Role of Pax3/7 in the tectum regionalization

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SUMMARY

Pax3/7 is expressed in the alar plate of the mesencephalon. The optic tectum differentiates from the alar plate of the mesencephalon, and expression of Pax3/7 is well correlated to the tectum development. To explore the function of Pax3 and Pax7 in the tectum development, we misexpressed Pax3 and Pax7 in the diencephalon and ventral mesencephalon. Morphological and molecular marker gene analysis indicated that Pax3 and Pax7 misexpression caused fate change of the alar plate of the presumptive diencephalon to that of the mesencephalon, that is, a tectum and a torus semicircularis were formed ectopically. Ectopic tectum in the diencephalon appeared to be generated through sequential induction of Fgf8, En2 and Pax3/7. In ventral mesencephalon, which expresses En but does not differentiate to the tectum in normal development, Pax3 and Pax7 misexpression induced ectopic tectum. In normal development, Pax3 and Pax7 expression in the mesencephalon commences after Otx2, En and Pax2/5 expression. In addition, expression domain of Pax3 and Pax7 is well consistent with presumptive tectum region in a dorsoventral axis. Taken together with normal expression pattern of Pax3 and Pax7, results of misexpression experiments suggest that Pax3 and Pax7 define the tectum region subsequent to the function of Otx2 and En.

Key words: Pax3, Pax7, En, Tectum, Chick

INTRODUCTION

It has been shown that isthmic region acts as an organizer for the mesencephalon and metencephalon, and that Fgf8 is a candidate organizing molecule (Martinez et al., 1991; Alvarado-Mallart, 1993; Marin and Puelles, 1994; Martinez et al., 1995; Crossley et al., 1996; Joyner, 1996). The optic tectum is a major component of the mesencephalic alar plate derivatives, and has been a focus of attention. Fgf8, Pax2/5 and En make a positive feedback loop for their expression to keep organizing activity, and contribute to initiate and maintain the tectum development (Song et al., 1996; Lun and Brand, 1998; Araki and Nakamura, 1999; Funahashi et al., 1999; Okafuji et al., 1999; Ristoratore et al., 1999; Shamim et al., 1999; Liu and Joyner, 2001). However, Grg4 may antagonize this positive feedback loop (Sugiyama et al., 2000). Now it is accepted that combination of Otx2, En and Pax2 defines the tectum region (Nakamura, 2001). Although molecular mechanisms that define the tectum region in the early developmental phase have been well studied, little attention has been paid to downstream factors for the tectum development.

Pax3 and Pax7 belong to the same Pax subfamily, and widely expressed in the nervous system and somites (Jostes et al., 1990; Goulding et al., 1991). In the nervous system Pax3 and Pax7 are expressed in the dorsal part of the neural tube (Jostes et al., 1990; Goulding et al., 1991). They are expressed in the whole region of the tectum anlage (Kawakami et al., 1997). Pax3 mutant mice, Splotch, show exencephaly and defects in myogenesis and neural crest cell differentiation (Epstein et al., 1991; Franz, 1993; Tremblay, 1995; Conway et al., 1997). Pax7 mutant mice also show the defects in myogenesis and neural crest cell differentiation but no defects in the central nervous system (Mansouri et al., 1996). Pax3 and Pax7 share their expression domain broadly in the central nervous system, which may explain functional redundancy and rather mild defects in mutant mice. Pax3 and Pax7 double mutant mice show severe exencephaly, spina bifida and defects in commissural neurons in the spinal cord, and die by E11.0 (Mansouri and Gruss, 1998).

As Pax3 and Pax7 are expressed in the mesencephalic alar plate, the possibility that they are involved in regionalization of the tectum has been suggested (Kawakami et al., 1997; Nomura et al., 1998). A tectum could be induced ectopically in the diencephalic region by transplantation of the isthmus or by misexpression of En, Pax2/5 or Fgf8. Pax7 was always induced by misexpression of Fgf8. Pax3 and Pax7 misexpression induced an ectopic tectum. Pax3 overexpression was carried out in the transgenic mice, in which Pax3 expression was...

Key words: Pax3, Pax7, En, Tectum, Chick
regulated by Hoxb4 promoter (Tremblay et al., 1996). In the transgenic mice, however, effects of Pax3 overexpression on the mesencephalon were not assessed as the Hoxb4 promoter does not assure expression in the mesencephalon. To explore the roles of Pax3/7 in tectum development, we carried out misexpression experiments of Pax3 and Pax7 in the diencephalon and ventral mesencephalon by in ovo electroporation. An ectopic tectum in the diencephalon and ventral mesencephalon was differentiated after Pax3 and Pax7 misexpression. A torus semicircularis, which derives from the caudal part of the mesencephalic alar plate and corresponds to the mammalian inferior colliculus, was also differentiated ectopically in the diencephalic region. Analysis of marker gene expression indicated fate change of the diencephalon occurred after sequential induction of Fgf8, En2 and Pax3/7. In normal development, Pax6 and Pax7 expression in the alar plate of the presumptive mesencephalon commences after Otx2, En and Pax2/5. Thus, results of misexpression experiments, together with normal expression patterns, suggests that Pax3 and Pax7 defines the alar plate of the mesencephalon subsequent to determination of the mesencephalon by Otx2, En and Pax2/5.

