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Early subdivisions in the neural plate define distinct competence for inductive signals

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SUMMARY

Regionalization of the embryonic brain is achieved through multi-step processes that operate sequentially and/or simultaneously. Localized sources of various signaling molecules act as organizing centers that pattern neighboring fields to create molecularly distinct domains. We investigated the mechanisms underlying the regionally distinct competence for two such organizing signals, Fibroblast growth factor 8 (Fgf8) and Sonic hedgehog (Shh), using chick embryos. First, we demonstrated that FGF receptor 1 (Fgfr1) and Fgfr3, expressed differentially in the developing brain, possess an equivalent potential to induce the regionally distinct Fgf8-responsive genes, depending on the anterior-posterior dimension of the brain. Next we found that homeodomain transcription factors Six3 and Irx3 can alter the regional responses to both Fgf8 and Shh in the forebrain. Six3 confers the ability to express *Bf1*, a gene essential for the telencephalon and eye development, and *Nkx2.1*, which is required for development of the hypothalamus. In contrast, Irx3 confers the ability to express En2 and *Nkx6.1* in response to Fgf8 and Shh, respectively. Furthermore, an alteration in the region-specific response to Fgf8 upon misexpression of *Irx3* resulted in transformation of diencephalic and possibly telencephalic tissues into the optic tectum. Finally, we demonstrated that *Six3* and *Irx3* can mutually repress their expression, which may contribute to the establishment of their complementary expression domains in the neural plate. These repressive interactions are specific, as *Six3* did not repress *Gbx2*, and *Irx3* did not disturb *Otx2* expression. These findings provide evidence that the early embryonic forebrain is demarcated into two domains with distinct genetic programs, which argues against the authentic telen-diencephalic subdivision.

Key words: Forebrain, Chick embryo, Organizing signal, Regionalization, Fgf8, Sonic hedgehog, Competence

INTRODUCTION

There is accumulating evidence that a number of inductive interactions play crucial roles in the specification of cell fates and regulation of regionally divergent histogenesis in the developing central nervous system (CNS) [reviewed in Edlund and Jessell (Edlund and Jessell, 1999)]. For instance, a secreted glycoprotein, Sonic Hedgehog (Shh), emanating from the axial mesendoderm and the ventral midline of the neural tube, has been shown to pattern the ventral territory of the developing neural tube (Chiang et al., 1996) [reviewed in Briscoe and Ericson (Briscoe and Ericson, 1999)]. In fact, a number of signaling molecules, such as members of Fibroblast growth factor (FGF), Transforming growth factor beta (TGFβ), Hedgehog (HH) and Wnt families, are expressed in a regionally restricted manner in the developing brain. These localized sources of signaling molecules are thought to pattern the neighboring fields to create molecularly distinct domains that lead to generation of various tissues in the brain. There is evidence that an inductive signal regulates the expression of distinct sets of transcription factors, depending on its concentration (Roelink et al., 1995; Ericson et al., 1997), so that cells with specific properties are located at certain ranges of distance from a signaling center.

On the other hand, regionally distinct competence for the same signaling molecules also plays an important role in generating further complexities. The way cells respond to an organizing signal depends somehow on their intrinsic properties. For instance, it has been shown that the midline-derived signal or Shh induces distinct ventral neuronal phenotypes along the entire neuraxis [reviewed in Lumsden and Krumlauf, and Tanabe and Jessell (Lumsden and Krumlauf, 1996; Tanabe and Jessell 1996)]. Shh induces motor neurons at the level of the spinal cord, whereas at the forebrain and midbrain levels it induces the hypothalamic neurons and tyrosine hydroxylase-positive neurons, respectively, instead (Roelink et al., 1995; Marti et al., 1995; Ericson et al., 1995; Hynes et al., 1995; Shimamura and Rubenstein, 1997). Furthermore, Nakagawa et

