materials and Methods). A recombinant Pax6 protein interacted with the different oligonucleotides containing putative Pax6 binding sequences, including the oligonucleotide containing the E1.1 sequence (Fig. 3B and data not shown). It is of note that formation of a complex with the E1.1 sequence required a higher amount of Pax6 protein than when another Pax6 binding site found in the *Ngn2* locus, E3.2, or the consensus Pax6 binding sequence were used, and even then, the amount of complex formed was lower (Fig. 3B, left panel). The E1.1 complex was dissociated in the presence of an antibody to Pax6 but not to Pax2, and recombinant Pax3 or Pax8 did not form complexes, demonstrating the specificity of the interaction of the E1.1 sequence with Pax6 (Fig. 3B, right panel). Taken together, these results show that Pax6 can interact in vitro with a canonical binding sequence present in the E1 element.

The above data indicate that the E1.1 sequence bind Pax6 in vitro, but with relatively low affinity. To determine if the E1.1 sequence can bind Pax6 protein in vivo and recruit it at a promoter, a concatemer of 4 copies of E1.1 was cloned in front of the basal *βglobin* promoter driving expression of *lacZ* (Yee and Rigby, 1993). The capacity of this construct, designated *4×E1.1βglobinlacZ*, to recruit Pax6 and activate the *βglobin* promoter, was compared with that of a similar construct containing a concatemer of 4 copies of the E3.2 sequence (*4×E3.2βglobinlacZ*). These two constructs were electroporated into the neural tube of HH stage 10-12 chicken embryos, which were tested for βgal expression 6 hours later. While the *4×E3.2βglobinlacZ* construct was efficiently activated in a large number of cells, the *4×E1.1βglobinlacZ* construct drove βgal expression in only very few cells (Fig. 3Ca,c), suggesting that *4×E3.2βglobinlacZ*, and not *4×E1.1βglobinlacZ*, can be activated by the low levels of Pax6 protein present in the early neural tube. To determine whether *4×E1.1βglobinlacZ* can be activated by higher concentrations of Pax6, the construct was co-electroporated with a *CMVPax6* expression vector. A significant increase in the number of βgal-positive cells was observed in this experiment when compared with the electroporation of *4×E1.1βglobinlacZ* alone (compare Fig. 3Ce with 3Ca), indicating that this construct can be activated by high concentrations of Pax6 protein. In contrast, the activity of the *4×E3.2βglobinlacZ* construct was not significantly enhanced when co-electroporated with *CMVPax6*. Together, this data indicates that the E1.1 sequence corresponds to a low affinity binding site for Pax6, while E3.2 is a site with higher affinity, thus confirming the results obtained in vitro (Fig. 3B).

The Pax6 binding site is required for the activity of the E1 element

The activity of the E1 element is known to be dependent on Pax6 (Scardigli et al., 2001) (Fig. 2) and we have identified a Pax6 binding site in E1 (Fig. 3), which suggests that occupation of this site by Pax6 may be important for E1 activation. To test this possibility, we disrupted the E1.1 site and tested the effect of this mutation on E1 activity in transgenic mice. Nucleotide

substitutions were introduced into the core sequence of the Pax6-binding site (see Materials and Methods), and the mutated E1 element (mtE1) was inserted in front of the basal *hsp68* promoter driving expression of *lacZ*. In E10.5 embryos carrying the *mtE1hsplacZ* transgene, β-gal activity was greatly diminished in the neural tube, in comparison with embryos carrying a wild-type E1 construct (compare Fig. 4B,D with 4A,C; *n*=4). However, mutation of the E1.1 sequence did not completely abolish E1 activity, even though E1 activity is completely dependent on *Pax6* function (Scardigli et al., 2001). The residual activity of mtE1 in the neural tube was observed at a dorsoventral position similar to that of wild-type E1 (Fig. 4D) (Scardigli et al., 2001). In contrast, mtE1 was completely inactive in the telencephalon, similar to what is observed with wild-type E1 in the absence of *Pax6* (Fig. 4B). Together, these results demonstrate that the Pax6 binding site present in E1 has