MATERIAL AND METHODS

Expression vectors

Partial fragment of chick Pax3 cDNA was isolated from E3 chick brain library, and then this fragment was fused to a C-terminal fragment isolated by 3’RACE to get the full length of Pax3 cDNA. The full-length of chick Pax7 cDNA was isolated from E3 chick cDNA by reverse transcription PCR. Primers for N- and C-terminal fragments are 5’-TGTGACATAGCCGAAAACCT-3’ and 5’-CTTT-GCTCTGTCGGTTGCTGA-3’, respectively. These fragments were fused to StuI site to make a full length of Pax7. Pax3 was used in electroporation at stage 10 (Hamburger and Hamilton, 1951) as a gift from Dr Kondoh), which has Rous sarcoma virus enhancer and chicken β-actin promoter.

In ovo electroporation

Fertilized chicken eggs from a local farm were incubated at 38°C. Pax3, Pax7 and Pax7-HA expression vector (3.0 μg/ml), β-galactosidase expression vector (MiwZ) (a gift from Dr Kondoh) and the green fluorescence (GFP) expression vector (pEGFP-N1, Clontech) (0.5 μg/ml), were transfected to chick embryos by in ovo electroporation at stage 10 (Hamburger and Hamilton, 1951) as previously described (Funahashi et al., 1999). A GFP expression vector was co-transfected to check the efficiency of electroporation. Transfection to the ventral mesencephalon was carried out at stage 13.

Electroporation of this condition does not induce defects in the embryos (Funahashi et al., 1999; Nakamura et al., 2000).

In situ hybridization

Whole-mount in situ hybridization was performed as described by Bally-Cuijff et al. (Bally-Cuijff et al., 1995) or by Stern (Stern, 1998). In situ hybridization for sections was carried out as described by Ishii et al. (Ishii et al., 1997). Probes for Fgf8, Lim1 and Pax6 were described previously (Araki and Nakamura, 1999; Matsuunaga et al., 2000). For Pax3 probe, partial fragment, isolated from cDNA library of E3 chick brain, was used. These fragments were inserted in pBluescript II SK (-) (Stratagene). After linearization, digoxigenin (DIG)-labeled antisense RNA was generated by T3 or T7 RNA polymerase (Stratagene) (Funahashi et al., 1999). For detection, alkaline phosphatase (ALP)-conjugated anti-DIG sheep-polyclonal antibody (Roche Molecular Biochemicals) was used, and 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoly-phosphate (BCIP) (Roche Molecular Biochemicals) were used for the coloring. In some cases, NBT staining was washed out by incubating in dimethylformamide (DMF) at 55°C.

Immunohistochemistry

Anti-Pax6 rabbit polyclonal antibody (provided by Dr N. Osumi), anti-Pax7 monoclonal antibody (Developmental Studies Hybridoma Bank; Kawakami et al., 1997), anti-En2 monoclonal antibody, 4D9 (American Type Culture Collection; Patel et al., 1989) and anti-HA rabbit polyclonal antibody (Berkeley Antibody Company) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Jackson Immuno Research Laboratories) was used as the secondary antibody. For double staining on sections, Alexa488-conjugated anti-rabbit IgG antibody (Molecular Probes) and Cy3-conjugated anti-mouse IgG antibody (Jackson Immuno Research Laboratories) were used as secondary antibodies.

Histology

Embryos were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline), embedded in Technovit (Kuler), serially sectioned at 5 μm, and stained with Hematoxylin and Eosin. Tiling images were automatically composed by MCID Image analyzer (Imaging Research Inc). β-galactosidase activity was detected on whole-mount embryos as previously described (Katahira et al., 2000).

Tracing retinal fibers with HRP

For tracing of the retinal fibers 30% HRP solution, dissolved in PBS, was injected into the eye using a glass micropipette by air pressure. At 24 hours after injection, the brain was dissected, and the HRP-positive fibers were stained with p-cresol-diaminobenzidine method (Streit and Reubi, 1977). After observation of the fiber trajectory, the specimen was embedded in Technovit, and sagittal sections of 5 μm were prepared.

