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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 where the ectopic tectum structure was formed (Nomura et al., 1998; Araki and Nakamura, 1999). On the other hand when the tectum development was repressed by *Shh* misexpression, *Pax7* expression was repressed in the dorsal mesencephalon (Watanabe and Nakamura, 2000). Therefore, we suspected that Pax3 and Pax7 would work to define the identity of the alar plate of the mesencephalon, and that *Pax3* and *Pax7* misexpression would induce an ectopic tectum. *Pax3* overexpression was carried out in the transgenic mice, in which Pax3 expression was 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 semicricularis, which derives from the caudal part of the mesencephalic alar plate and corresponds to the mammalian inferior corriculus, 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, *Pax3* 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′-TTGTGACATAGCCCGAAAACTT-3′, 5′-TCTT-GCTCGTCGGTTGCTGA-3′ and 5′-AGGCGGACCACTTTCACT-GC-3′, 5′-TTGCTGGAGTGGGTTGTTGG-3′, respectively. These fragments were fused at *Stu*I site to make a full length of *Pax7*. *Pax7* was also fused with HA-tag to make *Pax7-HA*. These fragments were inserted in pMiwIII, a derivative of pMiwSV (Suemori et al., 1990; 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-Cuif et al. (Bally-Cuif 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; Matsunaga 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-indolyl-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 (Kulter), 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 metencephalon (Fig. 2).

Pax7 misexpression caused a morphological change in the diencephalon at 48 hours after electroporation (*n*=34/56). 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 viii 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, whole 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; Grindley et al., 1997; Mastick 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

	Time (hours)						
	6	12	18	24	36	48	96
Pax6		– (3/3)	↓ (2/4)	↓↓ (4/4)		↓↓ (2/2)	↓↓ (4/4)
Fgf8	– (6/6)	↑ (1/4)	↑↑ (5/5)	↑↑ (7/7)	↑↑ (3/3)	↑ (4/9)	– (4/4)
En2			– (3/3)	– (4/4)	↑↑ (7/7)	↑↑ (9/13)	↑ (4/4)
Pax3		– (3/3)	↑ (1/3)	↑ (5/8)		↑ (1/7)	↑↑ (4/4)

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.

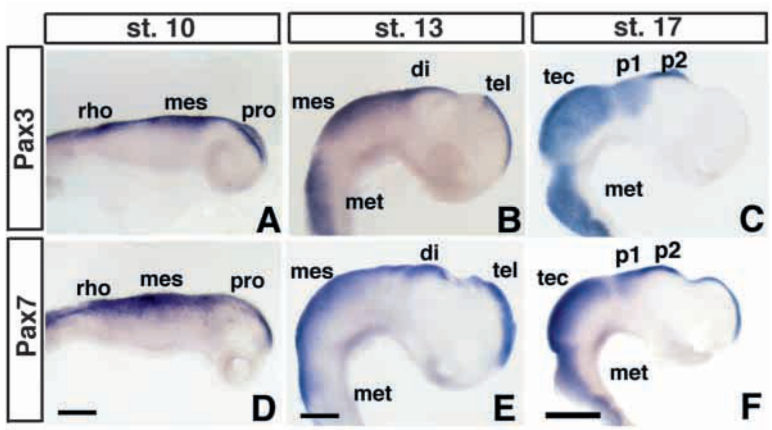

Fig. 1. Normal expression patterns of *Pax3* and *Pax7* in the prosencephalon and the mesencephalon. Whole-mount in situ hybridization for *Pax3* (A-C) and *Pax7* (D-F) at 10-somite stage (A,D), 18-somite stage (HH13) (B,E) and E2.5 (HH17) (C,F). *Pax3* and *Pax7* are expressed near the dorsal midline 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.

