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Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate

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SUMMARY

Regionalization of a simple neural tube is a fundamental event during the development of central nervous system. To analyze in vivo the molecular mechanisms underlying the development of mesencephalon, we ectopically expressed Engrailed, which is expressed in developing mesencephalon, in the brain of chick embryos by in ovo electroporation. Misexpression of Engrailed caused a rostral shift of the di-mesencephalic boundary, and caused transformation of dorsal diencephalon into tectum, a derivative of dorsal mesencephalon. Ectopic Engrailed rapidly repressed *Pax-6*, a marker for diencephalon, which preceded the induction of mesencephalon-related genes such as *Pax-2*, *Pax-5*, *Fgf8*, *Wnt-1* and *EphrinA2*. In contrast, a mutant Engrailed, En-2(F51→E), bearing mutation in EH1 domain, which has been shown to interact with a co-repressor, Groucho, did not show the phenotype induced by wild-type Engrailed. Furthermore, VP16-Engrailed chimeric protein, the dominant positive form of Engrailed, caused caudal shift of di-mesencephalic boundary and ectopic *Pax-6* expression in mesencephalon. These data suggest that (1) Engrailed defines the position of dorsal di-mesencephalic boundary by directly repressing diencephalic fate, and (2) Engrailed positively regulates the expression of mesencephalon-related genes by repressing the expression of their negative regulator(s).

Key words: *Engrailed*, Transcriptional repressor, *Pax-6*, Mesencephalon, Di-mesencephalic boundary, In ovo electroporation, Chick

INTRODUCTION

Regionalization in the early development of the central nervous system that is the basis for the structure of mature brain has been studied intensely (Xu et al., 1995; Maconochie et al., 1996; Millet et al., 1999). *Engrailed* (*En*), homologue of *engrailed* (*en*) in *Drosophila*, is initially expressed along the entire anteroposterior axis in the early mesencephalon (Davis et al., 1991; Gardner and Barald, 1992; Shamim et al., 1999), but later there is a gradient in its expression (Gardner et al., 1988; Gardner and Barald, 1992). *en*/*En* genes encode homeodomain-containing transcription factors and have been shown to cause 'active' transcriptional repression in vitro (Han and Manley, 1993; Hanks et al., 1998). While *Drosophila* En protein has been shown to function as a transcriptional repressor in vivo (Schwartz et al., 1995; Smith and Jaynes, 1996; Serrano et al., 1997; Siegler and Jia, 1999), it has been demonstrated recently that *Drosophila* En can also function as a transcriptional activator with a cofactor Extradenticle (Exd) in vivo (Serrano and Maschat, 1998).

En has been shown to play a pivotal role in early development of mesencephalon; disruption of *En-1* causes severe defects in the mesencephalon (Wurst et al., 1994). Other mesencephalon-related or isthmus-related genes, *Pax-2*, *Pax-5*, *Fgf8* and *Wnt-1*, have also been shown to play important roles for the development of mesencephalon (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; Krauss et al., 1992; McMahon et al., 1992; Urbanék et al., 1994, 1997; Crossley et al., 1996; Favor et al., 1996; Lee et al., 1997; Schwartz et al., 1997; Lun and Brand, 1998; Funahashi et al., 1999; Okafuji et al., 1999). Misexpression of *Pax-2* or *Pax-5* or transplantation of Fgf8 bead in diencephalon resulted in the ectopic induction of a tectum (Crossley et al., 1996; Funahashi et al., 1999; Okafuji et al., 1999), which derives from dorsal mesencephalon in normal embryo.

Although the phenotype of *En* knockout mice indicates that *En* is essential for the development of mesencephalon, this could be because *En* is indispensable either for mesencephalon precursor cells to acquire mesencephalic fate, or for the growth/survival of the mesencephalic cells, or for both. To gain more detailed knowledge on the function of En in early development of brain, we employed in ovo electroporation to ectopically express En in the developing chick brain. We show that ectopic En resulted in rostral shift of di-mesencephalic boundary and converted the fate of diencephalic cells to mesencephalic fate. We also show that En immediately repressed *Pax-6*, a molecular marker for early diencephalon, which preceded ectopic induction of mesencephalon-related genes in diencephalon. Finally, we report the dominant negative effect caused by a mutated En-bearing mutation in

EH1 domain (Logan et al., 1992; Smith and Jaynes, 1996) or VP16-En chimeric protein. We discuss the roles of En in the early development of mesencephalon.

MATERIALS AND METHODS

Expression vectors

pMiwSV is a kind gift from Yoshio Wakamatsu (Tohoku University) and Hisato Kondoh (Osaka University), and pCA-GAP-GFP(S65A) is from Junichi Miyazaki (Osaka University) and Koki Moriyoshi (Kyoto University) (Wakamatsu et al., 1997; Niwa et al., 1991; Moriyoshi et al., 1996). Isolation of chick *En-1* and *En-2* cDNA was described previously (Itasaki and Nakamura, 1996). To make pMiw-En-1-HA and pMiw-En-2-HA, HA-tagged chick *En-1* and *En-2* cDNA were cloned into blunt-ended *Pst*I site of the multicloning site of pMiwSV (Fig. 1A), which has 'Miw promoter', consisting of chick β-actin promoter and RSV enhancer (Suemori et al., 1990). To make pMiw-En-2(F51→E)-HA (Fig. 1B), TTC, which corresponds to Phe51 in pMiw-En-2-HA, were substituted with GAG and subcloned into blunt-ended *Pst*I site of pMiwSV. To make pMiw-VP16-En-2(C133)-HA (Fig. 1B), VP16 activation domain was ligated with En-2(C133)-HA, which encodes C-terminal portion of chick En-2 from Leu133, using *Eco*RI linker, and subcloned between *Hin*dIII and *Bgl*II site of pMiwII, an improved version of pMiwSV with more convenient multicloning site. pMiwZ is a control vector bearing *lac*Z gene downstream of Miw promoter (Suemori et al., 1990).