RESULTS

Expression patterns of Pax3 and Pax7 in the prosencephalon and mesencephalon

We first examined expression patterns of Pax3 and Pax7 in the prosencephalon and mesencephalon. At stage 10, Pax3 and Pax7 expression was detected near the dorsal midline in the neural tube (Fig. 1A,D). Pax3 and Pax7 expression extended laterally in the alar plate of the mesencephalon at stage 13 (Fig. 1B,E), and by E2.5 (stage 17), the expression extended more to cover the alar plate of the mesencephalon and the p1 (prosomere1) region (Fig. 1C,F). We adopted the neuromeric criteria presented by Rubenstein et al. (Rubenstein et al., 1994). Pax3 and Pax7 were also expressed in the roof plate anterior to p2, but in the anterior midline of the telencephalon only Pax7 was expressed at stage 17 (Fig. 1F). Around stage 20, Pax3 and Pax7 were expressed strongly in the alar plate of the mesencephalon, which is now conspicuous as the tectal swelling. Thereafter Pax3 and Pax7 were expressed in the ventricular zone and mantle layer of the tectum (data not shown; Kawakami et al., 1997). Expression of Pax3 and Pax7 was also observed in neural crest cells, which shows dot-like staining in Fig. 1A,D.

Pax3 and Pax7 misexpression caused fate change of the diencephalon to the tectum

To examine the role of Pax3 and Pax7 in tectum development,
**Pax3** and **Pax7** expression vectors were transfected in the right side of the neural tube from the telencephalon to the mesencephalon (Fig. 2).

**Pax7** misexpression caused a morphological change in the diencephalon at 48 hours after electroporation (n=3/4/56). An ectopic swelling was generated in the diencephalic region especially in p2 region (Fig. 2A,B). But in p1, swelling was not formed so that the ectopic swelling did not continue to the tectum proper, being interrupted at p1 (Fig. 2A-F). Co-transfection of **Pax7** and lacZ expression vector showed that p1 region did not make swelling even though p1 region had been well transfected (Fig. 2A,B).

**Lim1** is a good marker expressed in the pretectum (Mastick et al., 1997; Matsunaga et al., 2000). **Lim1** expression remained in the p1 region after **Pax7** misexpression, though expression domain a little narrowed, which indicates that most of the pretectal region retained even after **Pax7** misexpression (n=5/5) (Fig. 2G-J).

At E6.5 (stage 27, 5 days after electroporation of **Pax3** expression vector), morphological change was more remarkable (Fig. 2C,D). Histologically, the ectopic swelling was generated in the diencephalic region (Fig. 2E) (n=4/5). High power micrograph of this area clearly shows that the ectopic swelling consists of laminar structure that is characteristic of the tectum, though cytoarchitectonic differentiation in the ectopic tectum was behind of the tectum proper (Fig. 2F). In the tectum proper, three layers were prominent in addition to the neuroepithelial layer, and in the ectopic swelling two layers were discernible in addition to the neuroepithelial layer (Fig. 2F).

To examine if the ectopic tectal swelling can receive retinal fibers, we looked at fiber trajectory of retinal axons at E13.5 after **Pax7** misexpression. Fiber tracing with HRP showed that most of retinal fibers innervated the ectopic tectum (n=3/3) (Fig. 2K,L). Sagittal sections of this specimen (Fig. 2M-R) show that the ectopic swelling contains well differentiated tectal structure though cytoarchitectonic differentiation was behind of normal tectum. According to LaVail and Cowan (LaVail and Cowan, 1971), the tectum of E12-14 has 12 layers. In the ectopic tectum (Fig. 2P), cell-dense layers of vi and vili are conspicuous; SO (stratum opticum) was easily recognized because retinal fibers had been labeled as shown in Fig. 2L. As the tectum proper at the experimental side was deprived of retinal fibers (Fig. 2L), superficial layers were poorly differentiated (Fig. 2O) as shown by Kelly and Cowan (Kelly and Cowan, 1972). The torus semicircularis (Fig. 2Q,R) that corresponds to the mammalian inferior colliculus (Puelles et al., 1994) was also formed ectopically. Taken together, the derivatives of the mesencephalic alar plate were differentiated ectopically. Pretectal nuclei persisted between the tectum proper and the ectopic tectum (Fig. 2N). The pattern of fiber trajectory and histology indicated that ectopic swelling acquired the character of the tectum.

**Pax7** represses **Pax6** expression and induces ectopic tectum by activating the gene cascade for tectum development

Next we examined effects on marker gene expression after **Pax7** misexpression. The results of timecourse analysis are summarized in Table 1.