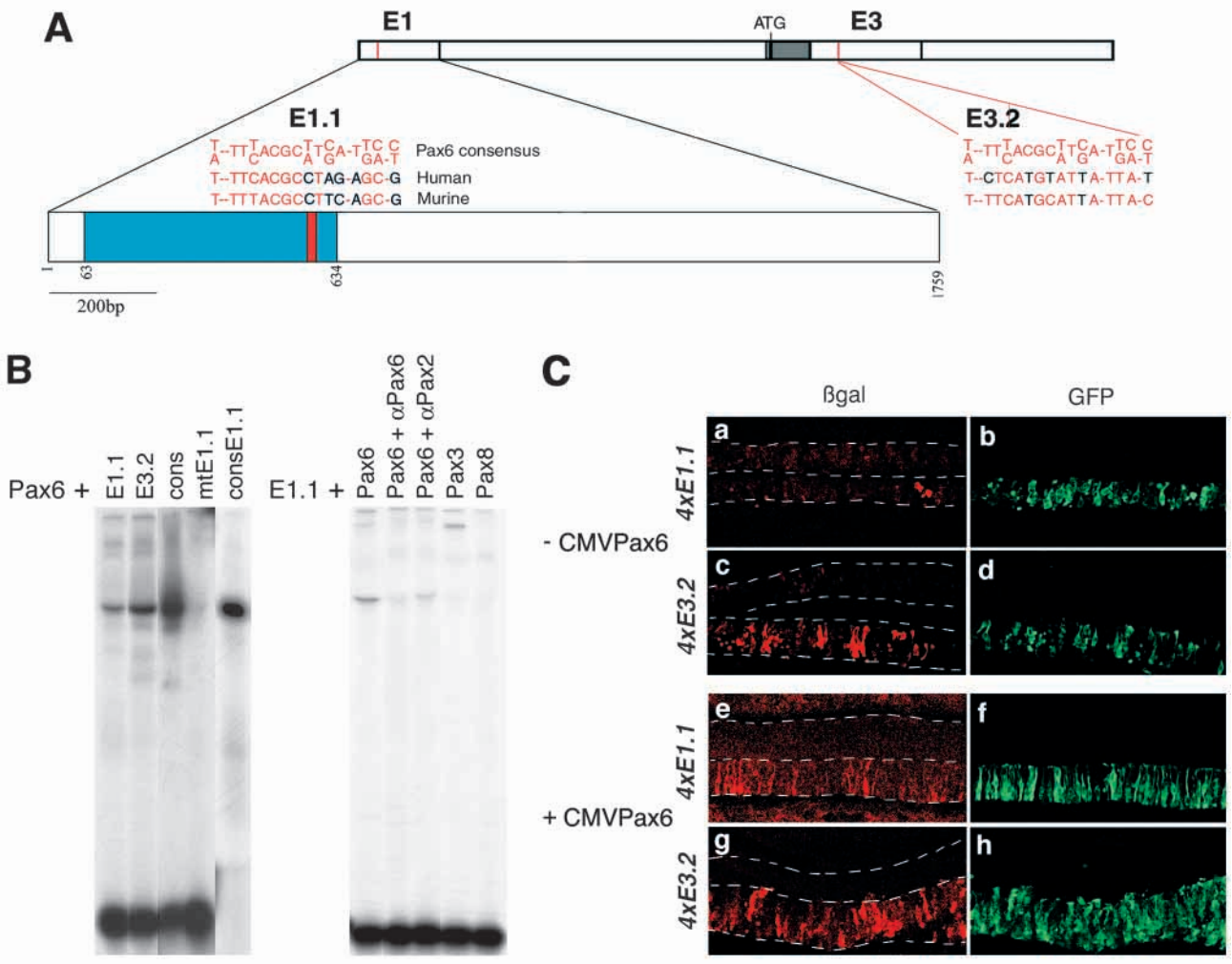

Fig. 3. Identification of Pax6 binding sites in *Ngn2* enhancers. (A) Schematic representation of the *Ngn2* locus showing the position of the E1 and E3 enhancers, the organization of the E1 enhancer, and the position of the E1.1 and E3.2 Pax6 binding sites, showing high similarity with a consensus binding sequence. The blue box represents a block of sequence in the E1 element showing high similarity (94%) between the murine and human *Ngn2* genes. The top sequences are the published consensus Pax6 binding site (Epstein et al., 1994; Czerny and Busslinger, 1995), the middle sequences are the Pax6 binding sites found in the human gene and the bottom sequences are the the same sites in the mouse gene. Red letters indicate conserved nucleotides between sequences in the *Ngn2* enhancers and the consensus binding sequence, and black letters indicate mismatches. (B, left panel) Electromobility shift assay performed with recombinant Pax6 protein and oligonucleotides containing the E1.1 and E3.2 sequences, the consensus Pax6 binding site as a positive control (cons), a mutated version of E1.1 as a negative control (mtE1.1), and an optimized version of E1.1 (consE1.1). Oligonucleotides corresponding to the sequences surrounding and including the E1.1 and E3.2 binding sites form a complex with Pax6 protein, but twice the amount of Pax6 protein was required to form a complex with E1.1 as compared to E3.2 or the consensus sequence. The smaller amount of complex formed with the E1.1-containing oligonucleotide suggests that the E1.1 sequence has a low affinity for Pax6. (B, right panel) The interaction of Pax6 and E1.1 is disrupted by incubation with an antibody to Pax6 but not to Pax2. Also Pax3 and Pax8 recombinant proteins do not form complexes with E1.1. The interaction of Pax6 with E1.1 is therefore specific. (C) Double labelling for β-gal (red, left panels) and GFP (green, right panels) on chick neural tubes 6 hours after electroporation with the constructs *4×E1.1-βglobinlacZ* (a,b,e,f), *4×E3.2-βglobinlacZ* (c,d,g,h) and *CMVPax6* (e-h). A *CMVGFP* vector was co-electroporated to control for transfection efficiency (b,d,f,h). The E3.2 concatemer efficiently drives β-gal expression in the early neural tube (c), where endogenous levels of Pax6 protein are low (see Fig. 1), while the E1.1 concatemer does not (a). Activity of the E1.1 concatemer is significantly enhanced in the presence of exogenous Pax6 protein (e), while activity of the E3.2 concatemer is not further increased. Dashed lines outline the neural tube.