al. (Nakagawa et al., 1996) reported that the neuroepithelial cell lines derived from different regions of the embryonic fore-midbrain each exhibit distinct responses to Shh. Likewise, Fgf8 or the isthmus-derived organizing signal, induces the midbrain and/or cerebellar phenotypes accompanied by expression of homeodomain transcription factor En2 posteriorly, whereas it induces Bf1, a winged-helix transcription factor essential for the telencephalon and eye development (Xuan et al., 1995; Huh et al., 1999) in the anterior forebrain (Martinez et al., 1991; Crossley et al., 1996; Shimamura and Rubenstein, 1997; Ye et al., 1998; Martinez et al., 1999). The border of the distinct responses to Shh and Fgf8 was assumed to be the zona limitans intrathalamica (ZLI) or the boundary between prosomere 2 and 3 (p2/3) (Rubenstein et al., 1998; Rubenstein and Beachy, 1998). However, questions still remain as to whether the boundaries of the distinct responses to Fgf8 and Shh indeed coincide, whether those differences are based upon the same properties of the responding cells, and how such differences are created during development.

In the hindbrain, Hox code of the anterior-posterior patterning system provides an identity to each rhombomeric compartment [reviewed in (Lumsden and Krumlauf, 1996)]. Within each rhombomere, several types of neurons are generated in a position-specific manner through the actions of organizing signals. For instance, the facial motor and contralateral vestibulo-acoustic efferent neurons in rhombomere 4 (r4) are specified by signals emanating from the midline tissues (Simon et al., 1995). *HoxB1* is thought to give identity to r4, since misexpression of this single gene was sufficient to transform r2 into r4, including ectopic generation of the facial motor neurons perhaps by Shh (Bell et al., 1999). Thus, so-called homeotic selector genes are able to control the region-specific responses to the organizing signals, suggesting a molecular explanation for the context-dependent actions of the organizing signals.

In this study, we carried out a series of experiments to gain insight into the mechanisms underlying the regionally distinct competence for the organizing signals in the rostral CNS. Giving first priority to the identification of molecules sufficient to regulate such properties, a restricted gene misexpression system facilitated by in vivo electroporation in chick embryos was employed. This experimental system enabled us to manipulate regional gene expression only in the neuroectoderm. This is particularly important, as misexpression in other tissues such as the mesodermal tissues implicated in the patterning of the CNS (Woo and Fraser, 1997; Muhr et al., 1997; Ensini et al., 1998; Koshida et al., 1998; Gould et al., 1998) may complicate the interpretation of given phenotypes. We identified two molecules that play pivotal roles in regulating the distinct competence for Fgf8 and Shh, thus providing clues for the further analysis of the upstream and downstream events in the regionalization of the brain. Finally, a revision of the subdivision of the developing forebrain is proposed.

MATERIALS AND METHODS

cDNA clones

Full-length clones of zebrafish *Six3* and chick *Irx3* were obtained as described (Kobayashi et al., 1998; Funayama et al., 1999), and those of chick *Pax2* and chick *Six3* were cloned by PCR. Other full-length cDNAs used in this study were kind gifts from following researchers: human *Fgfr1* and mouse *Fgfr3* (A. Rosenthal); mouse *Gbx2* and *Fgf8b* (G. Martin); mouse *Six3* (K. Kawakami); chick *Pax6* (K. Yasuda); chick *Shh* (C. Tabin). Probes for chick *Bf1*, *Gbx2*, *Emx2*, *Otx2*, *Nkx2.1* and *Nkx6.1* were kindly provided by Drs M. Noda, G. Martin, A. Simeone, L. Bally-Cuif, and J. Rubenstein, respectively.

DNA constructs

Mutations that cause the same amino acid replacement as seen in the thanatophoric dysplasia type I (Tavormina et al., 1995) were generated by PCR according to the instructions with a QuickChange™ site-directed mutagenesis kit (Staratagene). Primers used were: 5′CCAGCTGGATGTCGTGGAGTGTTCCCCTCACCGGCCC3′ and 5′GGGCCGGTGAGGGGAACACTCCACGACATCCAGCTGG3′ for *hFgfr1*; 5′CACTGGATGTGCTGGAGTGCTCCCCACACCGGCCC3′ and 5′GGGCCGGTGTGGGGAGCACTCCAGCACATCCAGTG3′ for *mFgfr3*. Those clones were sequenced to confirm the mutations and designated $Fgfr1^{TDI}$ and $Fgfr3^{TDI}$, respectively. A dominant negative form of *mFgfr3* was obtained from the *Hin*dIII-*Alw*44I fragment of the cDNA. A dominant transcriptional activator form of *zSix3* was generated by fusion of the VP16 transcription activation domain as described (Kobayashi et al., 2001). The wild-type and mutant *Fgfr*s and *zSix3*, as well as full-length cDNAs of *cSix3*, *mSix3*, *cIrx3*, *mGbx2*, *cPax2*, *cPax6*, *cShh* and *mFgf8b*, were inserted into the *Xho*I site of pCAGGS vector (Tokui et al., 1997) by blunt ligation for electroporation.

