Pax6 defines the di-mesencephalic boundary by repressing En1 and Pax2

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SUMMARY

Transcriptional factors and signaling molecules are responsible for regionalization of the central nervous system. In the early stage of neural development, Pax6 is expressed in the prosencephalon, while En1 and Pax2 are expressed in the mesencephalon. Here, we misexpressed Pax6 in the mesencephalon to elucidate the mechanism of the di-mesencephalic boundary formation. Histological analysis, expression patterns of diencephalic marker genes, and fiber trajectory of the posterior commissure indicated that Pax6 misexpression caused a caudal shift of the di-mesencephalic boundary. Pax6 repressed En1, Pax2 and other tectum (mesencephalon)-related genes such as En2, Pax5, Pax7, but induced Tcf4, a diencephalon marker gene. To know how Pax6 represses En1 and Pax2, we ectopically expressed a dominant-active or negative form of Pax6. The dominant-active form of Pax6 showed a similar but more severe phenotype than Pax6, while the dominant-negative form showed an opposite phenotype, suggesting that Pax6 acts as a transcriptional activator. Thus Pax6 may repress tectum-related genes by activating an intervening repressor. The results of misexpression experiments, together with normal expression patterns of Pax6, En1 and Pax2, suggest that repressive interaction between Pax6 and En1/Pax2 defines the di-mesencephalic boundary.

Key words: Pax6, En1, Pax2, Di-mesencephalic boundary, Chick, In ovo electroporation

INTRODUCTION

Much attention has been paid to molecular mechanisms underlying the regionalization of the central nervous system. Regionalization of the optic tectum, which differentiates from the alar plate of the mesencephalon, has been well analyzed. In the early stage of chick embryos, En1 and Pax2 are expressed in the entire mesencephalon down to the isthmus (Gardner and Barald, 1992; Okafuji et al., 1999; Shamim et al., 1999). Misexpression of either En1 or Pax2 in the diencephalon results in the fate change of the presumptive diencephalon to the tectum (Araki and Nakamura, 1999; Okafuji et al., 1999). Mice mutant in either En1 or Pax2 show defects in the mesencephalon (Wurst et al., 1994; Favor et al., 1996; Schwarz et al., 1997, 1999; Urbán et al., 1997). Otx2 is expressed in the rostral neural tube down to the isthmus (Simeone et al., 1992; Bally-Cuif et al., 1995; Millet et al., 1996). Otx2 mutant mice lack the prosencephalon and mesencephalon (Acampora et al., 1995). Misexpression of Otx2 in the metencephalon changes the fate of the alar plate to the tectum (Broccoli et al., 1999; Katahira et al., 2000).

It is accepted that isthmic region acts as an organizer of tectum formation, and Fgf8 is thought to play a key role (Alvarado-Mallart, 1993; Marin and Pulles, 1994; Crossley et al., 1996; Joyner, 1996). An Fgf8-soaked bead implanted into the diencephalon causes transformation of the presumptive diencephalon to the tectum (Crossley et al., 1996). It was recently shown that Fgf8, Pax2/5 and En form the positive feedback loop for their expressions (Song et al., 1996; Lun and Brand, 1998; Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999; Shamim et al., 1999). This feedback loop may maintain the differentiated state of the tectum and contribute to its rostrocaudal polarity (Lee et al., 1997; Picker et al., 1999).

Otx2 is expressed in the prosencephalon and mesencephalon, while Gbx2 is expressed in the metencephalon (Nis and Leutz, 1998; Shamim and Mason, 1998; Hidalgo-Sanchez et al., 1999). The caudal limit of Otx2 expression corresponds to that of the tectum (Bally-Cuif et al., 1995; Millet et al., 1996). Misexpression of Otx2 in the metencephalon resulted in a fate change of the alar plate to the tectum (Broccoli et al., 1999; Katahira et al., 2000). In contrast, misexpression of Gbx2 in the mesencephalon caused rostral shift of the caudal limit of the tectum (Millet et al., 1999; Katahira et al., 2000). Since expression domains of Otx2 and Gbx2 overlap and Otx2 and Gbx2 repress each other’s expression, it was proposed that repressive interaction between Otx2 and Gbx2 may determine the caudal limit of the tectum (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000).