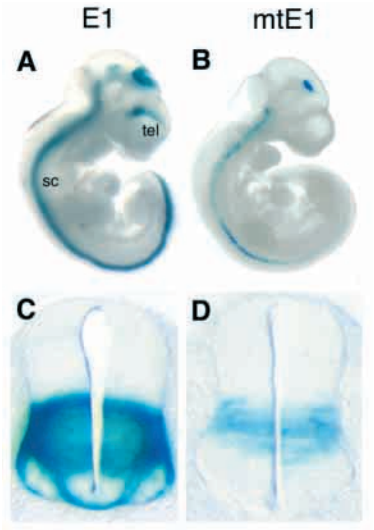

Fig. 4. Disruption of the E1.1 Pax6 binding sequence leads to a severe reduction of E1 enhancer activity. (A,B) Whole-mount X-gal staining of E10.5 mouse embryos carrying the control *E1hsplacZ* (A) and the mutated *mtE1hsplacZ* (B) transgenes. (C,D) Transverse sections through the same embryos at brachial levels. The mutation of the E1.1 sequence (B,D) leads to a severe decrease of transgene activity in the spinal cord (sc) and a complete loss of activity in the telencephalon (tel) as compared with the control transgenic embryo (A,C).

an essential role in governing the activity of the element in both spinal cord and telencephalon.

The size of the domains of activity of E1 depends on the level of expression of Pax6

The above data suggest that Pax6 activates E1 by directly binding to a conserved sequence present in this element. However, E1 is only active in vivo in regions where Pax6 reaches its highest concentration levels (Fig. 1). This suggests that E1 activity requires high levels of Pax6 expression, and that the borders of the domain of E1 activity are defined by a Pax6 concentration gradient. Alternatively, the restriction of E1 activity may be due to the requirement for another activator within the E1 domain, or the presence of a repressor in complementary regions. If the hypothesis that the Pax6 gradient is involved in E1 regulation is correct, then changing the concentration of Pax6 within its normal expression domain should be sufficient to modify the domain of activity of the enhancer. To examine the effect of increasing the concentration of Pax6 protein on E1 activity, the *E1βglobinlacZ* reporter construct was electroporated in the neural tube of HH stage 10-12 chicken embryos, and embryos were harvested 48 hours later. The time of harvesting corresponds to E3.5 or approximately HH stage 21-22, a stage of chick development equivalent to E10.5 in the mouse, by which time the dorsoventral gradient of Pax6 is established in the neural tube. In control experiments where *E1βglobinlacZ* was electroporated alone, 48 hours later β-gal expression was restricted to a medioventral domain of the chick spinal cord, similar to that observed in mouse embryos carrying an *E1hsplacZ* transgene (Fig. 4C, Fig. 5A). When a *CMVPax6* vector was co-electroporated with *E1βglobinlacZ*, β-gal was ectopically expressed by cells located outside this medioventral region but within the Pax6 expression domain. Double labeling for β-gal and Pax6 revealed that although these β-gal-positive cells were located in regions where Pax6 is normally expressed at low levels, they themselves expressed high levels of Pax6 from the electroporated *CMVPax6* vector (Fig. 5C-F). Thus, increasing Pax6 concentration is sufficient to activate E1 at ectopic locations in the Pax6 expression domain.

In another set of experiments, we used a transgenic mouse strain that carries multiple copies of a human YAC including the entire *PAX6* locus [designated *PAX6YAC* (Schedl et al., 1996)] to artificially increase Pax6 expression within its normal expression domain. The activity of E1 in this context was analyzed by crossing *E1hsplacZ* transgenic mice (Scardigli et al., 2001) with *PAX6YAC* mice. The progeny of this cross were harvested at E10.5 and E12.5 and analyzed for β-gal activity. A similar pattern of β-gal activity was observed in the spinal cord of embryos carrying the *E1hsplacZ* transgene, whether or not they also carried the *PAX6YAC* transgene (data not shown,