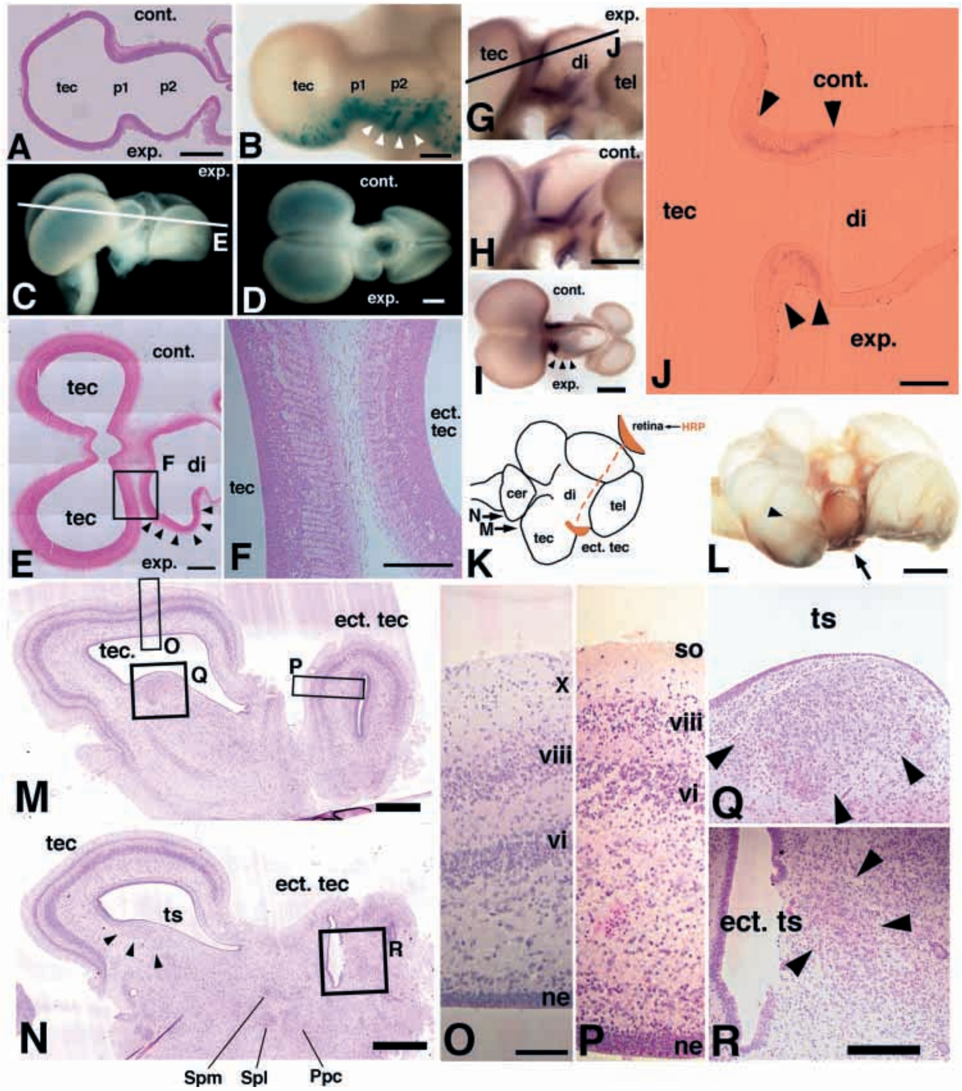

Fig. 2. *Pax3/7* misexpression changed the fate of the alar plate of the diencephalic P2 region to that of the mesencephalon. (A,B) Morphological change at 48 hours after electroporation of *Pax7*. Horizontal section. Hematoxylin and Eosin staining (A). X-gal staining of an embryo at 48 hours after co-electroporation 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 maldifferentiation 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