Pax6, which is expressed in the prosencephalon, is essential for the development of the diencephalon (Walther and Gruss 1991; Bally-Cuif et al., 1994; Stoykova et al., 1996, 1997; Grindley et al., 1997; Mastick et al., 1997; Warren et al., 1997). Pax6 mutant mice, Sey, show fate change of the pretectum, the diencephalon to the tectum (Crossley et al., 1996). It was the diencephalon causes transformation of the presumptive diencephalon to the tectum (Crossley et al., 1996).
caudal part of the diencephalon, to the mesencephalon (Mastick et al., 1997). En1 or Pax2 misexpression repressed Pax6 and caused the fate change of the dorsal diencephalon to the tectum (Araki and Nakamura 1999; Okafuji et al., 1999). We thus presumed that Pax6 represses En1 and Pax2 expression, and that repressive interaction between Pax6 and En1/Pax2 defines the rostral limit of the tectum. To prove this assumption, we ectopically expressed Pax6 in the mesencephalon by in ovo electroporation (Mastumatsu et al., 1997; Ogino et al., 1998; Funahashi et al., 1999; Momose et al., 1999). We showed that Pax6 misexpression repressed En1 and Pax2 expression and caused the fate change of the rostral tectal swelling to the diencephalon (pretectum). We also examined precise spatial and temporal expression patterns of Pax6, En1 and Pax2 in normal embryos. The results of Pax6 misexpression, together with normal expression patterns of Pax6, En1 and Pax2, suggest that repressive interaction of Pax6 and En1/Pax2 defines the di-mesencephalic boundary. Finally we showed that dominant-active Pax6 misexpression caused a similar but more severe phenotype than Pax6 misexpression. It is indicated that Pax6 acts as a transcriptional activator in the formation of the di-mesencephalic boundary.

MATERIALS AND METHODS

Pax6 expression vector

The full length chick Pax6 cDNA, which was kindly provided by Dr K. Yasuda, was inserted in pMiwIII, a derivative of pMiw (Suemori et al., 1990), which has Rous sarcoma virus enhancer and chicken β-actin promoter. Pax6 cDNA used in this experiment is the most prevalent isoform (Cvekl and Pattingrsky, 1996), which contains entire paired box, homeobox and S/T/P (serine-, threonine- and proline-enriched activation domain) but does not include a 14 amino acid insertion (5a) in the paired box.

The Pax6-VP16 construct is a fusion of a Pax6 fragment (corresponding to amino acids 1-311) and a VP16 fragment encoding the activation domain (Triezenberg et al., 1998). Pax6-EnR is a fusion of Pax6 (corresponding to amino acids 1-311), an En2 fragment (corresponding to amino acids 1-120, including the repressor domain), and HA-tag. These constructs were also inserted in pMiwIII.

Dil labeling

1,1'-dioctadecyl-3,3',3'-tetramethylindocarbo-cyanine perchlorate (Dil) was saturated in tetraglycol (Sigma) and injected by air pressure around the di-mesencephalic region.

In ovo electroporation

Fertilized chicken eggs from a local farm were incubated at 38°C. Pax6 expression vector (3.0 μg/ml) and the green fluorescence (GF) expression vector (pEGFP-N1, Clontech) (0.5 μg/ml) were transfected to chick embryos by in ovo electroporation as previously described (Funahashi et al., 1999). GF expression vectors were co-transfected to check the efficiency of electroporation. In ovo electroporation was carried out in the embryos at stage 7-8 or stage 10-11 (Hamburger and Hamilton, 1951). There were no differences in the phenotype between the embryos electroporated at stage 7-8 and stage 10-11. Most embryos were electroporated at stage 10-11 to obtain higher survival rate of the embryos.