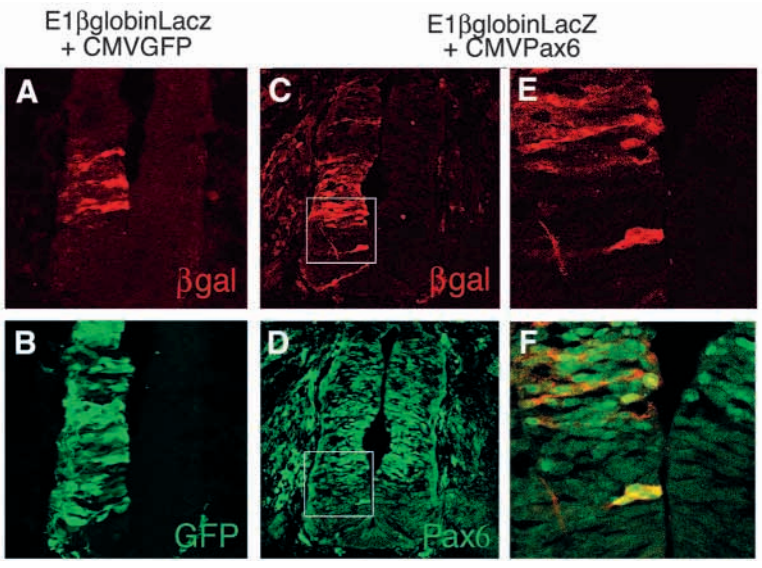

Fig. 5. Increasing Pax6 expression levels by electroporation induces ectopic activity of the E1 enhancer in the chick spinal cord. Labelling for β-gal (A,C,E,F), GFP (B) and Pax6 (D,F) on transverse sections of spinal cord, 48 hours after the electroporation of the *E1βglobinlacZ* construct (A-F), together with the tracer *CMVGFP* (A,B) or the *CMVPax6* construct (C-F). The activity of *E1βglobinlacZ* is restricted to the medioventral part of the spinal cord, in chick (A,B) as in mouse (Fig. 4C). High level of Pax6 protein delivered by electroporation (D) leads to ectopic activation of *E1βglobinlacZ* (C). E shows a higher magnification of the boxed area in C. F shows merged Pax6 and β-gal staining of the same enlarged area. Co-expression of β-gal and Pax6 shows that β-gal is induced in cells that belong to a domain of low Pax6 expression but that themselves express high Pax6 levels. Note that the α-Pax6 antibody used recognises both endogenous and exogenous proteins.

n=8). In contrast, the domain of β-gal activity was clearly expanded in the cerebral cortex of embryos carrying both the *E1hsplacZ* and *PAX6YAC* transgenes, when compared with embryos carrying *E1hsplacZ* alone (compare Fig. 6B,D,F with 6A,C,E; *n*=8). E1 activity in *E1hsplacZ; PAX6YAC* embryos was not restricted to the lateral cortex as in normal embryos, but had spread to a laterodorsal domain where Pax6 is normally only expressed at low levels (Fig. 6D,F). Thus, the size of the domain of E1 activity in the cerebral cortex depends on the level of expression of Pax6 in this region. Altogether, experiments carried out both in mouse and chick support the idea that the borders of the domain of activity of the E1 element are determined by the shape of the Pax6 gradient in the spinal cord and telencephalon.

The size of the domains of activity of E1 depends on the affinity of its Pax6 binding site

The E1 element contains a low affinity Pax6 binding site which is required for E1 activation (Fig. 4). Thus, a simple mechanism to account for the need for high concentrations of Pax6 to activate E1 (Figs 5 and 6), is that occupancy of this site can only take place when Pax6 reaches a sufficiently high concentration (see Fig. 3). Although other, more complex models can be invoked, such as a requirement for a cooperative interaction between Pax6 and other transactivators on the E1 element, we set out to test whether the response of E1 to particular concentrations of Pax6 protein is determined by the affinity of its binding site. One prediction of this hypothesis is that increasing the affinity of the site by modifying its sequence should allow E1 to respond to lower levels of Pax6, and thus expand E1 activity domains to sites where Pax6 expression levels are low. We thus modified the sequence of the E1.1 site to generate a site matching perfectly the published consensus Pax6 binding sequence (Epstein et al., 1994; Czerny and Busslinger, 1995) (see Materials and Methods). This E1.1 consensus sequence (consE1.1) has a higher affinity for Pax6 than the wild-type E1.1 sequence, as determined in a band shift assay (Fig. 3B, compare lanes E1.1 and consE1.1).

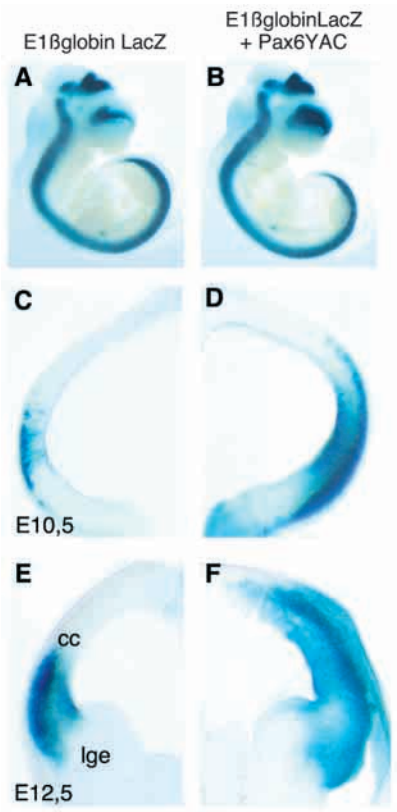

Fig. 6. The domain of E1 enhancer activity in the telencephalon is expanded when the dose of Pax6 is increased. (A,B) X-gal staining of E10.5 *E1hsplacZ* transgenic embryos in a wild-type background (A) and a *PAX6YAC* transgenic background (B). (C-F) X-gal staining of frontal sections of the telencephalon of E10.5 (C,D) and E12.5 (E,F) *E1hsplacZ* transgenic mice in the same genetic backgrounds. E1 activity is restricted to a lateral domain in the cerebral cortex (A,C), which extends ventrally up to the border with the lateral ganglionic eminence (E). In the presence of multiple copies of the human *Pax6* gene in *Pax6YAC* mice (Schedl et al., 1995), the domain of E1 activity is expanded both ventrally and dorsally (D,F). cc, cerebral cortex; lge, lateral ganglionic eminence.