In ovo electroporation

Conventionally raised chick eggs from local farms were incubated for appropriate period at 38°C. In ovo electroporation was performed as previously described (Funahashi et al., 1999) with some modification. Before electroporation, we usually injected DNA solution, which contains 1 μg/μl En (or its derivative) expression plasmid and 1 μg/μl GFP expression plasmid, into the lumen of neural tube. Embryos younger than stage 10 (Hamburger and Hamilton, 1951) were given three 50 mseconds 25 V rectangular pulses from an electroporator CUY21 (Tokiwa Co., Fukuoka, Japan) with electrodes CUY610 (ϕ, 0.5 mm; length, 1.0 mm; gap, 4 mm; Unique Medical Imada, Natori, Japan); older embryos were given four pulses. Embryos were fixed after an appropriate period of incubation at 38°C with 4% paraformaldehyde. Typical viability after the electroporation at stage 10 was about 80% at 24 h.a.e. (hours after electroporation) and was about 60% at 48 h.a.e.

At 1 h.a.e., there was no staining for ectopic En-2 on the experimental side (not shown). At 2 h.a.e., very faint staining for ectopic expression of En-2 protein became apparent and the intensity of the staining increased to near maximum level by 6 h.a.e. (Fig. 2A-C).

For GFP-tagging to diencephalic cells in Fig. 2F, we first introduced En expression plasmid without GFP expression plasmid into di-mesencephalic region. Immediately after that, we injected GFP expression plasmid, put the electrodes so that the GFP expression plasmid could be introduced into only diencephalon, and charged the pulses.

Detection of gene products

Most of fixed embryos were subjected to whole-mount in situ hybridization for analysis of the reaction of each gene to ectopic En-2. Whole-mount in situ hybridization was performed basically as previously described (Bally-Cuif et al., 1992), except that hybridization and washing were done at 65°C. After whole-mount in situ hybridization, embryos were immunostained with anti-En-2 monoclonal antibody 4D9 (Patel et al., 1989) and HRP (horse radish peroxidase)-conjugated anti-mouse antibody (Jackson, West Grove, PA, USA) as previously described (Funahashi et al., 1999). To distinguish the HRP immunostaining from the signal of whole-mount in situ hybridization, the color for in situ hybridization was destained after immunostaining. For destaining, embryos were washed in 100% methanol for 5 minutes twice, then incubated in dimethylformamide at 55°C for 3 hours. Chick *Pax-2* and *Pax-5* were isolated by PCR subtraction (Funahashi et al., 1999). Other probes for chick genes were isolated by genomic PCR (for *Wnt-1*) or RT-PCR with mRNA from embryonic day 3 chick embryos.

Histology and cytology

Some fixed specimens were embedded with Historesin (Leica, Heidelberg, Germany), serially sectioned at 5 μm, and stained by Haematoxylin and Eosin. Each photograph taken at high magnification was automatically composited by a tiling program, MCID (Imaging Research Inc., St. Catharines, Ontario, Canada). Nile blue staining was performed as previously described for detection of both necrosis and apoptosis (Jeffs and Osmond, 1992).

RESULTS

En caused rostral shift of di-mesencephalic boundary

On the experimental side of the embryo at 7 days after electroporation of En-2 expression plasmid, the di-mesencephalic boundary shifted rostrally and the tectal swelling expanded rostrally at the expense of caudal part of diencephalon (Fig. 2D-D‴, arrow). There was no increase in cell death in the diencephalon on the experimental side at 24 h.a.e. or at 48 h.a.e. (Fig. 2E,E′ and data not shown). We tagged diencephalic cells with GFP (Fig. 2F). The tagged region expanded by 48 h.a.e. and expressed *EphrinA2* (Fig. 2F′-F‴),

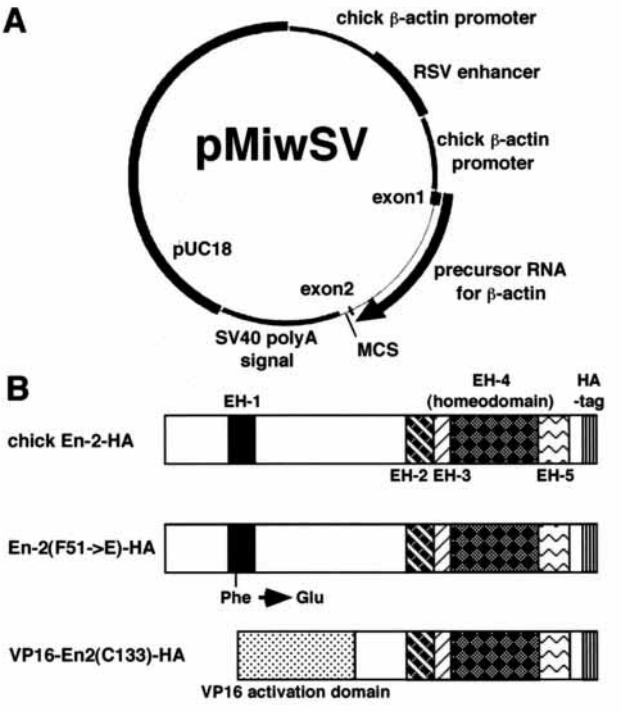

Fig. 1. The expression vector (*A*) and structure of chick En-2 and its derivatives (*B*) used in this study. Although all the data presented in this paper have been obtained with HA-tagged chick En or its derivatives, wild-type chick En-2 without HA-tag showed the same phenotype (not shown).