In situ hybridization

Whole-mount in situ hybridization was performed as described by Bally-Cuif et al. (1995) or Stern (1998). In situ hybridization for sections was carried out as described by Ishii et al. (1997). Briefly, samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and immersed in PBS containing 20% sucrose overnight, and embedded in OCT compound (Tissue-Tek) to make cryosections. Probes for En1, Pax2, Pax5 and Pax6 were described previously (Funahashi et al., 1999; Okafuji et al., 1999; Araki and Nakamura, 1999). An approximate 1.4 kb fragment of Lim1, which covers the whole open reading frame was obtained by RT-PCR from E2.5 chick embryonic brains. A partial clone of chick Tcf4 was isolated from a cDNA library of E3 chick brain (GenBank accession number: AB040438). These fragments were inserted in pbLuescript II SK (+) (Stratagene). After linearization, digoxigenin (DIG)- or fluorescein isothiocyanate (FITC)-labeled antisense RNA was generated by T3 or T7 RNA polymerase (Funahashi et al., 1999). Alkaline phosphatase (ALP)- or digoxigenin (DIG)- conjugated anti-DIG or FITC goat polyclonal antibody (Roche Molecular Biochemicals) was used. For double in situ hybridization, Fast Red TR/Naphthol AS/MX (Sigma FASTTM; Sigma) was used for the detection of the first signal, and 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used for the second signal. ALP, used in the first detection, was inactivated by incubating with 100 mmol/l glycine-HCl (pH 2.2) for about 15 minutes at room temperature. In some cases, fast red staining was washed out in ethanol. NBT staining was washed out by incubating in dimethylformamide (DMF) at 55°C.

Immunohistochemistry

Anti-Pax6 rabbit polyclonal antibody kindly provided by Dr N. Osumi (Inoue et al., 2000), anti-Pax7 monoclonal antibody (Developmental Studies Hyridoma Bank, DSHB, Kawakami et al., 1997), anti-En1 monoclonal antibody, 4G11 (DSHB, Ericson et al., 1997), anti-En2 monoclonal antibody, 4D9 (American Type Culture Collection, Patel et al., 1989), and anti-GAP-43 monoclonal antibody, GAP-7B10 (Sigma) were used as the primary antibodies. Horseradish peroxidase (HRP)- conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was used as the secondary antibody. In double staining for Pax6 and En1 on sections, Alexa-488-conjugated anti-rabbit IgG antibody (Molecular Probes) and Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) were used as the secondary antibodies.

Histology

Embryos were fixed in 4% paraformaldehyde in PBS, embedded in Technovit (Kulzer), serially sectioned at 5 μm, and stained with hematoxylin-eosin. Tiling images were automatically composed by MCID Image analyzer (Imaging Research Inc).

RESULTS

Expression pattern of Pax6 and En1, Pax2

Pax6 is repressed when the diencephalon transdifferentiates into the tectum by En or Pax2 misexpression (Araki and Nakamura., 1999; Okafuji et al., 1999). Pax2/Pax5 double knockout mice show complete deletion of the mesencephalon, and caudal shift of Pax6 expression and the posterior commissure (Schwarz et al., 1999). Sey mutant mice show fate change of the prectectum to the mesencephalon resulting in defects in the di-mesencephalic boundary (Mastick et al., 1997).

In normal development, Pax6 expression is first detectable at the 2-somite stage (stage 7+) in the neural plate of the presumptive prosencephalic region (Li et al., 1994; Bally-Cuif et al., 1995; Stern, 1998). Expression of Pax6 is restricted to the presumptive prosencephalic territory and has been suggested to be involved in territory formation (Rowitch et al., 1995;
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Araki and Nakamura, 1999; Okafuji et al., 1999; Shamim et al., 1999). En1 mRNA is first detectable at the 3-somite stage in the presumptive mesencephalic region (Shamim et al., 1999). Pax2 expression is first detectable at the ventral portion of the prospective mes-metencephalic boundary at the 4-somite stage (Okafuji et al., 1999). We thus assumed that repressive interaction between Pax6 and En1/Pax2 played a pivotal role in boundary formation between the diencephalon and mesencephalon. To address this issue, we first examined normal expression patterns of Pax6, En1 and Pax2, paying special attention to their spatial relations.

At the 6- or 7-somite stage (stage 8-9), Pax6 transcripts were detected in the prosencephalon, while En1 and Pax2 mRNAs were detected in the mesencephalon. Analysis of whole mount and sections indicate that expression of Pax6 and En1/Pax2 overlapped around the di-mesencephalic boundary (Fig. 1A-C,E-G). To show more precisely the extent of overlapping expression, double staining for Pax6 and En1 was carried out on a section of an 8-somite stage embryo (Fig. 1K-M).