The modified E1 element containing an optimized E1.1 sequence (consE1) was cloned in a *βglobinlacZ* vector to test its activity in chicken and mouse embryos. Constructs were electroporated into the neural tube of HH stage 10-12 chicken embryos, and first analyzed 6 hours later. As shown earlier, wild-type E1 drives β-gal expression in only a few cells at this stage because of the low endogenous level of Pax6 expression (Fig. 2G, Fig. 7A). The *consE1βglobinlacZ* construct was active in a larger number of cells (Fig. 7C). This result suggests that introduction of a high affinity Pax6 binding sequence into the E1.1 site results in efficient activation of the E1 element by the low level of Pax6 protein present in the early neural tube. To determine if a consensus Pax6 binding sequence could modify the activity of the E1 element, irrespective of where it was placed in the enhancer, we introduced this sequence into a different site (named E1.2) in the E1 element. The resulting mutated E1 element (named consE1.2) was cloned in the *βglobinlacZ* vector and its activity tested. The E1.2 site was chosen because, like E1.1, it contains a sequence with high similarity to the consensus Pax6 binding sequence. However, in contrast to E1.1, mutation of this sequence did not affect the overall activity of the E1 element (data not shown). The mutated consE1.2 element had very low activity in neural tubes harvested 6 hours after electroporation, similar to the wild-type E1 element (*n*=6; data not shown). This result suggests that the consensus Pax6 binding sequence must be inserted in an active Pax6 binding site in order to modify the response of the E1 element to Pax6.

To determine whether the *consE1βglobinlacZ* construct can also respond to low levels of Pax6 present in ventral and dorsal regions of the spinal cord at later stages, embryos electroporated at HH stage 10-12 where harvested 48 hours later. In control experiments, as expected, the *E1βglobinlacZ* construct was only active in a narrow medial domain of the spinal cord where Pax6 reaches its highest concentration (Fig. 1A-C, Fig. 7E), approximating the E1 domain in transgenic mouse embryos (Fig. 1A) (Scardigli et al., 2001). As predicted, the *consE1βglobinlacZ* vector was active in a broader domain that had expanded both dorsally and ventrally to regions expressing low levels of Pax6 protein (Fig. 7H,J). Thus, the consE1 element can be activated by low Pax6 concentrations because it contains a high affinity Pax6 binding sequence.

Together, these data suggest that the size of the E1 activity domain in the spinal cord, and specifically the position of its borders within the Pax6 concentration gradient, are determined by the affinity of a Pax6 binding site in E1.

We then examined whether the size of the E1 domain in the cerebral cortex is controlled by a similar mechanism. For this purpose, transgenic mouse embryos were generated with the *E1βglobinlacZ* and *consE1βglobinlacZ* constructs, and harvested at E11.5 and E12.5 to examine β-gal activity in the telencephalon. The domain of β-gal expression, which was restricted to the lateral cortex in all *E1βglobinlacZ* embryos examined (*n*=11; Fig. 8A,C,E) (see also Scardigli et al., 2001), was clearly expanded to the dorsolateral cortex in the majority of the embryos that carried the *consE1βglobinlacZ* constructs (2 out of 5 embryos examined at E11.5, and 4 out 6 embryos examined at E12.5; Fig. 8B,D,F). In the remaining embryos, the domain of β-gal expression was the same as in embryos carrying the control transgene (data not shown). Thus, as demonstrated in the chicken spinal cord, the presence of a high affinity Pax6 binding sequence at the E1.1 site results in activation of the E1 element in regions of the cerebral cortex where Pax6 concentrations are low. This indicates that the borders of E1 domain in the telencephalon are determined by the affinity of a Pax6 binding site.