Electroporation

Electroporation for Hamburger and Hamilton stage 8-10 (HH8-10) chick embryos was carried out in ovo as described previously (Funahashi et al., 1999). For younger stages (HH3-7), a method using New Culture (Stern, 1993) was developed. A 2 mm square platinum cathode was embedded in a thick silicon rubber disk glued at the center of a Petri dish, on which an embryo with a glass ring was placed. An anode of the same size was placed with a gap of 4 mm above the cathode, having the embryo inbetween. A culture method using paper rings described previously (Sundin and Eichele, 1992) was also employed by using similar electrodes but with a dam around the cathode. A 5 mg/ml DNA solution was injected into the space between the vitelline membrane and epiblast, and 10 V of DC pulses were then applied for 50 milliseconds 5 times by a CUY21 electroporator (TR Tech, Japan). The fraction of cells that expressed exogenous genes was more than 80% within a targeted area. When more than two genes were co-introduced, equal volumes of each DNA solution (5 mg/ml) were mixed unless otherwise stated. In all cases, pCAGGS-GFP plasmid (Momose et al., 1999) was co-electroporated by mixing 1/10 to the total DNA solution prior to injection. The sites of transgene introduction were monitored by the expression of GFP under an epifluorescence dissecting microscope (Leica MZFLIII) and/or immunohistochemistry or in situ hybridization for transfected genes.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was carried out as described previously (Shimamura et al., 1995). Whole-mount immunohistochemistry was performed as described (Shimamura and Takeichi, 1992). Monoclonal antibodies 4D9, 4C7 and PAX7 obtained from Developmental Studies Hybridoma Bank (Iowa, USA) were used for chick En2, Hnf3β, and Pax7, respectively. Rabbit antiserum against chick Irx3 was obtained by immunizing rabbits with a synthetic polypeptide (SFPQLGYQYIRPLYPAER) and affinity purified using a standard procedure. For histological analyses of long surviving specimens, 5 μm paraffin sections were made and stained for thionine by a standard protocol. Photomicrographs were taken with a CCD camera (Hamamatsu C5810), and captured images were assembled by Photoshop® software (Adobe).

RESULTS

Different FGF receptors are not responsible for the distinct responses to Fgf8 in the neural plate

It is known that FGF signaling is mediated via four different receptors. It has been reported that those four FGF receptors (FGFR) are expressed differentially in the developing chick nervous system (Wilke et al., 1997; Walshe and Mason, 2000). Those receptors as well as their multiple isoforms exhibit distinct binding affinities to various FGF ligands in vitro [reviewed in Johnson and Williams, and Ornitz et al. (Johnson and Williams 1993; Ornitz et al., 1996)]. While distinct activities of FGFR for differentiation and proliferation in skull development have been suggested (Iseki et al., 1999), their respective roles in brain development remain elusive. Thus, we first asked whether different FGFR mediate distinct actions of Fgf8 in the developing brain. To address this question, we generated a constitutively active FGFR. Collections of spontaneous mutations in *Fgfr1*, *Fgfr2* and *Fgfr3* loci have been identified in human genetic disorders, most of which lead to defects in bone development [reviewed in Webster and Donoghue (Webster and Donoghue, 1997)]. Among them, a missense mutation found in thanatophoric dysplasia type I (TDI) changed Arg248 to Cys of Fgfr3, and was shown to activate the signaling cascade in a ligand-independent manner (Tavormina et al., 1995; Naski et al., 1996). We chose *Fgfr3*, which was reported to be expressed in the midbrain but not in the forebrain in the developing chick brain (Wilke et al., 1997; Walshe and Mason, 2000), and *Fgfr1* as a control whose expression is found ubiquitously. The same amino acid substitution as in the *TDI* mutation was introduced into *Fgfr1* and *Fgfr3* cDNA, and HH8-10 chick embryos were transfected with those expression constructs by in ovo electroporation. When those constructs were misexpressed in the anterior mesencephalon and the posterior diencephalon, both activated receptors similarly induced En2 ectopically, a marker for the midbrain and anterior hindbrain (Fig. 1B,C; $Fgfr3^{TDI}$, n=33; $Fgfr1^{TDI}$, n=23). Those ectopic En2-expressing cells were strictly localized within cells expressed co-electroporated *GFP*, suggesting that these effects are cell-autonomous. In the specimens that survived until more advanced stages (approx. HH30), enlarged tecta at the expense of the posterior diencephalon were often observed (data not shown). We also observed an equivalent ability of the *Fgfr1* and *Fgfr3* in inducing *Bf1*, a marker gene for the anterior-dorsal prosencephalic structures, as described in the later section (Fig. 3G; data not shown). Neither of the wild-type *Fgfr*s induced En2 nor caused morphological alterations of the brain. These findings indicate that the actions of Fgf8 in the patterning of developing brain documented previously can indeed be mediated by FGFR, and the differential expression of *Fgfr1* and *Fgfr3* does not account for the regionally distinct responses to Fgf8.