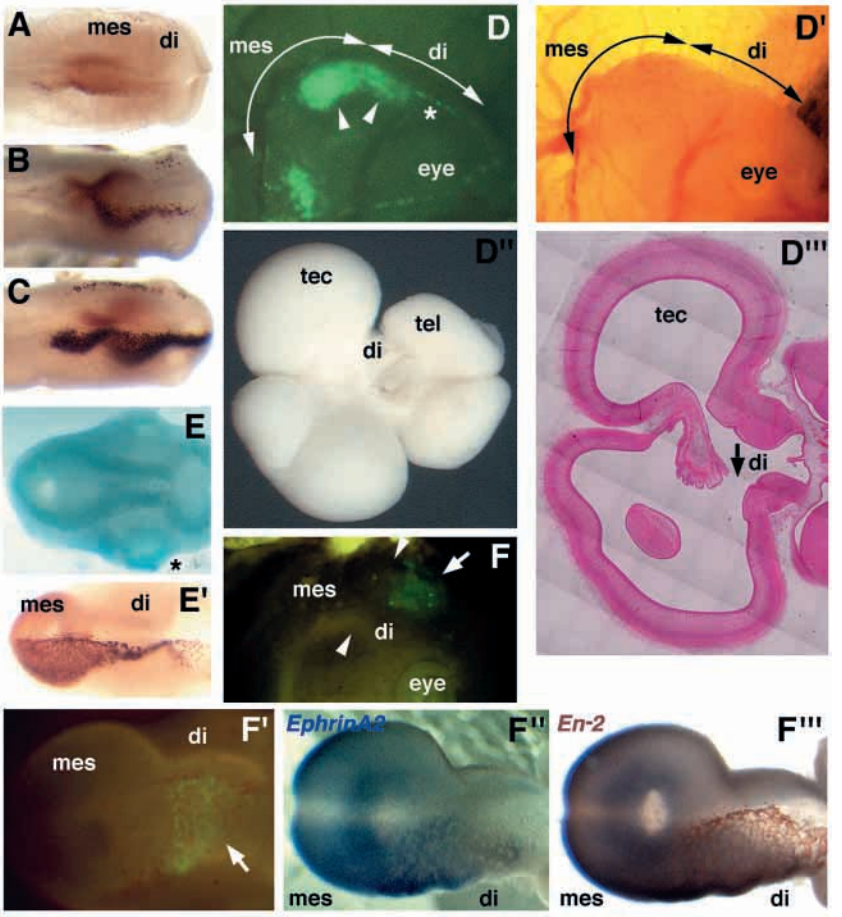

Fig. 2. Ectopic En-2 caused rostral shift of di-mesencephalic boundary and conversion of the cell fate of diencephalic cells into mesencephalic fate. (A-C) Expression of En-2 protein at 2 hours (A), 3 hours (B) and 6 hours (C) after electroporation. (A) At 2 h.a.e., weak ectopic chick En-2 (brown staining) was first detected (experimental side on the right). Symmetrical weak staining corresponds to endogenous En-2. The dispersed expression in the ectoderm of the control side was due to leakage of the plasmid solution from the neural tube. Although such leakage was observed in some embryos, the expression in ectoderm showed no effect on the development of the brain. (D-D‴) Ectopic En in the diencephalon can induce rostral shift of di-mesencephalic boundary and rostral expansion of mesencephalon at the expense of diencephalon. The embryo with ectopic En-2 in the diencephalon was fixed at 7 days after electroporation. (D) GFP fluorescence at 24 h.a.e. (D′) Bright-field photograph of D. To correlate the initial distribution of the electroporated plasmid in a living embryo and the morphological change at later stage of the same embryo, we co-electroporated pMiw-En-2-HA with pCA-GAP-GFP(S65A), a green fluorescence protein (GFP)-expression vector (Moriyoshi et al., 1996). Arrowheads show that the electroporated construct is expressed in the posterior diencephalon and the anterior mesencephalon. Patchy fluorescence in the extraembryonic tissue is marked with asterisk. (D″) 7 days after electroporation; (D‴) horizontal section of D″. Rostral shift of the di-mesencephalic boundary and rostral expansion of the mesencephalon at the expense of diencephalon is evident (arrow). (E,E′) There is no specific induction of cell death with ectopic En-2 (brown), illustrated with Nile Blue staining (light-blue staining). The embryo in E is slightly tilted to show normal cell death in one of the lenses (asterisk; García-Porrero et al., 1979) for the positive control. (F-F‴) Ectopic En-2 caused the conversion of cell fate in the prospective diencephalon to a mesencephalic one. Immediately after electroporation of En-2 expression plasmid, GFP expression plasmid was electroporated into the diencephalon. (F) Fluorescence (arrow) in the diencephalon at 24 h.a.e. Arrowheads shows the di-mesencephalic boundary. (F′) The fluorescence (arrow) at 48 h.a.e. (F″) The ectopically induced *EphrinA2* (blue staining) overlaps the GFP fluorescence in F′. (F‴) Ectopically expressed En-2 (brown staining). Electroporation was performed at stage 10 (Hamburger and Hamilton 1951) for A-C,E,F and stage 13 for D. Each set of photographs marked with same alphabet with/without primes is the same embryo in all figures. All embryos in this figure are oriented with rostral to the right. di, diencephalon; mes, mesencephalon; tec, tectum; tel, telencephalon.

a marker for dorsal mesencephalon (Flenniken et al., 1996; Logan et al., 1996; Shigetani et al., 1997). This suggests that ectopic expression of En-2 in the prospective diencephalon caused the cell fate of the prospective diencephalon to convert into a mesencephalic fate. The same phenotype was obtained after ectopic chick *En-1* misexpression (not shown; see also Shamim et al., 1999). Because *En-1* and *En-2* have been shown to be functionally redundant in the embryonic brain (Hanks et al., 1995) and an excellent anti-En-2 antibody is available (Patel et al., 1989), we used chick *En-2* for further experiments. pMiwZ, a control plasmid (Suemori et al., 1990), caused no morphological change in the brain (not shown).

To give more molecular support to this hypothesis that En causes transformation of diencephalon into mesencephalon, we looked at the effect on other molecular markers for diencephalon and mesencephalon. In the diencephalon of the experimental side, ectopic expression of *Pax-7*, another marker for the tectum (Kawakami et al., 1997), was induced (Fig. 3A-A″). On the contrary, *Pax-6*, a marker for diencephalon (Bally-Cuif and Wassef, 1994; Li et al., 1994), was repressed by ectopic En-2 (Fig. 3B-D′). Interestingly, *Pax-7* and *EphrinA2* were not induced in the ventral diencephalon (Fig. 3A,A′, arrows, and data not shown), although *Pax-6* was repressed even in the ventral diencephalon (Fig. 3D,D′). As *Pax-7* and *EphrinA2* are not expressed in ventral mesencephalon in normal embryos, this result suggests that ectopic En-2 did not perturb dorsoventral polarity of diencephalon.