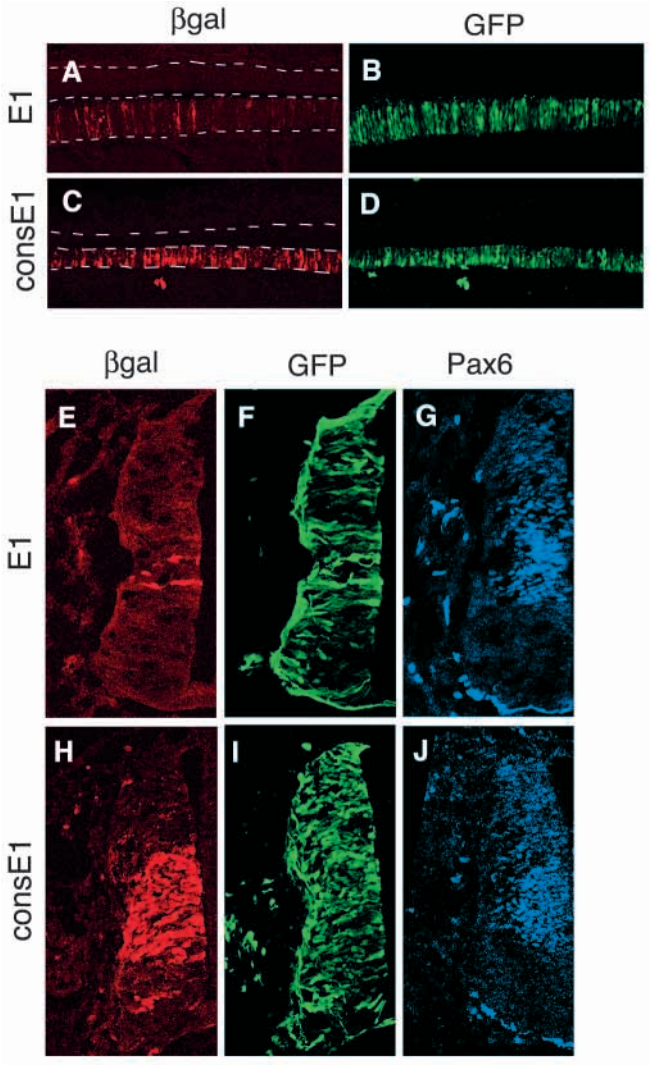

Fig. 7. Activity of the E1 enhancer is increased in the chick spinal cord when the low affinity Pax6 binding sequence has been replaced with a consensus binding site. Labelling for β-gal (A,C,E,H), GFP (B,D,F,I) and Pax6 (G,J), of chick neural tubes harvested 6 hours (A-D) or 48 hours (E-J) after electroporation with the constructs *E1βglobinlacZ* (A,B,E-G), *consE1βglobinlacZ* (C,D,H-J) and *CMVGFP* (B,D,F,I). In A-D, neural tubes are shown in dorsal views and the electroporated side is towards the bottom. Activity of the E1 element is low at this early stage (HH stage 13-15), and introducing a consensus Pax6 binding sequence at the E1.1 site significantly increases activity of the E1 element (C). The dashed lines outline the shape of the neural tube. In E-J, α-β-gal and α-GFP stainings were performed on the same transverse sections of spinal cord, and α-Pax6 staining on adjacent sections. Activity of the E1 element at this stage (HH stage 21-22) is confined to a medial domain of high Pax6 concentration (F,G), whereas the modified element *consE1* is active in a broader domain that includes cells expressing low Pax levels (I,J).

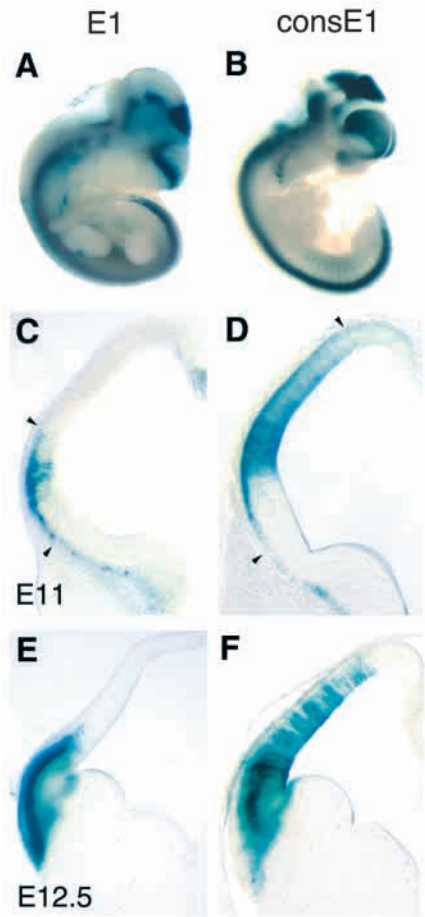

Fig. 8. Optimizing the sequence of the E1.1 binding site leads to an expansion of the domain of E1 activity in the cerebral cortex. X-gal staining of E11 transgenic embryos (A,B) and of frontal sections of the telencephalon of E11 (C,D) and E12.5 (E,F) embryos. Activity of the E1 element is restricted to the lateral cortex (A,C,E), and introduction of a consensus Pax6 binding sequence into the E1.1 site leads to an expansion of the activity of the element to a more dorsal domain (B,D,F). Arrowheads in C and D mark the dorsal and ventral limits of the E1 activity domain.

DISCUSSION

We have characterized the mechanism by which Pax6 regulates an enhancer of *Ngn2* in the ventral spinal cord and dorsal telencephalon, two regions of the embryonic CNS where *Ngn2* has a proneural role. We demonstrate that Pax6 is both necessary and sufficient to activate the E1 enhancer and that the strict dorsoventral borders of E1 activity are achieved through direct, low affinity interactions of Pax6 with a binding site in E1, resulting in the restriction of E1 activation to domains of high Pax6 expression. In this section we discuss the importance of the direct regulation of a proneural gene by a patterning gene, and the significance of concentration gradients and differential binding affinities cooperating to define gene expression patterns in the developing nervous system.

Direct regulation of the E1 enhancer by Pax6 involves binding to a single canonical site

Several studies have recently shown that Pax6 is a regulator of *Ngn2* expression in the presumptive cerebral cortex, and that activation of *Ngn2* is an important mechanism by which Pax6 specifies the dorsal fate of this territory (Fode et al., 2000; Stoykova et al., 2000; Toresson et al., 2000). We have extended these observations to another part of the embryonic CNS, the spinal cord, where we demonstrate that Pax6 is required for *Ngn2* expression in the p1 and p2 ventral progenitor domains (Fig. 2) (Scardigli et al., 2001). We provide several arguments supporting the idea that Pax6 regulates *Ngn2* expression in these two territories by directly binding to one of its enhancers, E1. First, the E1 element loses its activity in the lateral cortex and ventral spinal cord in a *Pax6* null mutant background, and reciprocally, forced expression of Pax6 in the neural tube leads to activation of E1 at ectopic locations within 6 hours of overexpression (Figs 2, 5) (see also Scardigli et al., 2001). Second, there is a single canonical and evolutionary conserved Pax6 binding site in the sequence of E1, and this site (named E1.1) binds Pax6 both in vitro and in neuroepithelial cells (Fig. 3). Finally, the specific disruption of E1.1 dramatically reduces the activity of E1 in the spinal cord and eliminates it altogether in the telencephalon (Fig. 4).