First, we looked at the effects of **Pax7** misexpression on **Pax6** expression. **Pax6** is expressed in the diencephalon and essential for its development (Walther and Gruss, 1991; Stoykova et al., 1996; Stoykova et al., 1997; Warren and Price, 1997). **Pax6** mutant mice, **Sey**, show fate change of p1 region to the mesencephalon (Mastick et al., 1997). Repression of **Pax6** by **Pax7** was detected by 15 hours after electroporation (n=1/3), and repression was observed in all the embryos examined at 24 hours after electroporation (n=4/4) (Table 1 and Fig. 3A-C). Repression of **Pax6** was remarkable in the p2 region (Fig. 3C). In the p1

### Table 1. Summary of the results of the timecourse analysis of marker gene expression in the diencephalon by **Pax7**-HA misexpression

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Upward arrows represent ectopic induction and downward arrows represent repression. Single arrows represent weak induction or repression. A bar indicates that induction or repression was not detected.

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Fig. 1. Normal expression patterns of **Pax3** and **Pax7** in the prosencephalon and the mesencephalon. Whole-mount in situ hybridization for **Pax3** (A-C) and **Pax7** (D-F) at 10-somite stage (A,D), 18-somite stage (HH13) (B,E) and E2.5 (HH17) (C,F). **Pax3** and **Pax7** are expressed near the dorsal midline of the neural tube and in the migrating neural crest cells in the mesencephalic region at stage 10 (A,D). Expression of **Pax3** and **Pax7** commences around stage 13 in the alar plate of the mesencephalon (B,E). In the alar plate, **Pax3** and **Pax7** are expressed posterior to p1/p2 boundary at stage 17. Expression around the roof plate extended rostrally beyond the p1/p2 boundary (C,F). **Pax7** is also expressed in anterior midline of the telencephalon, but **Pax3** is not expressed there. Scale bars: 500 μm in F; 200 μm in D.E. di, diencephalon; mes, mesencephalon; met, metencephalon; p1, prosomere 1; p2, prosomere 2; pro, prosencephalon; rho, rhombencephalon; tel, telencephalon.
**Fig. 2.** Pax3/7 misexpression changed the fate of the alar plate of the diencephalic P2 region to that of the mesencephalon. (A,B) Morphological change at 48 hours after electroporation of Pax7. Horizontal section. Hematoxylin and Eosin staining (A), X-gal staining of an embryo at 48 hours after co-electroporation of Pax7 and lacZ expression vector (B). White arrowheads show the ectopic swelling in the diencephalon. Note that the ectopic swelling is formed in the p2 region, although expression vectors were uniformly transfected from the telencephalon to the mesencephalon. (C-F) Morphological change at 5 days after electroporation of Pax3. View from the experimental side (C), dorsal view (D). Horizontal section at the plane indicated in C. Hematoxylin and Eosin staining (E). (F) A high power micrograph of boxed area in (E). Arrowheads on E indicate the ectopic swelling in the diencephalon. Note that ectopic swelling (ect. tec) has the laminar structure similar to the tectum proper (tec). Differentiation of laminar architecture in the ectopic swelling is behind of tectum proper. (G-J) In situ hybridization for Lim1 to show that pretectum is retained after Pax7 misexpression. View from the experimental side (G), view from the control side (H) and dorsal view (I) of an E4.0 embryo. (J) Horizontal section at the plane indicated in G. The control side is printed in reverse for the comparison. Lim1 expression in the pretectum is detected (between arrowheads on panel J). (K-R) Retinal fiber trajectory and histology of E13.5 embryos after Pax7 misexpression. Schematic drawing of retinal fiber trajectory (K), and lateral view (L). Horseradish peroxidase was injected into the left eye at E12.5 and the embryo was sacrificed at 24 hours later (E13.5, 12 days after Pax7 misexpression). Most of retinal fibers innervate the ectopic swelling (arrow), and some of them innervate the tectum proper (arrowhead). After observation of retinal fiber trajectory, the specimen was embedded in Technovit, and sagittal sections were stained with Hematoxylin and Eosin. Approximate planes of M,N are indicated in K. Panels P-R are higher magnifications indicated on M,N. The tectum proper (O) does not contain stratum opticum (SO) as is inferred from L, which may resulted in maldevelopment of superficial layers (layer x is barely discernible). The ectopic tectum (P) contains SO, and layers vi, vii, viii, ix are discernible, though its differentiation is behind the normal tectum. Torus semicircularis at the proper site and ectopically differentiated in the diencephalon are shown in Q,R, respectively. We could identify pretectal nuclei (nucleus spiriformis lateralis (Spl) and medialis (Spm), and nucleus principalis precommissuralis (Ppc)). We adopted the criterion of the tectum layers of LaVail and Cowan (LaVail and Cowan, 1971). Scale bars: 2.0 mm in L; 1.0 mm in D.H.I; 500 μm in A,B,E,J,M,N; 200 μm in F,R; 100 μm in O. cont., control side; cer, cerebellum; exp., experimental side; tec, tectum; ect. tec, ectopic tectum; ts, torus semicircularis; ect. ts, ectopically differentiated torus semicircularis.