On the other hand, we found that neither of the activated receptors induced En2 anterior to the ZLI, even when they were expressed anteriorly, which is reminiscent of *Fgf8* (Fig. 1C-F; data not shown for *Fgfr3*). These findings are consistent with the previous observation that the anterior forebrain is not competent to express En2 in response to the isthmus-derived organizing signal(s) or Fgf8 (Martinez et al., 1991; Crossley et al., 1996; Shimamura and Rubenstein, 1997; Martinez et al., 1999).

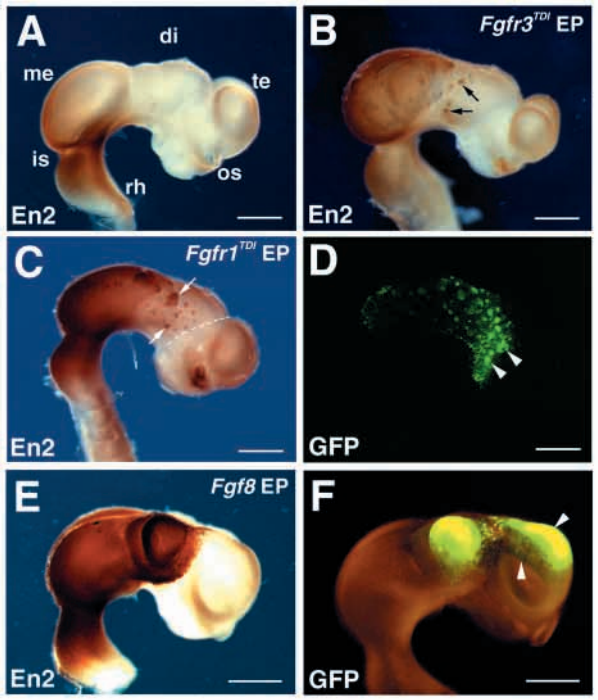

Fig. 1. Induction of En2 by FGF signaling. Dissected brains from HH19-21 chick embryos whole-mount immunostained for En2. Electroporation (EP) was done at HH8 with *GFP* only (A), constitutively active *Fgfr3* (B), constitutively active *Fgfr1* (C) and *Fgf8b* (E) in the fore-midbrain region. Areas of the transgene expression were monitored by expression of *GFP* co-electroporated (D,F). Ectopic En2 expression is detected in the posterior diencephalon (arrows in B,C), and bordered by the zona limitans intrathalamica (ZLI; dashed lines in C), although the transgenes are expressed more anteriorly (arrowheads in D,F). Bars, 0.5 mm. di, diencephalon; is, isthmus; me, mesencephalon; os, optic stalk; rh, rhombencephalon; te, telencephalon.