Superimposed images clearly show that the cells at the boundary region express both Pax6 and En1 (Fig. 1M, arrows). At stage 10, Pax6 expression was detected in the prosencephalon. En1 and Pax2 were expressed in the mesencephalon and rostral metencephalon in an almost overlapping manner, although Pax2 expression was regressing from the rostral mesencephalon. Serial sections of embryos at the 12-somite stage show that the expression domain of Pax6 and En1/Pax2 were segregated (Fig. 1H-J). Double staining for Pax6 and En1 confirmed that Pax6 and En1 expressions were segregated completely by the 11-somite stage (Fig. 1N-P).

To identify the position of the presumptive di-mesencephalic boundary in the early stage, we labeled a small population of cells at the boundary region (Fig. 1D, white arrow) and traced it until stage 12 (Fig. 1D’, white arrow). Dil labeling experiments clearly showed that the di-mesencephalic
boundary was formed at the site where Pax6 and En1/Pax2 expressions overlap (compare Fig. 1D with 1A-C).

Caudal shift of the di-mesencephalic boundary by Pax6 misexpression

To examine the role of Pax6 in di-mesencephalic boundary formation, we next misexpressed Pax6 in the presumptive di-mesencephalic region. Pax6 expression vector was transfected only in the right side of the neural tube from the diencephalon to the metencephalon in this study (Figs 2A, 5B). Pax6 misexpression caused caudal extension of the diencephalon (D, arrowheads). On the control side, the diencephalic structure consists of the thin neuroepithelial layer and the thick mantle layer and the rostral part of the tectal swelling consists of a thick neuroepithelial layer and two thin layers outside the neuroepithelial layer. On the experimental side, the rostral part of the tectal swelling consists of a thin neuroepithelial layer and thick mantle layer (D, arrowheads). (E-M) In situ hybridization for Lim1 (E-I) at E4.0 (HH23) and immunohistochemical staining for Pax7 (L-M) at E6.5 (HH 29). H, I and M are high power magnification micrographs of the area indicated in G and L. The area indicated by arrowheads in M is where Pax7-positive cells are detected in the mantle layer. (F,J) View from the experimental side, (E) view from the control side, (K) dorsal view. On the control side, Lim1-positive cells are found in the pretectum, whereas on the experimental side Lim1-positive cells are found in the rostral part of the tectal swelling (indicated by arrowheads in F and G). (H,M) These cells are in the mantle layer. (LM) In the pretectum, Pax7 is expressed in the neuroepithelial layer and mantle layer. (M) On the experimental side, the Pax7-positive mantle layer extends more caudally. Arrows indicate the approximate site of transition from the diencephalon to the mesencephalon, and arrowheads indicate caudal extension of the diencephalon. In all panels except for E, the experimental side is down and rostral is to the right. The lines in B, E and J indicate the approximate planes of D, G and L, respectively. Scale bars are 1.0 mm (B,F,J), 500 μm (D,G,L), 250 μm (M), and 100 μm (I). cont, control side; exp, experimental side; met, metencephalon; mg, marginal layer; ml, mantle layer; ne, neuroepithelial layer; tec, tectum.

(Fig. 2C). Histological examination, however, revealed that Pax6 misexpression indicated a caudal shift of the di-mesencephalic boundary (n=4/5) (Fig. 2D). At this stage, on the control side, the rostral part of the tectum consists of a thick neuroepithelial layer and two layers outside the neuroepithelium. The di-mesencephalon consists of a thin neuroepithelial layer, a thick mantle layer and a marginal layer. On the control side, the morphological transition from the diencephalon to the tectum corresponds well to the site where tectal swelling begins (Fig. 2D, arrow). In contrast, on the experimental side, the most rostral part of the tectum swelling