region, Pax6 expression was not so much affected even when Pax7 had been misexpressed strongly (Fig. 3A-C). Double staining by immunocytochemistry with specific antibodies for Pax6 and Pax7 revealed that Pax6 was repressed in the cells that had expressed Pax7, which indicates that repression is in a cell-autonomous manner \((n=4/4)\) (Fig. 3D-H). Pax7 also repressed Tcf4, which is a dorsal diencephalon marker gene (data not shown).

Then, we checked effects on tectum-related genes. Fgf8 induction by Pax7 was detected in some embryos by 12 hours after electroporation (Table 1), and in all the embryos by 24 hours after electroporation (Table 1). Induction of Fgf8 was detected earlier than repression of Pax6 (Table 1). However, if we take it into consideration that detection of repression is much more difficult than that of induction, induction of Fgf8 and repression of Pax6 by Pax3/7 may have occurred around the same time. Induction of Fgf8 was always detected rostral to the p1/p2 boundary, that is, in the p2 region, but not in the p1 region \((n=7/7)\) (Fig. 3I-N). Double staining for Fgf8 and Pax7 shows that Fgf8 was induced in the Pax7-expressing cells, that is, in a cell autonomous manner (Fig. 3L-N). En2 induction was not detected even at 24 hours after electroporation (Table 1)
Fig. 3. Effects of Pax7 on expression of tectum-related genes. (A-H) Cell autonomous repression of Pax6 by Pax7. In situ hybridization for Pax6 (blue), the control (A), and the experimental embryos (C) at 24 hours after electroporation. Immunostaining for Pax7 (brown) was also carried out on the same embryo (B). Repression of Pax6 is rarely detected in the p1 region (C). (D-H) Double immunocytochemical staining for Pax6 (green) and Pax7 (red) 24 hours after Pax7 electroporation. (F,G) High power magnifications of boxed areas in D,E, respectively. (H) A combined image. Pax6 is repressed in the cells that express Pax7 (arrowheads, F-H), indicating that Pax6 is repressed by Pax7 in a cell autonomously. (I-N) Induction of Fgf8 by Pax7. In situ hybridization for Fgf8 (blue, I,K), and immunostaining for Pax7 (brown) was added on the same embryo (J) at 24 hours after electroporation. (L,M) Higher power micrographs of the area indicated in J,K, respectively. (N) A micrograph of the same field as M, showing only Pax7 expression. This figure was taken after staining for Fgf8 was washed away. These figures indicate that induction of Fgf8 expression by Pax7 is in a cell autonomous manner. The arrows in L-N indicate the same position. (O-S) En2 induction via Fgf8 after Pax7 misexpression. In situ hybridization for Fgf8 (blue, O,P), and immunostaining for En2 (brown) on the same embryo (Q) at 48 hours after electroporation. (R,S) High power micrographs of boxed area in P,Q, respectively. The arrows in R,S indicate ectopic expression of Fgf8. Arrowheads in S indicate ectopic expression of En2. En2 induction was always detected around Fgf8-expressing cells. (T-X) En2 induction after Pax3 misexpression. In situ hybridization for Pax3 (blue, T,U), and immunostaining for En2 (brown) on the same embryo (V). (W,X) High power micrograph of boxed area in U,V. En2 induction was detected adjacent to the Pax3-misexpressing cells, which indicate that En2 was induced in a same manner as after Pax7 misexpression. A,I,O,T are printed in reverse for the comparison. Scale bars: 500 μm in C,K,Q,V; 200 μm in E; 25 μm in H,N,S,X.

1). By 36 hours after electroporation, En2 was ectopically induced in the alar plate of the diencephalon. Ectopic En2 induction was observed in p2, but not in p1 in the embryos of 48 hours after electroporation (n=9/13) (Fig. 3O-S). Double staining for Fgf8 and En2 shows that En2 was induced in the cells around those in which Fgf8 had been ectopically induced, suggesting that En2 was induced by Fgf8. After Pax3 misexpression, En2 was also induced around the Pax3-expressing cells, which indicates that En2 was induced in non-cell autonomous manner (n=3/4).

Repression of Otx2 by Pax7 was not recognized at 24 hours after electroporation (n=3/3). Ectopic expressions of En1 and Pax2 was not detected in the diencephalic region (data not shown). Pax5 induction was very weakly detected at 48 hours after electroporation, but not detected before 48 hours after electroporation (data not shown).