Misexpression of transcription factors altered the regionally distinct responses to Fgf8

It was reported that members of the vertebrate homolog of *Drosophila iroquois* complex (*iro-C*) gene family (*Irx*) are expressed posterior to the ZLI (Bosse et al., 1997). In *Drosophila*, *iro-C* genes are thought to be the part of a 'pre-pattern' that governs the localized expression of the proneural *achaete-scute* genes that determine the sites at which neural precursors arise (Gómez-Skarmeta et al., 1996; Leyns et al., 1996). Recent analysis also showed that they function as a dorsoventral compartment selector gene in *Drosophila* eye discs (Cavodeassi et al., 1999). We have cloned a chick homolog of *Irx* genes (*Irx3*) and confirmed that the anterior margin of the *Irx3*-expressing domain is delineated by the ZLI or p2/3 boundary that is recognized morphologically and molecularly (Fig. 2A-C). We asked then whether *Irx*3 determines the competence for Fgf8 signaling specific to the posterior forebrain. When the *Irx3* gene was electroporated anterior to the ZLI, ectopic patches of En2-expressing cells were observed (Fig. 2D; n=29/30). While those ectopic En2-positive cells were usually found somewhat sporadically, several bulges with clusters of En2-positive cells were occasionally formed (Fig. 2D; n=4). Interestingly, these ectopic En2-positive cells were found only in the vicinity of the *Fgf8*-