Ectopic En-2 also caused a change in mesencephalic morphology; narrowing of the posterior mesencephalon (Fig. 3E,E′), which became apparent at 24 h.a.e. (Fig. 3H′, arrow). Since *Pax-2* (Fig. 3H,H′), *Fgf8* (not shown) and *Wnt-1* (Fig. 3E,E′), molecular markers for the isthmus, were ectopically expressed in the ectopic En-2-positive region, the isthmic activity that narrows the neural tube may have shifted rostrally and caused such a morphological change.

Repression of Pax-6 preceded the induction of mecencephalon-related genes

To investigate the interactions of the genes after misexpression of En-2, we analyzed the time course of their repression/induction after electroporation. It was difficult to detect clear repression of *Pax-6* by ectopic En-2 at 2 h.a.e. (not shown) partly because *Pax-6* expression in early diencephalon has weak unevenness (see control side in Fig. 3B,C), and partly because the number of ectopic En-2-positive cells is small at this stage. At 3 h.a.e., repression of *Pax-6* could be detected (Fig. 3B,B′), so repression occurred less than 2 hours after ectopic expression of En-2 protein since detectable ectopic expression of electroporated En-2 begins between 1 and 2 h.a.e. (Fig. 2A). At 6 h.a.e., the repression was much clearer (Fig. 3C,C′). On the contrary, induction of *Pax-2*, *Pax-5* or *Fgf8* was not observed in the diencephalon before 18 h.a.e. (Fig. 3G,G′ and data not shown). Thus, repression of *Pax-6* is an event independent of the ectopic induction of mesencephalon-related genes (isthmus/tectum-related genes) in diencephalon after En-2 misexpression.

In the mesencephalon, their ectopic induction was first observed at 9 h.a.e. (Fig. 3F,F′ and data not shown). The dorsal midline was devoid of induction of *Pax-2*, *Pax-5* and *Fgf8* even when ectopic En-2 was expressed over the dorsal midline (Fig. 3G,G′ and data not shown). Around 24 h.a.e. the ectopic expression of *Pax-2*, *Pax-5* and *Fgf8* reached maximum level in both diencephalon and mesencephalon (Fig. 3H,H′,I,I′). Their ectopic expression in the diencephalon and mesencephalon disappeared by 48 h.a.e. except for ectopic *Fgf8* near isthmus (data not shown). The onset of ectopic *EphrinA2* expression in the diencephalon and mesencephalon induced by En-2 corresponded to that of *Pax-2*, *Pax-5* or *Fgf8* (data not shown). In contrast to the transient expression of isthmus-related genes, ectopic expression of tectum-related genes such as *EphrinA2* and *Pax-7* in diencephalon was observed as late as 60 h.a.e. (Fig. 3A-A″ and data not shown), which is reminiscent of its continuous expression in normal mesencephalon (Logan et al., 1996; Kawakami et al., 1997; Shigetani et al., 1997). Ectopic induction of *Wnt-1* was first detectable at 9 h.a.e. around the endogenous *Wnt-1*-positive chevron-shaped domain in dorsal prosomere 2 (P2) (see Rubenstein et al. (1994) for the nomenclature), which seems to be susceptible to express *Wnt-1* ectopically (arrows in Fig. 3J,J′; Sugiyama et al., 1998). The ectopic *Wnt-1* in the mesencephalon or prosomere 1 (P1) was first detected at 18 h.a.e. (Fig. 3K,K′), and it lasted until 48 h.a.e. (Fig. 3L,L′). In diencephalon, the induction of *Wnt-1* began earlier than that of *Pax-2*, *Pax-5* or *Fgf8* (compare Fig. 3F,F′ and J,J′; data not shown). This may be a reflection of the fact that *Wnt-1* is endogenously expressed in early diencephalon. Time-course analyses are summarized in Fig. 4.

While all mesencephalon-related genes analyzed in this study were induced ectopically in diencephalon by En-2 by 18 h.a.e., obvious morphological differences in the diencephalon between the experimental side and the control side were not recognized at 24 h.a.e. (Fig. 3H,H′,I,I′). At 48 h.a.e., rostral shift of di-mesencephalic boundary and rostral expansion of tectal swelling was observed on the experimental side (Fig. 3L,L′).

Dominant negative effect by mutated En

The fact that En can function as both transcriptional repressor and activator in *Drosophila* (Schwartz et al., 1995; Smith and Jaynes, 1996; Serrano et al., 1997; Serrano and Maschat, 1998; Siegler and Jia, 1999) prompted us to test in vivo whether En functions as a transcriptional repressor during early brain development. We misexpressed either of two types of mutated En-2 protein, En-2(F51→E) or VP16-En-2(C133). In En-2(F51→E), phenylalanine residue in EH1 domain, which has been shown to interact with a co-repressor Groucho (Jiménez et al., 1997; Tolkunova et al., 1998), was substituted with glutamic acid (Fig. 1B). Such substitution has been shown to cause defects in its repressive activity in vivo in *Drosophila* (Smith and Jaynes, 1996). When En-2(F51→E) was expressed in diencephalon, shift of the di-mesencephalic boundary, induction of ectopic tectum, repression of *Pax-6*, or induction of *Pax-2*, *Pax-5*, *Pax-7*, *Fgf8*, *Wnt-1* or *EphrinA2* did not occur (Fig. 5A-B′, and data not shown). Interestingly, when En-2(F51→E) was expressed in mesencephalon, the size of the tectal swelling was reduced (Fig. 5A‴), and endogenous *Pax-7* and *EphrinA2* were weakly repressed (Fig. 5B,B′ and data not shown). In addition, expression of *Pax-2* and *Fgf8* in the isthmus was also repressed (not shown).