Fig. 2. Caudal shift of the di-mesencephalic boundary by Pax6 misexpression. (A-D) Morphology after Pax6 misexpression. Transfection was confirmed by GFP at 24 hours after electroporation (A), and the embryos were fixed at E5.5 (HH27) (B-D). Lateral view (B), dorsal view (C). Horizontal section stained with hematoxylin-eosin (D). Transfection occurred only in the right side of the neural tube from the diencephalon to the metencephalon (A). Pax6 misexpression caused caudal extension of the di-mesencephalon (D, arrowheads). On the control side, the di-mesencephalic boundary consists of the thick neuroepithelial layer and the thin mantle layer and the rostral part of the tectal swelling consists of a thick neuroepithelial layer and two thin layers outside the neuroepithelial layer. On the experimental side, the rostral part of the tectal swelling consists of a thin neuroepithelial layer and thick mantle layer (D, arrowheads). (E-M) In situ hybridization for Lim1 (E-I) at E4.0 (HH23) and immunohistochemical staining for Pax7 (L-M) at E6.5 (HH 29). H, I and M are high power magnification micrographs of the area indicated in G and L. The area indicated by arrowheads in M is where Pax7-positive cells are detected in the mantle layer. (F,J) View from the experimental side, (E) view from the control side, (K) dorsal view. On the control side, Lim1-positive cells are found in the pretectum, whereas on the experimental side Lim1-positive cells are found in the rostral part of the tectal swelling (indicated by arrowheads in F and G). (H,M) These cells are in the mantle layer. (LM) In the pretectum, Pax7 is expressed in the neuroepithelial layer and mantle layer. (M) On the experimental side, the Pax7-positive mantle layer extends more caudally. Arrows indicate the approximate site of transition from the diencephalon to the mesencephalon, and arrowheads indicate caudal extension of the di-mesencephalon. In all panels except for E, the experimental side is down and rostral is to the right. The lines in B, E and J indicate the approximate planes of D, G and L, respectively. Scale bars are 1.0 mm (B,F,J), 500 μm (D,G,L), 250 μm (M), and 100 μm (I). cont, control side; exp, experimental side; met, metencephalon; mg, marginal layer; ml, mantle layer; ne, neuroepithelial layer; tec, tectum.

Fig. 3. The effect of Pax6 misexpression on fiber trajectory of the posterior commissure. The embryo was fixed at E4.0 (HH23) and stained with anti-GAP-43 antibody. (A) Control side, (B) experimental side, (C) dorsal view. (A) is printed as the mirror image for comparison with B. Ectopic fibers are detected in the tectal swelling (B, arrowheads). Ectopic fibers in the rostral portion of the tectal swelling cross the roof plate (C, arrow), but fibers in the middle portion of the swelling curve caudally, to avoid the roof plate (C, arrowheads). pc, the posterior commissure. Scale bar, 0.5 mm (C).
consistent of a thin neuroepithelial layer, a thick mantle layer and a marginal layer, indicating that the structure of the diencephalon expanded caudally (Fig. 2D, arrowheads).

Caudal shift of the di-mesencephalic boundary after Pax6 misexpression was confirmed by its effect on Lim1 and Pax7 expression. Both genes are expressed in the pretectum so that En1 was repressed in the cells in which Pax6 was misexpressed, indicating that repression is cell-autonomous.

Next we looked at fiber trajectory of the posterior commissure. The posterior commissure is an important landmark for the di-mesencephalic boundary (Mastick et al., 1997), and distinguished by immunostaining with anti-GAP-43 antibody (Fig. 3A,B). M. Nakafuku, personal communication). On the experimental side, GAP-43-positive fibers were discerned ectopically on the rostral mesencephalon (Fig. 3B, arrowheads) (n=7/10). These ectopic fibers originated from the ventral diencephalon, curved caudally, and extended to the rostral mesencephalon. Some of these fibers crossed the roof plate in the rostral region of the tectum (Fig. 3C, arrow). Ectopic fibers near the middle region of the tectum could not cross the roof plate, and they curved caudally to keep a required distance from the roof plate (Fig. 3C, arrowheads). Fiber trajectory of the posterior commissure also indicates caudal extension of the diencephalon.

**Down-regulation of tectum-related genes and up-regulation of Tcf4 by Pax6 misexpression**

We have shown that Pax6 misexpression caused a caudal shift of the di-mesencephalic boundary. Since we assume that the di-mesencephalic boundary is set through repressive interaction between Pax6 and En1/Pax2, we further looked at the effect of Pax6 misexpression on En1 and Pax2 expression. In normal development, expression of En1 and Pax2 regresses caudally, and come to localize in the isthmic region by E2.5. We electroverted embryos at stage 7-8 and examined the effect before the repression of En1/Pax2 expression. Repression of En1 (n=7/8) and Pax2 (n=9/11) was clearly detected by 12 hours after electroporation (data not shown). To examine repression by Pax6 more precisely, we carried out double staining for Pax6 and En1. Repression of En1 was not detected at 6 hours after electroporation (n=3/3) (Fig. 4D,E), but detected at 12 hours after electroporation (n=3/3) (Fig. 4G,H). Double staining for Pax6 and En1 revealed that En1 was repressed in the cells in which Pax6 was misexpressed, indicating that repression is cell-autonomous.