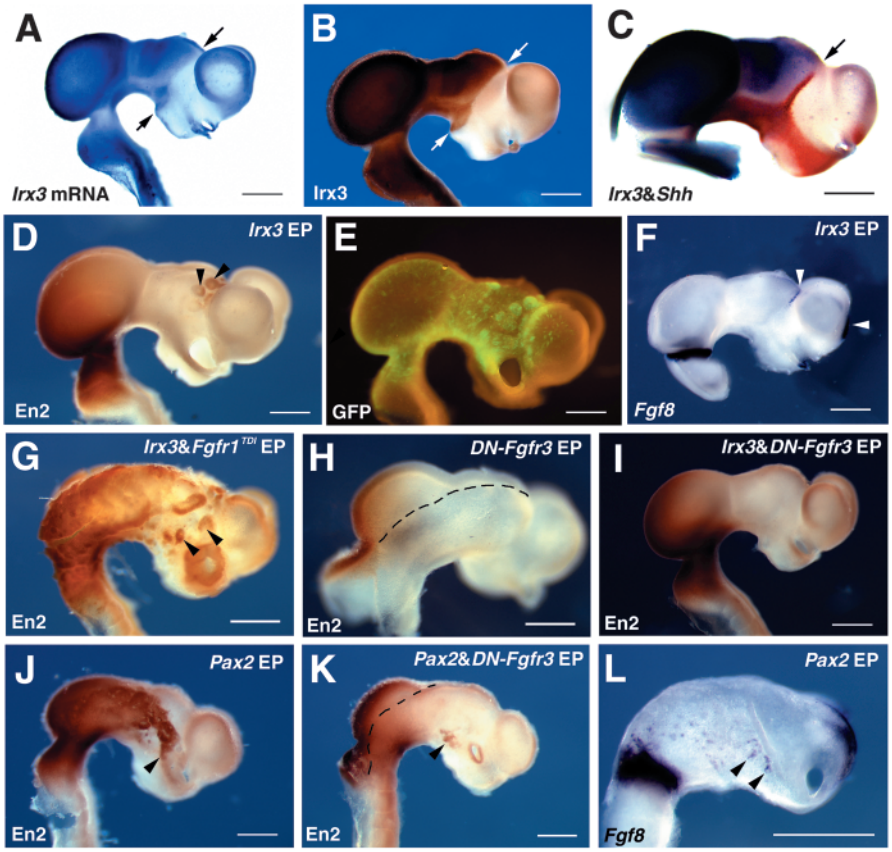

Fig. 2. *Irx3* provides the posterior competence for Fgf8. Distribution patterns of *Irx3* mRNA (A) and its protein (B) in HH21 embryo. (C) Two-color in situ hybridization for *Irx3* (purple) and *Shh* (red) in an HH19 dissected brain. The anterior border of the *Irx3*-expressing domain is delineated by the ZLI in which *Shh* is expressed (arrows in A-C). HH16-21 dissected brains were immunostained for En2 (D,G,H-K) or hybridized for *Fgf8* (F,L). Electroporation was done at HH8 with *Irx3* (D-F), *Irx3* and constitutively active *Fgfr1* (G), dominant-negative *Fgfr3* (H), *Irx3* and dominant-negative *Fgfr3* (I), *Pax2* (J,L) and *Pax2* with dominant-negative *Fgfr3* (K). The expression vectors for the dominant-negative *Fgfr3* and *Irx3* or *Pax2* were mixed at 9:1 prior to injection. H is an oblique posterior view of the specimen, and the dorsal midline is indicated by dashed lines in H and K, highlighting that the right side (electroporated) of the midbrain region has shrunk and the expression of En2 is severely downregulated. Note that ectopic En2 expression (arrowheads in D) is found only in the vicinity of the *Fgf8*-expressing sites (arrowheads in F), despite broad expression of the transgenes as visualized by GFP fluorescence (E). Bars, 0.5 mm.

expressing sites, despite the widespread expression of the transgene. *Fgf8* is abundantly expressed in the roof plate of the telencephalon, and subsequently in a dorsal portion of the ZLI by HH19 in the forebrain (Crossley et al., 1996) (Fig. 2F). This observation is consistent with the idea that *Irx3* regulates the response to Fgf8 (Fig. 2D-F). To further verify this possibility, we first examined whether the constitutively active FGFR enhances induction of En2 by *Irx3*. When $Fgfr3^{TDI}$ and *Irx3* were co-electroporated, many patches of En2-expressing cells were detected anterior to the ZLI, including regions distant from the *Fgf8*-expressing sites, such as the ventral thalamus and hypothalamus (Fig. 2G; *n*=16/16). Finally, we co-electroporated a truncated *Fgfr3*, which has been shown to block FGF signaling in a number of experimental systems