In VP16-En-2(C133), dominant positive En-2, N-terminal portion including EH1 domain was substituted with the transcriptional activation domain of VP16 protein (Fig. 1B; Triezenberg et al., 1988). VP16-En-2(C133) caused similar effects as En-2(F51→E); in the diencephalon, *Pax-6* was not repressed, and *Pax-2*, *Pax-5*, *Pax-7*, *Fgf8*, *Wnt-1* or *EphrinA2* was not induced (not shown); in the mesencephalon, the endogenous expressions of *Pax-2*, *Pax-7*, *Fgf8* or *EphrinA2* were repressed (Fig. 5C,C′ and data not shown) and the size of the tectal swelling was reduced (Fig. 5D,D′). In addition to these phenotypes, caudal shift of the di-mesencephalic boundary was observed in VP16-En-2(C133)-expressing embryos (Fig. 5E,E′, arrows). In severe cases, the size of the tectal swelling was so reduced that the di-mesencephalic boundary was indistinct (Fig. 5D,D′). In mesencephalon (Fig. 5D,D′,E,E′) and dorsal prosencephalon (Fig. 5F,F′, arrowheads) VP16-En-2(C133) caused ectopic induction or upregulation of *Pax-6*. However, we could not detect induction of *Pax-6* near mes-metencephalic boundary (Fig. 5D,D′) even when we performed electroporation at earlier stages (Fig. 5E,E′; stage7^{+}-8^{-}; Hamburger and Hamilton, 1951). Surprisingly, endogenous *Pax-6* was repressed by VP16-En-2(C133) in the ventral diencephalon (Fig. 5F-F″, arrows). This might be a secondary effect caused by the induction of other negative regulator(s), against *Pax-6* by VP16-En-2(C133) (see Discussion).

DISCUSSION

En defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate

In this study, we concentrated on the phenotype of the dorsal diencephalon and mesencephalon induced by En because dorsal mesencephalon is manifested by tectal swelling, molecular markers (*Pax7* and *EphrinA2*) are obtainable, and the data of past transplantation experiments are available. We

have shown that wild-type En induced rostral shift of di-mesencephalic boundary and that VP16-En(C133) induced caudal shift of the boundary. Since *En* is expressed along the entire anteroposterior axis in early mesencephalon (Fig. 6A) (Davis et al., 1991; Gardner and Barald, 1992; Shamim et al., 1999) and En seems to be able to repress *Pax-6* directly (see below), we infer that *En* defines the position of the rostral mesencephalic boundary in normal development by repressing diencephalic fate in dorsal brain (Fig. 6A).

What then determines the rostral limit of *En*-positive domain in the early developing brain? Several studies suggest that vertical signals from prechordal mesendoderm or notochord are essential for the *En* expression (Hemmati-Brivanlou and Harland, 1989; Hemmati-Brivanlou et al., 1990; Ang and Rossant, 1993; Darnell and Schoenwolf, 1997). Recently, Shamim et al. (1999) indicated that transient Fgf4 in early notochord functions as the vertical signal and that the rostral limit of the *Fgf4* expression, that is, the rostral limit of notochord, determines the rostral limit of the *En* expression in neural plate.

While the rostral limit of early *En*-positive region corresponds to rostral boundary of mesencephalon (Davis et al., 1991; Gardner and Barald 1992; Shamim et al., 1999), its caudal limit does not correspond to the caudal boundary of mesencephalon. Instead, the caudal limit of *Otx2*-positive region has been shown to correspond to caudal boundary of the tectum by transplantation experiment (Millet et al., 1996), and misexpression of *Otx2* in metencephalon, where *En* is expressed endogenously, induced ectopic tectum (Broccoli et al., 1999; Katahira et al., 1999). These results, together with our result that misexpressed En induced ectopic tectum in the dorsal diencephalon where *Otx2* is expressed endogenously, would suggest that the region where expression of *Otx2* and *En* overlaps in developing brain becomes mesencephalon (Fig. 6A).

After Nakamura et al. (1986) showed that the diencephalon can transdifferentiate into the tectum when transplanted into the posterior mesencephalon, it has been shown that the isthmus works as an organizer for the tectum (references in Shamim et al., 1999), and that the candidate of the organizing molecule is Fgf8 (Crossley et al., 1996; Lee et al., 1997; Shamim et al., 1999). Recently, *Fgf8* has been shown to form a positive feedback loop with *Pax-5* (Funahashi et al., 1999). In these experiments, En was induced in the host diencephalon (Gardner and Barald, 1991; Martinez et al., 1991; Crossley et al., 1996; Lee et al., 1997; Funahashi et al., 1999; Shamim et al., 1999). Since En seems to repress diencephalic fate as we showed in this study, ectopic induction of the tectum by such experimental manipulation in the diencephalon may depend on the repressive activity of En.

Pax-6 may be a direct target of En in early developing brain

We have shown that wild-type En repressed endogenous *Pax-6*, which seems to be a direct effect, and that VP16-En(C133) induced ectopic *Pax-6* in mesencephalon. As the di-mesencephalic boundary in *Pax-6* mutant mice is unclear (Grindley et al., 1997; Warren and Price, 1997), *Pax-6* also seems to be involved in the formation of di-mesencephalic boundary. The fact that *Pax* genes are involved in the control of cell proliferation (for review, see Dahl et al., 1997) and cell adhesion (Chalepakis et al., 1994; Holst et al., 1997; Stoykova et al., 1997; Meech et al., 1999), both of which are major determinants of tissue morphology, strongly support the idea. Thus, the repression of *Pax-6* is one of the pivotal roles of En in the positioning of di-mesencephalic boundary.

The phenotype caused by En-2(F51→E) or VP16-En(C133) suggests that En acts as a transcriptional repressor in early diencephalic and mesencephalic region. This is the first report that vertebrate En acts as a transcriptional repressor in vivo. While some genes have been proven to be direct targets of En in *Drosophila* (Saenz-Robles et al., 1995; Schwartz et al., 1995; Serrano et al., 1997, 1998; Siegler and Jia, 1999), targets of En in vertebrate have not yet been identified. Because of their mutually exclusive expression patterns (Bally-Cuif and Wassef, 1994; Burrill et al., 1997; Plaza et al., 1997), *Pax-6* is one of the primary candidates for the target gene of En in vertebrates. We detected the repression of *Pax-6* within 2 hours of the onset of ectopic En expression (compare Figs 2A and 3B,B′), and the repression preceded the induction of mesencephalon-related genes (Fig. 4).