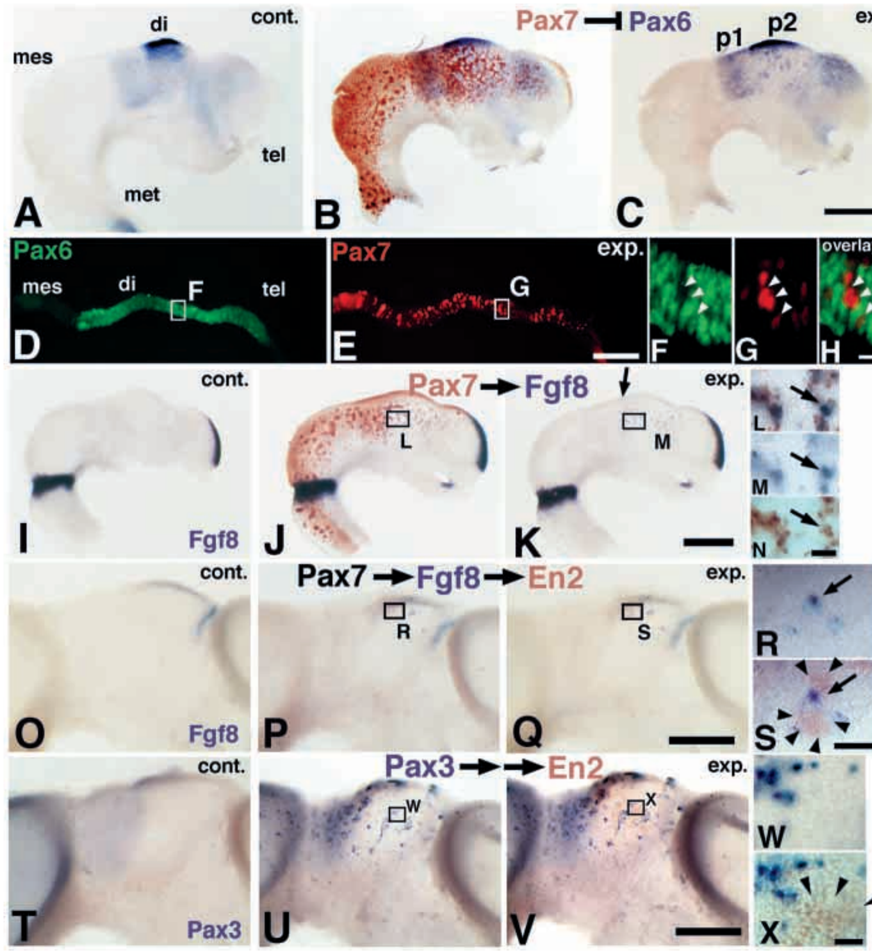

Fig. 3. Effects of Pax7 on expression of tectum related genes. (A-H) Cell autonomous repression of *Pax6* by Pax7. In situ hybridization for *Pax6* (blue), the control (A), and the experimental embryos (C) at 24 hours after electroporation. Immunostaining for Pax7 (brown) was also carried out on the 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 indicated 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*. (Fig. 3R,S). 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. Timecourse 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