These data provide strong evidence that the interaction of Pax6 with its cognate binding site E1.1 is important for the activity of E1, but they do not exclude the possibility that additional mechanisms are involved. A second canonical Pax6 binding site (E1.2) is indeed present in the E1 sequence, but this site is unlikely to have a significant role in E1 activation, as it is not conserved in the human Pax6 locus, and its disruption does not affect the activity of E1 in the spinal cord or telencephalon (data not shown). However, E1 conserves a residual activity in the spinal cord when E1.1 is mutated, and this residual activity must also be Pax6 dependent since E1 is completely inactive in the ventral neural tube of *Sey* mutant embryos (Scardigli et al., 2001). Thus, Pax6 can weakly activate E1 without interacting with the E1.1 sequence, suggesting either that it binds weakly to non-canonical sequences in E1, as reported for the δ-cristallin gene (Kamachi et al., 2001), or that it can regulate E1 without directly binding DNA, possibly through interactions with other DNA binding factors.

There are multiple examples of Pax6 target genes that require synergistic interactions between Pax6 and co-factors in order to be efficiently transcribed (Simpson and Price, 2002). We do not know whether this is also the case for the regulation of the E1 element, but the very high conservation between mouse and man of a large block of sequence in which the Pax6 binding site is embedded (504 conserved nucleotides out of 534, see Fig. 3) strongly suggests that the activity of E1 involves binding of many factors other than Pax6. We have also provided evidence, from the comparison of the activity of the E1.1 and E1.2 sites, that the context of the Pax6 binding site in E1.1 is important. In particular, the activity of E1 can be increased or reduced by changing the affinity of the E1.1 sequence for Pax6, whereas similar manipulations of the E1.2 sequence have little or no impact on E1 activity (data not shown). Thus, the fact that the E1.1 site has an important role in E1 activity, and E1.2 does not, is not because E1.2 has a lower ability to recruit Pax6 to the enhancer. More likely, this reflects differences in the environment of E1.1 and E1.2 sequence such as the proximity to binding sites for co-factors with which Pax6 must interact to activate E1.

The same mechanism controls E1 activity in the spinal cord and telencephalon

A striking finding of this study is that the same mechanism is employed to control the expression of *Ngn2* in progenitor domains located in two distant regions of the embryonic CNS, the ventral spinal cord and the dorsal telencephalon. Similarities in the molecular mechanisms that pattern the spinal cord and telencephalon along their dorsoventral axis have been noted before, and include common inductive signals such as Sonic Hedgehog and bone morphogenetic proteins, related intrinsic determinants, including HD proteins of the Pax and Nkx families, and bHLH proteins of the Mash and Ngn families, and in particular the establishment by Pax6 of boundaries between adjacent progenitor domains, through cross-regulatory interactions with the HD proteins Nkx2.2 in the spinal cord, and Nkx2.1 and Gsh2 in the telencephalon (Wilson and Rubenstein, 2000; Briscoe and Ericson, 2001; Schuurmans and Guillemot, 2002). The activity of E1 in both spinal cord and telencephalon thus probably reflects a common role of Pax6 in these two territories. It must be noted however, that E1 is not active in all domains of high Pax6 expression [e.g. the retina) (Marquardt et al., 2001)], suggesting that regional determinants may act as co-factors, as discussed above, to constrain Pax6 function and restrict E1 activity along the anteroposterior axis of the neural tube.

We have also observed differences in how E1 is regulated in the spinal cord and telencephalon that are worth noting. In particular, both the introduction of a high affinity Pax6 binding sequence into the E1.1 site, and the analysis of E1 activity in the presence of increased dosage of Pax6, resulted in ectopic activation of E1 in the telencephalon, but not the spinal cord of transgenic mice. One explanation could be that the concentration gradients of Pax6 are different in these two territories, with a steeper Pax6 gradient in the spinal cord possibly limiting the expansion of E1 activity even with a modified element that responds to lower concentrations of Pax6. Alternatively, the increased Pax6 gene dosage in *PAX6YAC* mice, which carry 5 to 7 copies of the entire human Pax6 locus (Schedl et al., 1996), could be sufficient to modify the concentration gradient of Pax6 in the cerebral cortex but

not in the spinal cord, if different mechanisms controlling Pax6 expression levels operate in the two territories.