It has been shown that *Pax-6* gene positively autoregulates itself (Plaza et al., 1993). Plaza et al. (1997) suggested that En protein can cut the positive autoregulatory circuit of *Pax-6* gene by binding directly to the paired domain of Pax-6 protein in vitro. However, Grindley et al. (1997) suggested that such autoregulation of the *Pax-6* gene seems to be confined to the posterior P1 in developing brain because disappearance of *Pax-6* expression in the diencephalon is restricted to the posterior P1 in *Pax6*$^{Sey\text{-}1Neu}$ mouse, in which the Pax-6 protein lacks a transactivation domain (Hill et al., 1991; Glaser et al., 1992, 1994). Thus, the repression of *Pax-6* by ectopic En-2 in most parts of diencephalon in this study is not due to the direct interaction between En-2 and Pax-6 proteins, but may be due to the direct transcriptional repression of *Pax-6* gene by En-2. However, further analysis of the transcriptional control unit in *Pax-6* gene is required for a final conclusion.

En positively regulates mesencephalon-related genes by repressing the expression of other hypothetical negative regulator(s)

The fact that misexpressed En caused transformation of dorsal diencephalic cells to tectal cells suggests that *En* is sufficient for the mesencephalic precursor cells to acquire tectal fate. At the molecular level, En ectopically induced *Pax-2*, *Pax-5*, *Fgf8*, *Wnt-1* and *EphrinA2* in diencephalic and mesencephalic regions. The de novo activation of these mesencephalon-related genes in diencephalic and mesencephalic regions by En indicates that the molecular machinery for positive regulation of the mesencephalon-related genes still exists in the entire dorsal diencephalic and mesencephalic region at the stage when En was ectopically expressed. Although expressions of *Pax-2*, *Pax-5*, *Fgf8*, *Wnt-1* and *EphrinA2* begin later than that of *En-1* in chick embryos (Shamim et al., 1999; Funahashi et al., 1999; Okafuji et al., 1999; I. A., unpublished data), de novo activation of these genes in diencephalon induced by *En* does not necessarily mean that *En* is involved in their initial activation (see Lun and Brand, 1998; Shamim et al., 1999). However, it is probable that *En* functions to maintain their expression in normal embryos. The function of *En* on *Pax-7*