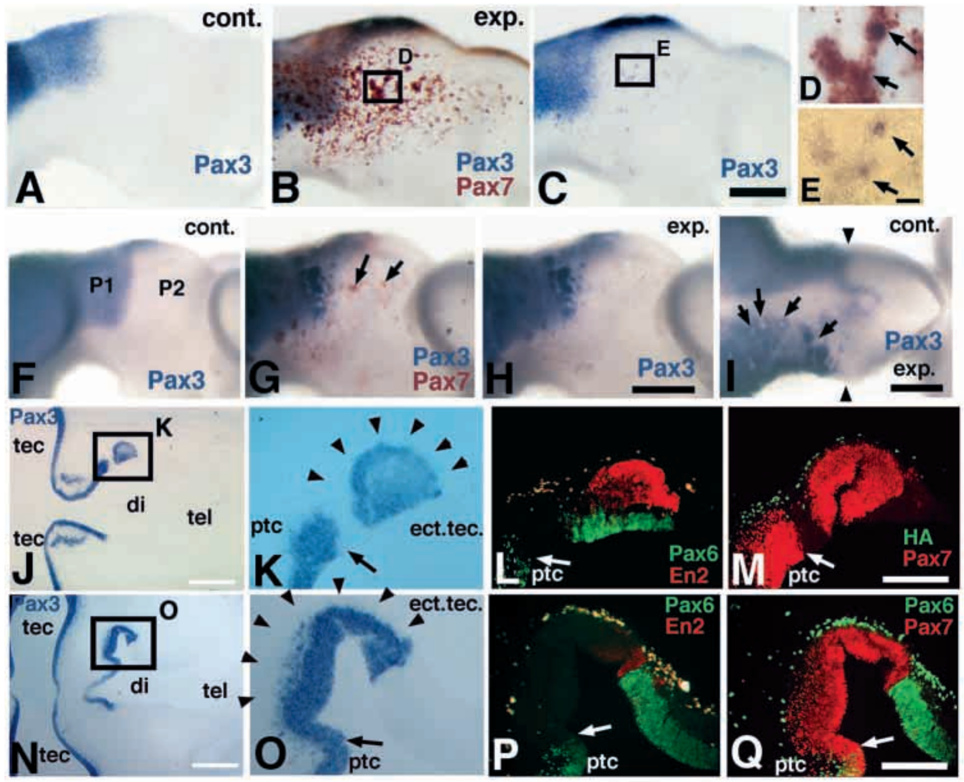

Fig. 4. Interrelationship between *Pax3* and Pax7. (A-I) In situ hybridization for *Pax3* (blue) (A,C,E,F,H,I) and in situ hybridization for *Pax3* and immunohistochemistry for Pax7 (brown) (B,D,G) 24 hours (A-E) and 48 hours (F-I) after electroporation. View from the control side (A,F), view from the experimental side (B,C,G,H), dorsal view (I). The control side is printed in reverse (A,F). High power magnification of boxed area in B,C (D,E). Corresponding arrow in D,E indicates the same position. Arrows in G indicate exogenous expression of Pax7. Arrows in I indicate repression of endogenous *Pax3* expression by Pax7. Arrowheads in I indicate the line corresponding to p1/p2 boundary. (J-Q) Serial sections of the embryo at 96 hours after electroporation. (J-M) An embryo in which the swelling in the diencephalon is small; (N-Q) an embryo in which the swelling had enlarged. (J,N) In situ hybridization for *Pax3*. (L,P,Q) Double staining by immunohistochemistry for Pax6 (green) and for En2 (L,P, red), for Pax7 (Q, red). (M) Immunohistochemistry for HA-tag (green) and for Pax7 (red). (K,O) High power magnifications indicated in J,N. Areas surrounded by arrowheads on panels K and O indicate ectopic swelling, where Pax3 expression was induced. Arrows in K-M,O-Q indicate the approximate position of the boundary between the ectopic swelling and the pretectum. Exogenous Pax7 expression which is identified by HA-tag had disappeared at 96 hours after electroporation (M). Green cells in M are mesenchymal cells (stained for HA). En2 is expressed in almost entire region of the ectopic swelling with rostral-high and caudal-low level when ectopic swelling is small (L). En2 expression is almost absent by the time the ectopic swelling enlarged, leaving at the rostral tip of the swelling (P). Pax7 was induced in the ectopic swelling, making a sharp boundary with Pax6 (Q). This boundary is consistent with the Pax6/En2 boundary (P). Scale bars: 500 μm in H,I,J,N; 250 μm in C; 200 μm in M,Q; 50 μm in E. ptc, pretectum.

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 misexpressed *Pax3* and *Pax7* 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′,I′). *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).