Next we looked at inter-relationship between Pax3 and Pax7. Pax3 was induced ectopically in the diencephalon by 18 hours after electroporation of Pax7 (Table 1; Fig. 4A-E). Induction was very weak at first, and higher magnification indicates that the induction is cell autonomous (Fig. 4F-I). By 96 hours after electroporation, when the ectopic swelling became conspicuous, Pax3 expression became strong and covered the whole ectopic swelling (Fig. 4J, N). Serial sections showed that Pax3 was expressed in the ectopic swelling, where En2 had been ectopically induced and Pax6 was repressed (Fig. 4K,L,O-Q). Pax7 also covered whole the ectopic swelling (Fig. 4M,Q). To distinguish induced and exogenous Pax7 expression, we misexpressed HA-tagged Pax7. The ectopic swelling was not stained immunocytochemically by anti-HA antibody at 96 hours after electroporation (Fig. 4M). As expression vector adopted in the present study assures transient expression, it is conceivable that Pax7 expression by introduced expression vector disappeared by this stage. Thus, Pax7 expression seen in the ectopic swelling was not exogenous one, but expression from the host Pax7 gene. Temporal analysis shows that Pax3 was weakly induced by Pax7 at 18 and 24 hours after electroporation, then Pax3 expression nearly disappeared by 48 hours after electroporation. Then by 96 hours after electroporation, Pax3 expression covered whole the ectopic swelling. These results indicates that there are two modes in Pax3 induction by Pax7.
in the ectopic swelling; one is direct induction by Pax7, and the other is indirect and may be induced after the feedback loop of tectum organizing molecules had been turned on.

**Pax7 misexpression expands the territory of the tectum ventrally**

Shh misexpression in the dorsal mesencephalon resulted in repression of Pax7 expression and fate change of the tectum to the tegmentum (Watanabe and Nakamura, 2000). Pax7 expression is well correlated with tectum development in a dorsoventral axis (Nomura et al., 1998). Therefore, we analyzed effects on marker gene expression for the tectum and Pax7 misexpression in the ventral mesencephalon and analyzed effects on dorsoventral axis.

At E6.5, the tectum and the tegmentum can be distinguished histologically: the tectum shows specific laminar structure as shown previously. After Pax7 misexpression, tectal structure extended ventrally ($n=3/6$) (Fig. 5A-C). Next we checked effects on marker gene expression for the tectum and tegmentum. Induction of Pax3 in the ventral mesencephalon was observed at 24 hours after electroporation of Pax7 ($n=3/3$) (Fig. 5G,H). Higher power micrographs indicate that induction of Pax3 by Pax7 is cell autonomous (Fig. 5H,1). Pax3 misexpression also expanded the tectal domain ventrally (Fig. 5D-F).

Ectopically induced Pax3 expression by Pax7 disappeared near the floor plate, and Pax3 expression domain expanded ventrally corresponding to that ventral expansion of the tectal swelling by 48 hours after electroporation ($n=4/6$) (Fig. 5K,L). As Shh, a ventralizing factor, emanates from the floor plate, re-specification along dorsoventral axis may have taken place. These results together with those in diencephalon suggest that morphological change in the ventral mesencephalon occurred earlier than that in the diencephalon. In accordance with this idea, differentiation of the ectopic tectum in the diencephalon is behind of the tectum proper (Fig. 2E,F), while no such differentiation gap was found when tectal structure extended ventrally (Fig. 5C,F).