A direct regulatory link between neural patterning and neurogenesis

The generation of neurons by progenitors in the embryonic nervous system involves two distinct processes: the commitment of multipotent progenitors to a neuronal fate, resulting in their differentiation into neurons, and the specification of progenitors identity, resulting in the differentiation of neurons of a particular subtype. A number of studies suggest that these two processes are coupled at several levels. First, proneural bHLH genes, the major regulators of neuronal commitment in multipotent progenitors, are also involved in the specification of neuronal identity (Anderson, 1999; Brunet and Ghysen, 1999; Bertrand et al., 2002). In particular, proneural genes have been shown to control some aspects of the neuronal phenotype, such as the neurotransmission profile, through the regulation of downstream HD genes that directly activate genes encoding biosynthetic enzymes for neurotransmitters (Hirsch et al., 1998; Lo et al., 1998; Parras et al., 2002). Second, the regulation of the proneural genes themselves appears to be intimately linked with the regionalization of the neural tube, as these genes are expressed in restricted neuroepithelial domains with well-defined dorsoventral borders. Some of the genes that are involved in partitioning the neuroepithelium in dorsoventral progenitor domains have recently been shown to control the expression of proneural genes in these territories. For example, the HD protein Phox2b acts as a patterning gene to specify the identity of branchiomotor neuron progenitors in the hindbrain, and it simultaneously promotes the neuronal differentiation of these progenitors by upregulating the expression of the proneural genes *Ngn2* and *Mash1* (Dubreuil et al., 2002). A control of proneural gene expression by neural patterning genes has also been reported in *Drosophila* (e.g. Calleja et al., 2002). It is likely to be a general feature of neural development in both invertebrates and vertebrates.

This work provides the first demonstration that a proneural gene is directly regulated by a patterning gene in vertebrates, suggesting that neural patterning and neurogenesis may generally be tightly linked. It is likely that multiple patterning genes are involved in the generation of the complex expression patterns of proneural genes. Indeed, Pax6 is essential for the regulation of only one of the four known enhancer elements of *Ngn2* (Scardigli et al., 2001). Recent work suggests that in *Drosophila*, regulators of proneural genes act hierarchically rather than in a combinatorial manner, so that the number of direct transcriptional activators is actually very small (Calleja et al., 2002). Further studies are necessary to determine whether this holds true for vertebrate proneural genes.

The role of a Pax6 concentration gradient in the regulation of *Ngn2*

Our results demonstrate that the E1 element is regulated by high levels of Pax6 protein. This element is only active in domains of the spinal cord and telencephalon where the concentration of Pax6 reaches sufficient levels, i.e. a medioventral domain of the spinal cord, and a lateral domain of the cerebral cortex (Fig. 1). E1 can be ectopically activated in regions where Pax6 concentration is normally low but has been artificially raised either by electroporation of a Pax6 expression construct (Fig. 7), or by introduction of multiple copies of a yeast artificial chromosome containing the *Pax6* gene (Fig. 6).

Pax6 has therefore an essential role in determining the size of the domain of activity of one of the enhancers of *Ngn2*, and it may thus be involved in establishing borders of *Ngn2* expression, in particular at the sulcus limitans in the spinal cord and at the striatal-cortical border in the telencephalon. Although it is well established that Pax6 is an important regulator of neural cell fates (Ericson et al., 1997; Takahashi and Osumi, 2002), the significance of its non-uniform, graded expression along the dorsoventral axis of the spinal cord, has remained unclear. We present evidence that this gradient is involved in controlling the spatial pattern of expression of one of its targets, *Ngn2*.

The concentration gradient of Pax6 in the neocortex, from high rostrolateral to low caudomedial, has been shown to be important for its regionalisation in distinct areas, as shown by the analysis of Pax6 mutant embryos in which rostral cortical areas contract while caudal areas expend (Bishop et al., 2000). The HD protein Emx2 and the nuclear receptor COUP-TFI, are also distributed in gradients across the neocortex, and mutant analysis has similarly implicated these factors in regionalisation of this territory (Bishop et al., 2000; Mallamaci et al., 2000; Zhou et al., 2001b). How concentration gradients of transcription factors translate into discrete cortical areas having unique molecular, architectonic and functional properties is currently not known. Our results on the regulation of the E1 enhancer suggest that factors such as Pax6 and Emx2 could directly activate the expression of target genes involved in specification of area identity in restricted domains of the neocortex.

The role of the low affinity Pax6 binding site in establishing the domain of E1 activity

Our results support a model whereby the ability of the E1 element to only respond to high concentrations of Pax6 protein is due to the presence of a low affinity binding site occupied only when the concentration of Pax6 reaches a high level. The low affinity of the E1.1 sequence was demonstrated by the following observations. Compared with a consensus Pax6 binding sequence, the E1.1 sequence only forms a small amount of complex with recombinant Pax6 protein in vitro (Fig. 3). Moreover, 4 tandem copies of E1.1 cannot recruit enough Pax6 protein to efficiently activate a basal promoter in a context where Pax6 is expressed at low levels as in the early neural tube, whereas the same construct is activated by high levels of exogenous Pax6 protein (Fig. 3). Evidence that the low affinity of the E1.1 site underlies the property of the E1 element to respond solely to high Pax6 levels, is that increasing the affinity of this site results in an expansion of the E1 domain into regions of low Pax6 expression (Figs 6, 7).

In invertebrate species several examples are known of transcription factors activating only a subset of their target genes at a particular concentration. For example, the transcription factor PHA-4 has been shown to sequentially activate a number of pharyngeal genes in *C. elegans*, through the progressive increase in PHA-4 concentration during development, and the presence in target genes of binding sites with different affinities for PHA-4 (Gaudet and Mango, 2002).

Thus, the affinity of binding sites determines a temporal pattern of gene expression in this case, and a spatial expression pattern in the case of the interaction between Pax6 and E1. Other mechanisms, such as cooperative DNA binding, have been implicated in the establishment of gene expression patterns by gradients of transcription factors. Further study of the regulation of *Ngn2* should determine whether diverse strategies are similarly used to establish the complex expression patterns of proneural genes.

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