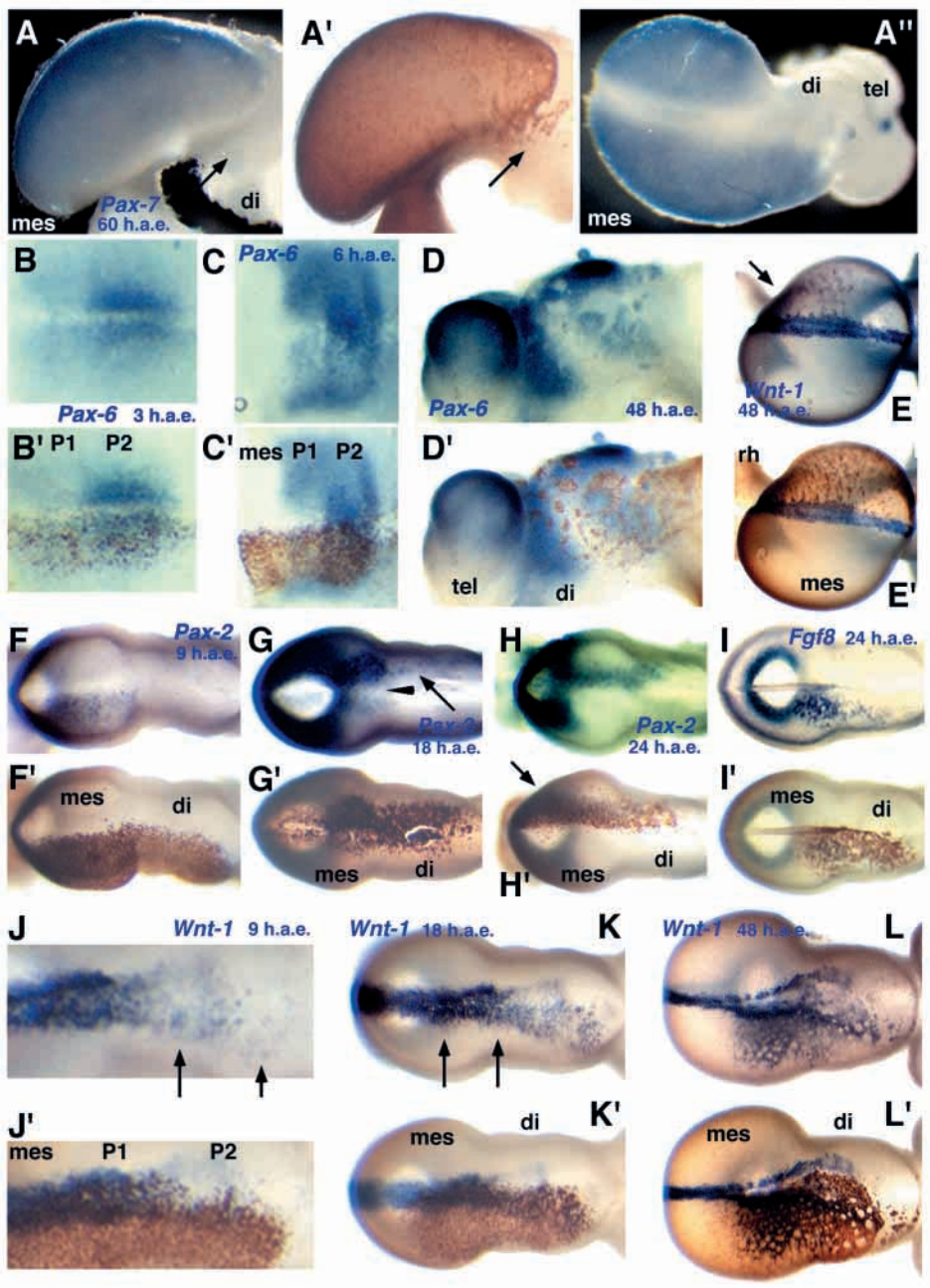

Fig. 3. Time-course analysis of the reaction of each gene in di-mesencephalic region to misexpressed En-2. (A-A″) Ectopic *Pax-7* (blue staining) was observed in the rostrally expanded tectal swelling induced by misexpressed En-2 (brown staining) at 60 h.a.e. Ectopic En-2 in the ventral diencephalon (arrow in A′) did not induce *Pax-7*. (B,B′) Weak repression of endogenous *Pax-6* (blue) by misexpressed En-2 (brown) in the diencephalon was detected at 3 h.a.e. (C,C′) Repression became clear by 6 h.a.e. (D,D′) Repression of endogenous *Pax-6* (blue) by misexpressed En-2 (brown) in the diencephalon at 48 h.a.e. (E,E′) En-2 caused narrowing (arrow) in posterior mesencephalon. Blue staining in E corresponds to *Wnt-1* expression. (F,F′) Ectopic *Pax-2* (blue) induced by En-2 (brown) in the mesencephalon was first detected at 9 h.a.e. (G,G′) Ectopic *Pax-2* (arrow) induced by En-2 (brown) in the diencephalon was first detected at 18 h.a.e. The dorsal midline was devoid of induction of *Pax-2* (arrowhead). (H,H′) Ectopic *Pax-2* (blue) induced by En-2 (brown) at 24 h.a.e. (I,I′) Ectopic *Fgf8* (blue) induced by En-2 (brown) at 24 h.a.e. (J,J′) Ectopic *Wnt-1* or upregulation of endogenous *Wnt-1* in the diencephalon by En-2 (brown) was first detected at 9 h.a.e. (arrows). (K,K′) Ectopic *Wnt-1* induced by En-2 (brown) in the mesencephalon and P1 was first detected at 18 h.a.e. (arrows). (L,L′) Ectopic *Wnt-1* (blue) induced by misexpressed En-2 (brown) at 48 h.a.e. Rostral expansion of tectal swelling (lower side) was clearly observed at this stage. Electroporation was performed at stage 13-14 except for (A,D) in which electroporation was performed at stage 10-11. (B,C) Flat mounts. (G) Color for whole-mount in situ hybridization (blue staining in G) was destained after HRP immunostaining (brown staining in G′). All embryos in this figure, except D,D′, are oriented with rostral to the right. di, diencephalon; mes, mesencephalon; P1, prosomere 1; P2, prosomere 2; rh, rhombencephalon; tel, telencephalon.

also seems to be the maintenance of the expression in the tectum because the initial expression of *Pax-7* extends along the entire dorsal brain, and later the expression becomes restricted to the tectum (Kawakami et al., 1997).

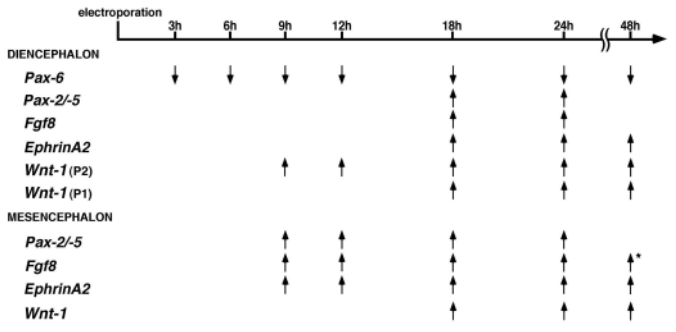

Fig. 4. Summary of the results of the time-course analysis of the reaction of each gene in diencephalon and in mesencephalon to misexpressed En-2. Upward arrows mean the ectopic induction and downward arrows mean the repression. Ectopic *Fgf8* at 48 h.a.e. (marked with asterisk) was restricted near the isthmus

While our data suggest that En acts as a transcriptional repressor in early di-mesencephalic region, En ectopically induced *Pax-2*, *Pax-5*, *Fgf8*, *Wnt-1* and *EphrinA2* both in the mesencephalon and in the diencephalon. The simplest explanation for this superficial discrepancy is that En functions as a transcriptional repressor of the gene(s) for other putative intermediate negative factor(s) (designated X in Fig. 6B), which inhibit the expression of mesencephalon-related genes (e.g. *Pax-2*, *Fgf8*) when there is no Engrailed. Thus, when there is En repressive activity, the expression of the putative negative