Next, we analyzed effects of Pax6 misexpression on the tectum-related genes such as En2, Pax5 and Pax7. En2 is expressed in the tectum in a gradient which is high caudally and low rostrally. It has been shown that En confers caudal characteristics to the tectum (Itohaki et al., 1996; Logan et al., 1996; Shigetani et al., 1997). Pax5 is expressed at the isthmus, and is thought to be a factor that maintains the isthmic organizing activity (Funahashi et al., 1999). En2 (n=9/9), Pax5 (n=6/6) and Pax7 (n=7/7) were repressed by Pax6 misexpression (Fig. 5C,F,I). Higher magnification micrographs show no overlapping expression of Pax6 and marker genes for the mesencephalon, suggesting that the repression is in a cell-autonomous manner (Fig. 5B, 5E, 5H, 5I). Pax6 misexpression also repressed Wnt1 and EphrinA2 (data not shown).

The effects of Pax6 misexpression on Tcf4 expression was also examined. Tcf4 is expressed in the alar plate of the diencephalon in an early developmental phase, and a good marker for the dorsal diencephalon (Cho et al., 1998). At stage 17, on the control side Tcf4 is specifically expressed in the dorsal diencephalon (Fig. 5I). In the experimental side, Tcf4 expression was induced ectopically in the mesencephalon by 24 hours after electroporation (n=5/5) (Fig. 5L), although the induction was not detected at 12 hours after electroporation.
A dominant-active form of Pax6 caused more severe boundary shift

It was demonstrated that Pax6 can function as a transcriptional repressor as well as an activator (Duncan et al., 1998). To know whether Pax6 represses En1 and Pax2 directly or indirectly by activating some repressors in our system, we examined the effect of dominant-active and dominant–negative forms of Pax6 (Pax6-VP16, Pax6-EnR, respectively). In these constructs, the paired domain and homeodomain of Pax6 were fused with the activation domain of VP16 (Triezenberg et al., 1988) and the repression domain of En2 (eh1 domain) (Logan et al., 1992; Smith and Jaynes, 1996), respectively.

Dominant-active Pax6 misexpression induced a similar but more severe phenotype than Pax6 misexpression. At E5.5 (HH 27) (96 hours after electroporation), tectal expansion was smaller on the experimental side (Fig. 6B). The fate change of the rostral mesencephalon to the diencephalon was clear; the neuroepithelial layer on the experimental side in the rostral region of the tectal swelling was thinner than that on the control side (Fig. 6C, arrowheads) (n=4/4). Pax6-VP16 also induced Tcf4 expression (data not shown) and repressed En1 (n=5/5) and Pax2 (n=4/4) (Fig. 6D,E).

On the other hand, dominant-negative Pax6 (Pax6-EnR) misexpression induced an opposite phenotype to Pax6 misexpression: expansion of the size of the tectum (Fig. 6G,H), rostral shift of the di-mesencephalic boundary, and reduction of the pretectum area (n=3/3) (Fig. 6H, arrowheads). Pax6-EnR misexpression also caused rostral expansion of En1 (n=6/12) and Pax2 (n=5/9) expression (Fig. 6I,J).

**DISCUSSION**

We have shown that, (1) the expression domains of Pax6 and En1/Pax2 overlap around the presumptive di-mesencephalic region at an early stage, (2) the di-mesencephalic boundary is formed at the site where Pax6 and En1/Pax2 expression overlap, (3) Pax6 misexpression represses tectum-related genes, and causes a fate change of the rostral part of the tectal swelling to the pretectal tissue, and (4) dominant-active form of Pax6 misexpression causes a similar but more severe phenotype than wild-type Pax6 misexpression. The possible role of Pax6 in the formation of the di-mesencephalic boundary is discussed below.