**Lim1** is expressed in the ventral mesencephalon at E4.0, and is thought as a good marker for the tegmentum (Watanabe and Nakamura, 2000). At E4.0 (60 hours after electroporation), Lim1 expression in the ventral mesencephalon made two stripes on the control side; broad lateral stripe and narrow medial stripe (Fig. 5M). On the experimental side, broader stripe was completely missing although narrow stripe remained and the position of narrow stripe did not shift ventrally ($n=2/5$) (Fig. 5M). On the control ventral side, Pax6 expression band is discernible (Agarwala, 2001). The Pax6 expression band disappeared after Pax3 misexpression ($n=3/4$) and after Pax7 misexpression ($n=4/4$, Fig. 5N).
Fig. 5. Pax3/7 misexpression caused fate change of the tegmentum to the tectum. (A-C,D-F) Morphology after Pax7 and Pax3 misexpression at E6.5 and E7.5, respectively. View from the control side (A,D), and view from the experimental side (B,E). A,D are printed in reverse for comparison. (C,F) Horizontal section (the plane is indicated in A,D) stained with Hematoxylin and Eosin. Arrows indicate the boundary between the tectum and tegmentum. The arrowhead indicates the ventral midline. The figures indicate that the tectal structure expanded ventrally after Pax7 and Pax3 misexpression. (G-I) Pax3 induction in the ventral mesencephalon after Pax7 misexpression. In situ hybridization for Pax3 (H), and addition of immunohistochemical staining for Pax 7 (I) on an E2.5 embryo at 24 hours after electroporation. View from the control side, printed in reverse (G). View from the experimental side (H,I). (H, I) High power micrographs of boxed areas in H, I, respectively. The arrow in H, I indicates the same position. (K-N) Ventral view of E3.5 embryos after Pax7 misexpression. In situ hybridization for Pax3 (blue, K) and addition of immunohistochemical staining for Pax7 (brown, L). Pax3 is induced in the tegmentum region (K,L). Arrows in K,L indicate oculomotor nerve roots. Note that the distance from the ventral midline to the oculomotor nerve root is the same on the control and the experimental side, but the distance from the ventral midline to the ventral limit of the Pax3-expressing domain is narrower on the experimental side than on the experimental side (K,L). (M,N) In situ hybridization for Lim1 (blue) and addition of immunohistochemical staining for Pax7 (brown) on an E4.0 embryo, viewed ventrally, at 60 hours after electroporation (M). Lateral band of Lim1 expression in the tegmentum has disappeared on the experimental side (M). In situ hybridization for Pax6 (blue) and immunohistochemical staining for Pax7 (brown) after Pax7 misexpression (N). Pax6 expression arch was also repressed after Pax7 misexpression (N). In K-N, right-hand side is the control side. Scale bars: 1.0 mm in A,D; 500 μm in C,F,L-N); 200 μm in G; 50 μm in H′. di, diencephalon; L, lateral stripe of Lim1 expression; M, medial stripe of Lim1 expression; met, metencephalon; tgm, tegmentum.

**DISCUSSION**

We have shown that (1) in normal development Pax3 and Pax7 expression covers the mesencephalic alar plate; (2) Pax3 and Pax7 misexpression caused fate change of the alar plate of the diencephalic p2 region to that of the mesencephalon, but the p1 region was less affected by Pax3/7 and retained its original fate; (3) Pax3 and Pax7 misexpression resulted in repression of Pax6 expression, and induction of tectum-related genes such as Fgf8, En2 and Pax3 and Pax7 in the diencephalon in a sequential order; (4) Pax3 and Pax7 misexpression in the ventral mesencephalon resulted in repression of tegmentum marker gene and ventral expansion of the tectal territory; and (5) Pax3 and Pax7 misexpression exerted similar effects. As we can use specific antibody for Pax7, we mainly misexpressed Pax7 throughout experiments. Possible function of Pax3 and Pax7 in tectum development is discussed below.

**Pax3/7 induced ectopic tectum in the diencephalon and ventral mesencephalon**

The present study has shown that Pax3 and Pax7 induced ectopic swelling in the diencephalon, and that the swelling had the laminar structure characteristic of the tectum and the torus semicircularis. The ectopically differentiated tectal structure could receive retinal fibers. As torus semicircularis was also differentiated, it was concluded that the alar plate of the presumptive diencephalon changed its fate to the mesencephalic alar plate by Pax3 or Pax7 misexpression. As for dorsoventral axis, the tectal swelling expanded ventrally (see Fig. 5C). One may raise a question if this ventral expansion of the tectum is caused by transformation of the dorsal tegmentum or by overgrowth of the tectum. If the former is the case, dorsal part of the tegmentum may be lost, and if the latter is the case, the tegmental structure is condensed relatively. We paid attention to the Lim1 and Pax6 expression in the ventral mesencephalon. Lim1 is expressed in two stripes; broad lateral stripe and narrow medial stripe (see Fig. 5M). Pax6 is expressed in a band of arch, as suggested by Agarwala et al. (Agarwala et al., 2001). After Pax7 misexpression broader stripe of Lim1 expression was completely missing although narrow stripe remained almost intact. Arch of Pax6 expression was also disappeared (see Fig. 5N). The results indicate that dorsal tegmentum region was missing but ventral tegmentum region was almost intact. Thus, we concluded that ventral expansion of the tectal swelling may not be due to increasing or decreasing of cell proliferation but to fate change of the tegmentum to the tectum.
In normal development, \textit{Pax3} and \textit{Pax7} are expressed almost in an overlapping manner in the mesencephalic alar plate, and \textit{Pax3} and \textit{Pax7} misexpression caused very similar phenotype. These results indicate functional redundancy of \textit{Pax3} and \textit{Pax7} in tectum development as suggested previously from the phenotype of mutant mice (Epstein et al., 1991; Mansouri et al., 1996; Mansouri et al., 1998). Our misexpression study showed that in the dorsal mesencephalon and p1 region, \textit{Pax3} and \textit{Pax7} repressed each other’s expression. At the same time, some regulation mechanism to balance total expression level of \textit{Pax3} and \textit{Pax7} by downregulating or by upregulating each other’s expression may exist. In \textit{Pax3} mutant mice \textit{Pax7} was upregulated in the somite and neural tube (Borycki et al., 1999).