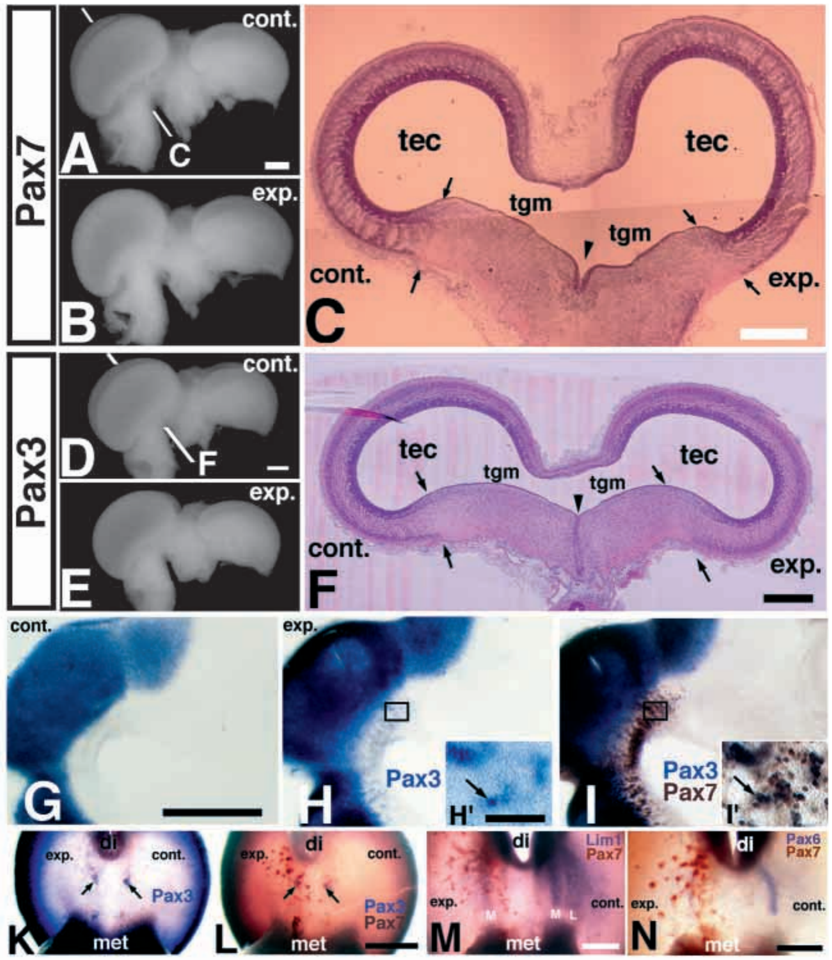

Fig. 5. *Pax3/7* misexpression caused fate change of the tegmentum to the tectum. (A-C,D-F) Morphology after *Pax7* and *Pax3* misexpression at E6.5 and E7.5, respectively. View from the control side (A,D), and view from the experimental side (B,E). A,D are printed in reverse for comparison. (C,F) Horizontal 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, *Pax3* and *Pax7* are expressed almost in an overlapping manner in the mesencephalic alar plate, and *Pax3* and *Pax7* misexpression caused very similar phenotype. These results indicate functional redundancy of *Pax3* and *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, Pax3 and Pax7 repressed each other's expression. At the same time, some regulation mechanism to balance total expression level of *Pax3* and *Pax7* by downregulating or by upregulating each other's expression may exist. In *Pax3* mutant mice *Pax7* was upregulated in the somite and neural tube (Borycki et al., 1999).

Pax7 repressed *Pax6* expression in the alar plate of the p2 region, which may mean that Pax7 repressed diencephalic property to cause re-specification of gene expression cascade toward mesencephalic development. In the p1 region, *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 *Pax3/7* and *Pax6* are expressed normally in the p1 region. Pax3/7 and 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 Pax3/7 in tectum development

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

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

It was suggested that the brain vesicle should express *Otx2*, *En* and *Pax2* to acquire the property to differentiate into the tectum (Nakamura, 2001). We would like to add *Pax3* and *Pax7*, expressed in the mesencephalic alar plate, for the tectum development. *En*, *Otx2* and *Pax2* are expressed or once expressed in both the alar plate and the basal plate of the mesencephalon. In this scenario, addition of Pax3 or 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 *Pax3* and *Pax7* misexpression

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