**Caudal extension of the diencephalon by Pax6 misexpression**

We showed that Pax6 misexpression repressed the tectum-related genes. Moreover, histological examination suggested that the rostral part of the tectal swelling exhibited the structure of the diencephalon resulting from a caudal shift of the di-mesencephalic boundary. Labeling of the di-mesencephalic boundary by DiI after Pax6 misexpression showed that the expansion of the diencephalic character was due to the fate change of the anterior part of the mesencephalon, but not due to the increased proliferation of the diencephalic cells nor to the decreased proliferation of the mesencephalic cells (our unpublished data). The caudal shift of the di-mesencephalic boundary was more clearly shown by the change in expression patterns of the pretectum-specific molecules, Lim1 and Pax7.

**Fig. 5. Repression of En2, Pax5 and Pax7 and induction of Tcf4 by Pax6 misexpression.**

(A–C’) Double staining for Pax6 (blue) and En2 (brown), (D–F’) for Pax6 (red) and Pax5 (blue), (G–I’) for Pax6 (blue) and Pax7 (brown), and (J–K’) for Pax6 (red) and Tcf4 (blue). (B’,C’,E’,F’,H’,I’,K’) are high power magnification of boxed areas of (B,C,E,F,H,I,K), respectively. (B,E,H,K) View from the experimental side; (A,D,G,J) view from the control side. Arrowheads in B’,E’,H’ and C’,F’,I’ indicate the same position, respectively. To show repression clearly, the color for Pax6 was washed away in (C,F,I,L). In the Pax6-expressing cells, En2, Pax5 and Pax7 expression is repressed. (K’) Tcf4 expression is induced around the cells that misexpress Pax6 strongly. Arrowheads indicate cells that express Pax6 strongly. Arrows indicate Tcf4-expressing cells. Scale bars are 0.5 mm (C,F), 0.25 mm (I,L), 50 μm (B’,E’,H’) and 10 μm (K’).
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Fig. 6. Effects of dominant-active and -negative forms of Pax6. (A,F) Lateral view, (B,G) dorsal view. (C,H) Hematoxylin-eosin staining at E 5.5 (96 hours after electroporation). The lines in A and F are the approximate planes of C and H. (B,C) The dominant-active Pax6 caused a more severe phenotype than wild-type Pax6. (C) The rostral region of the tectal swelling is transformed to the pretectum-like tissue (arrowheads). The size of the tectum is markedly reduced. (H) Dominant-negative Pax6 misexpression caused reverse effects. The right-hand-side is the experimental side, and rostral is up. (D,E) Repression of En1 and Pax2 by the dominant-active Pax6. Whole-mount in situ hybridization for En1/Pax2 by the dominant-negative Pax6. Whole-mount in situ hybridization for En1 (I) and for Pax2 (J). Rostral limit of En1 and Pax2 expression domains (arrowheads in J) shifted rostrally on the experimental side. Embryos were fixed 12 hours after electroporation (D,E,I,J). The experimental side is down, and rostral is to the right. Scale bars are 1.0 mm (A,F), 500 μm (C,H) and 0.25 mm (D,E,I,J).

Fig. 7. A model of how the di-mesencephalic boundary is formed. (A) Pax6 is induced in the prosencephalon by some unknown factor. (B) En1 and Pax2 are induced presumably by the axial signal such as Fgf4. Expression domain of Pax6 and that of En1/Pax2 overlap around the di-mesencephalic boundary. (C) Repressive interaction of Pax6 and En1/Pax2 may determine the di-mesencephalic boundary. Pax6 and En1/Pax2 repressed each other, but the mechanism is different. Pax6 repressed En1/Pax2 indirectly through negative regulators. On the other hand, En1 repressed Pax6 directly (Araki and Nakamura, 1999). En1 and Pax2 are in a positive feedback loop and maintain their expression each other. (D) Expression domains of Pax6 and En1/Pax2 are segregated completely, and finally the di-mesencephalic boundary is formed.

Lim1, which is expressed in the mantle layer of the pretectum at E4.0 (HH23) in normal embryos, extended caudally to the rostral part of the tectal swelling. Pax7 is expressed both in the tectum and pretectum but the expression pattern is different. In the pretectum, Pax7 is expressed in the neuroepithelial layer and mantle layer. After Pax6 misexpression, the Pax7-positive mantle layer extended caudally into the rostral part of the tectal swelling. In addition, the posterior commissure, the trajectory of which is seen in the pretectum, extended caudally after Pax6 misexpression. All these results indicate that Pax6 repressed tectum-related genes resulting in the caudal shift of the di-mesencephalic boundary.