\textit{Pax7} repressed \textit{Pax6} expression in the alar plate of the p2 region, which may mean that \textit{Pax7} repressed diencephalic property to cause re-specification of gene expression cascade toward mesencephalic development. In the p1 region, \textit{Pax6} expression was not affected so much, and most of it differentiated according to its original developmental program, that is, p1 differentiated into the diencephalic pretectum. This difference may be attributed to that both \textit{Pax3/7} and \textit{Pax6} are expressed normally in the p1 region. \textit{Pax3/7} and \textit{Pax6} repress each other’s expression (Matsunaga et al., 2000; present study), but in p1 region there may be some mechanism for each of them to escape from other’s repressive effect. As a consequence, forced expression of these genes would not exert strong effect, and would result in separation of ectopically differentiated tectum from the tectum proper at the p1 region.

**Role of \textit{Pax3/7} in tectum development**

The ectopically introduced \textit{Pax7} may turn off the gene expression cascade toward the diencephalic differentiation, by repressing \textit{Pax6} expression. At the same time, or rather earlier induction of \textit{Fgf8} expression took place. \textit{Fgf8} was broadly induced, and subsequently \textit{En2} expression was induced in the p2 region, not in the p1 region. \textit{Fgf8} and \textit{Otx2} are mutually repressive in their expression (Liu et al., 1999; Martinez et al., 1999; Katahira et al., 2000), but weak signal of \textit{Fgf8} does not affect \textit{Otx2} expression (Sato et al., 2001). In the present study, \textit{Otx2} expression was not affected by ectopically induced \textit{Fgf8} so that ectopic swelling in the p2 region may have differentiated into the tectum. If \textit{Fgf8} signal is strong enough to repress \textit{Otx2} expression in the diencephalon, cerebellum would ectopically differentiate (Martinez et al., 1999; Sato et al., 2001). Fate change to the tectum occurred in the region where ectopic \textit{En} was induced, that is, p2 region differentiated into the tectum, but p1 region kept its original fate after \textit{Pax3/7} misexpression. Expression of \textit{Lim1}, pretectum marker gene, and the persistence of the pretectal nuclei also indicated that the pretectum still remained. The results of the present study, those of \textit{En2} misexpression (Araki and Nakamura, 1999) and those in \textit{En1/2} double mutant mice that show complete deletion of the mesencephalon (Liu and Joyner, 2001) all indicate that the neural tissue should express \textit{En} to differentiate into the tectum.

The results of the present study indicate that the tissue should express \textit{Pax3} and \textit{Pax7} followed by \textit{En} expression to differentiate into the tectum. Exogenously introduced \textit{Pax3} or \textit{Pax7} might not be directly involved in the transformation of presumptive diencephalic alar plate to the mesencephalic property. Introduced \textit{Pax3/7} may first turn off the gene expression program towards diencephalon, then may induce \textit{Fgf8} and \textit{En} expression. At first, \textit{En} expression was found on the entire ectopic swelling, but then, confined to the rostral border of the swelling (see Fig. 4J-Q), which may indicate that the rostrocaudal polarity of the ectopic tectum was established in a mirror image to the tectum proper. Following \textit{En} expression, \textit{Pax3} and \textit{Pax7} expression covered whole the ectopic swelling. The results of transfection experiments with HA-tagged \textit{Pax7} expression vector suggested that expressions of \textit{Pax3} and \textit{Pax7}, covering whole the ectopic swelling, were not expressed from the transfected expression vector, but from the embryonic gene induced sequentially after \textit{En} expression (see Fig. 4L,M). These results together with the chronological order of expression of these genes in normal development indicate that \textit{Pax3} and \textit{Pax7} may take part in mesencephalic development after the \textit{En} expression.

It was suggested that the brain vesicle should express \textit{Otx2}, \textit{En} and \textit{Pax2} to acquire the property to differentiate into the tectum (Nakamura, 2001). We would like to add \textit{Pax3} and \textit{Pax7}, expressed in the mesencephalic alar plate, for the tectum development. \textit{En}, \textit{Otx2} and \textit{Pax2} are expressed or once expressed in both the alar plate and the basal plate of the mesencephalon. In this scenario, addition of \textit{Pax3} or \textit{Pax7} expression at the basal plate may be enough to change its fate to the tectum. This assumption well explains the results that the morphological change was detectable earlier in the basal plate of the mesencephalon than in the diencephalon after \textit{Pax3} and \textit{Pax7} misexpression.

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