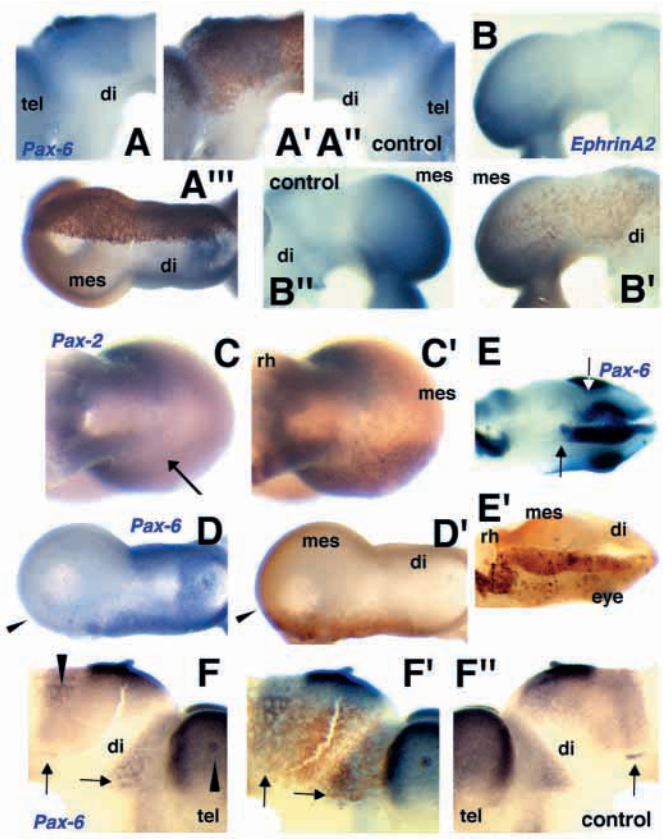

Fig. 5. Phenotypes in the embryos in which mutated En was misexpressed. (A-A″) Repression of Pax-6 (blue staining) by En-2(F51→E) (brown staining) was not observed. (A″) Control side. (A‴) Reduction of the size of the tectal swelling by En-2(F51→E). (B-B″) Weak repression of endogenous *EphrinA2* (blue) by En-2(F51→E) (brown). (B″) Control side. (C,C′) Repression of endogenous *Pax-2* (arrow) in the isthmus by VP16-En-2(C133) (brown). *(D,D′)* VP16-En-2(C133) (brown) induced ectopic *Pax-6* (blue) in the mesencephalon. The ectopic induction was restricted to the anterior mesencephalon. Arrowhead shows the position of the dorsal midline at the caudal mesencephalon. Note that the dorsal midline was bent because of the severe reduction of the size of the tectal swelling. (E,E′) The phenotype of an embryo in which electroporation of VP16-En-2(C133) (brown) was performed at earlier stage (stage 8⁻). Caudal shift of the di-mesencephalic boundary (arrows) and ectopic *Pax-6* (blue) were observed on the experimental side (lower side). (F,F′,F″) VP16-En-2(C133) (brown) caused upregulation of endogenous *Pax-6* (blue) in the dorsal prosencephalon (arrowheads). In the ventral diencephalon repression of *Pax-6* was observed (arrows). (D,E) Color for whole-mount in situ hybridization (blue) was destained after HRP immunostaining (brown). Electroporation was performed at stage 10-12 in A,B,D,F and at stage 13-14 in C. (A,B,D,F) Fixed at 48 h.a.e.; (C,E) fixed at 24 h.a.e. All embryos in this figure except A,A′,B″,F″ are oriented with rostral to the right. di, diencephalon; mes, mesencephalon; rh, rhombencephalon; tel, telencephalon.

factor(s) are repressed, and the expression of mesencephalon-related genes is induced by transcriptional activator(s), which exist ubiquitously in di-mesencephalic region (not shown in Fig. 6B for simplicity). This idea is supported by the result that it took longer time for the induction of these genes (first detected at 9 h.a.e.) than for the repression of *Pax-6* (3 h.a.e.).

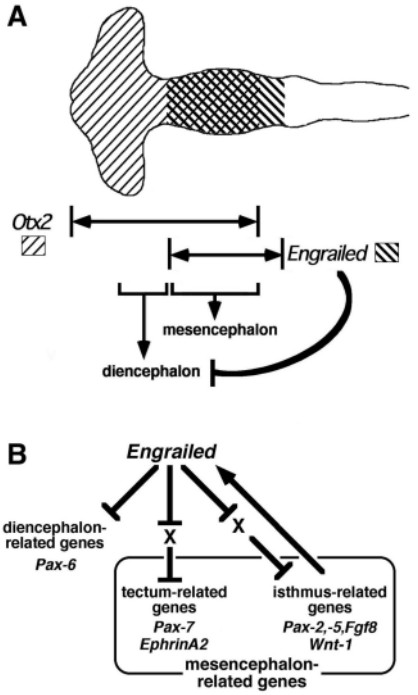

Fig. 6. Model on the function of *En* in the early development of mesencephalon. (A) At the cellular level, *En* represses the diencephalic fate and positively regulates the mesencephalic fate in dorsal di-mesencephalic region. Since *Otx2* has been also shown to be necessary for the development of the tectum (Broccoli et al., 1999; Katahira et al., 1999), the region in which expression of *Otx2* and *En* overlap may be fated to become mesencephalon. (B) At the molecular level, En represses diencephalon-related genes and positively regulates mesencephalon-related genes by directly repressing the expression of other negative regulator(s) for mesencephalon-related genes (designated as X). X directly or indirectly downregulate mesencephalic genes when there is no En. When there is En repressive activity, the expression of the putative negative factor(s) are repressed, and the expression of mesencephalon-related genes is induced by transcriptional activator(s), which exist ubiquitously in di-mesencephalic region (not shown in Fig. 6B for simplicity). *Pax-2*, *Pax-5*, *Fgf8* and *Wnt-1* are known to positively regulates *En* (Krauss et al., 1992; Crossley et al., 1996; Danielian and McMahon, 1996; Favor et al., 1996; Lee et al., 1997; Schwartz et al., 1997; Urbanék et al., 1997; Lun and Brand, 1998; Reifers et al., 1998; Sugiyama et al., 1998; Funahashi et al., 1999; Okafuji et al., 1999; Shamim et al., 1999). The analysis of transgenic mice suggests that *Pax-2*/ *Pax-5*/ *Pax-8* may bind to *En-2* promoter (Song et al., 1996).

In the diencephalon, it took much longer time to induce *Pax-2*, *Pax-5*, *Fgf8* and *EphrinA2* (first detected at 18 h.a.e.) than in the mesencephalon (9 h.a.e.). In the diencephalon, rearrangement of gene expression toward the development for mesencephalon may occur before the expression of these mesencephalon-related genes, and it will take a longer time for these genes to be expressed.

Since Pax-6 has been shown to be able to function also as a transcriptional repressor in vitro (Duncan et al., 1998), Pax-6 may be one of such negative regulators in diencephalon. However, the mesencephalon-related genes were ectopically induced by wild-type En and they were downregulated by VP16-En in the mesencephalon where there is no *Pax-6*

expression. Thus, in the mesencephalon there must be other negative regulator(s), the expression of which is repressed by En, if our model is correct. Analysis of mesencephalic phenotype in embryos in which Pax-6 is misexpressed, and the identification of the hypothetical negative factor(s), will advance our knowledge of the molecular mechanism of mesencephalic differentiation.

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