Expression of the tectum-related genes was repressed in the whole of the tectal region by Pax6 misexpression. But the fate change occurred only in the rostral part of the tectal swelling. A possible explanation is that the expression vector we used assures transient expression, so that repression of tectum-related genes may be transient. One example to support this assumption is that Pax7 was completely repressed at 24 hours after electroporation, but that its expression was almost restored by 48 hours after electroporation (data not shown). Therefore it may be plausible that expression of the tectum-related genes are re-organized by the organizing signal from the isthmus, so that only the rostral tectal swelling is transformed.

**The di-mesencephalic boundary is defined by interaction of Pax6 and En1/Pax2**

In the present study, Pax6 misexpression repressed En1/Pax2 and caused the fate change of the rostral tectal swelling to the pretectum. Previously we reported that either En or Pax2/5 misexpression caused the fate change of the presumptive diencephalon to the tectum by repressing Pax6 expression (Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999). Araki and Nakamura examined the time course of the Pax6 expression after En2 misexpression. Since Pax6 was repressed immediately after the translation product of En2 appeared, they concluded that En2 represses Pax6 directly. In Pax2/5 double knockout mice, Pax6 expression and the posterior commissure expands caudally (Schwartz et al., 1999). However, the fate of the pretectum is changed to the mesencephalon in Pax6 mutant mice (Mastick et al., 1997). Thus, repressive interaction between Pax6 and En1/Pax2 may define the di-mesencephalic boundary. This notion is very consistent with the expression patterns of Pax6 and En1/Pax2 in normal development. Expression of Pax6 and En1/Pax2
overlaps at the di-mesencephalic boundary region in the early stages, but the overlapping region gradually reduces during development, and their expression domains are completely segregated by the 11-somite stage.

A similar mechanism is likely to work in defining the position of the mes-metencephalic boundary. *Otx2* is expressed in the presumptive prosencephalon and mesencephalon, while *Gbx2* is expressed in the presumptive metencephalon. At first their expression domains overlap around the mes-metencephalic boundary, and then through mutual repressive interaction, the mes-metencephalic boundary is defined (Broccoli et al., 1999; Hidalgo-Sanchez et al., 1999; Millet et al., 1999; Katahira et al., 2000).

**Pax6 indirectly represses the fate of the tectum by activating repressors**

Pax6 is reported to function as a repressor of the β-crystalline gene in lens fiber cells (Duncan et al., 1998). In the present study, we have shown that expression of the tectum-related genes is repressed by Pax6. It is of great interest whether Pax6 represses these genes directly or indirectly via activating other repressor molecules. The paired domain and homeodomain genes are repressed by Pax6. It is of great interest whether Pax6 acts as an activator of intervening repressors. Thus, it is reasonable to assume that Pax6 acts as an activator around the di-mesencephalic boundary region in the early stage.

**CONCLUSION**

We propose the following process for the formation of the di-mesencephalic boundary. First *Pax6* expression commences at the 2-somite stage in the prosencephalic region (Fig. 7A). Then *En1* is expressed at the 3-somite stage, and *Pax2* expression commences at the 4-somite stage (Okafuji et al., 1999). *En1* and *Pax2* expression cover the whole of the mesencephalon at the 5-somite stage. *En1* expression may be induced by a signal, such as Fgf4, from the axial mesoderm (Shamin et al., 1999). At these steps, *Pax6* and *En1/Pax2* expression overlap around the di-mesencephalic boundary (Fig. 7B). Pax6 is strongly induced in the rostral part of the di-mesencephalic boundary, while *En1/Pax2* are strongly induced in the caudal part. Repressive interaction between *Pax6* and *En1/Pax2* may define the di-mesencephalic boundary. Negative regulators induced by Pax6 may repress mesencephalon-related genes. En is suggested to repress *Pax6* directly (Araki and Nakamura, 1999) (Fig. 7C). Through the repressive interaction, the di-mesencephalic boundary may be finally determined corresponding to the border of *Pax6* and *En1/Pax2* expression domains (Fig. 7D).

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