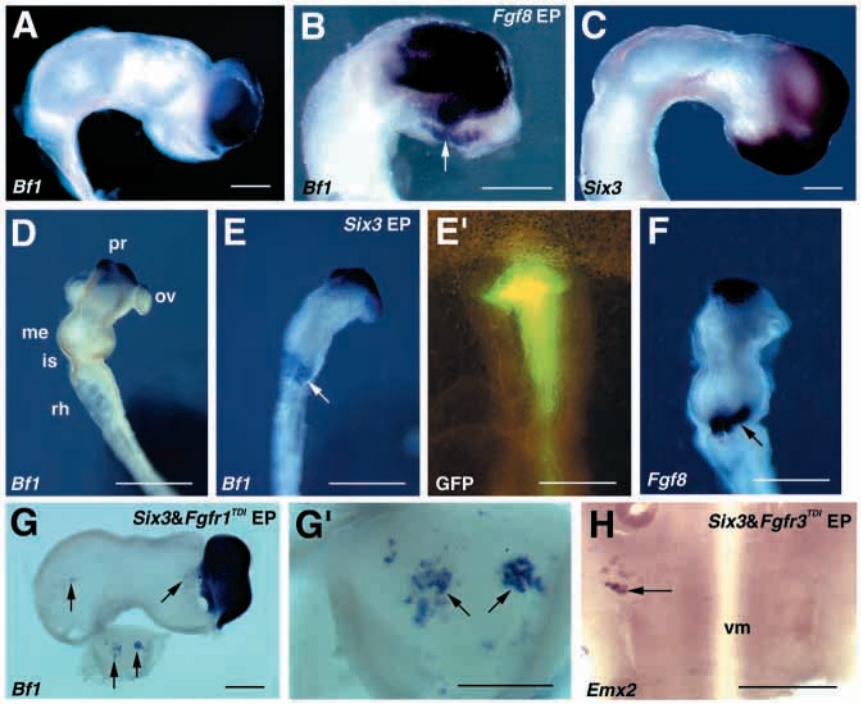

Fig. 3. *Six3* confers the anterior competence for Fgf8. Dissected brains from HH19 embryos that had been electroporated at HH8 with *GFP* (control; A) and *Fgf8b* (B) were in situ hybridized for *Bf1*. (C) Normal expression of *Six3* in a HH19 dissected brain. Ectopic *Bf1*-expressing domains are detected in the hypothalamic region (arrow in B). Dissected brains from HH13 embryos were stained for *Bf1* (D, control; E, *Six3*-misexpressed) and for *Fgf8* (F, normal embryo). Note an ectopic *Bf1*-expressing domain at the mid-hindbrain boundary where *Fgf8* is expressed (arrows in E,F). Embryos were cultured by New's method and electroporation was done at HH5. Note the efficient introduction of exogenous genes into the entire brain (E′). (G,H) HH25 dissected brain to which *Six3* with constitutively active *Fgfr1* (G,G′) or *Fgfr3* (H) were introduced at HH10, stained for *Bf1* (G,G′) and *Emx2* (H). The specimen was flat-mounted to expose the anterior hindbrain region, showing the experimental side on the left (H). Ectopic *Bf1*- or *Emx2*-expressing cells are detected (arrows in G,G′,H). Bars, 0.5 mm. is, isthmus; me, mesencephalon; ov, optic vesicle; pr, prosencephalon; rh, rhombencephalon; vm, ventral midline.

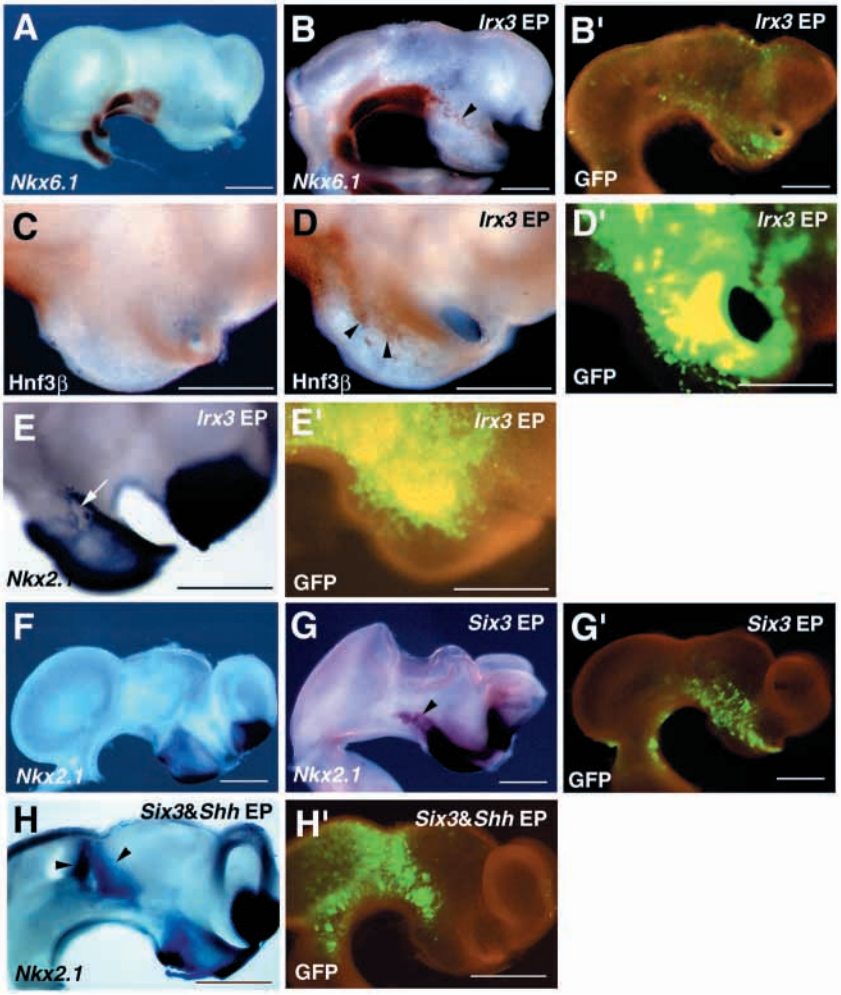

Fig. 4. *Six3* and *Irx3* alter the regional responses to Shh. HH21 dissected brains normal (A,C,F), *Irx3*- (B,D,E), *Six3*- (G) and *Six3* and *Shh*-misexpressed (H), were in situ hybridized for *Nkx2.1* (E-H) or *Nkx6.1* (A,B), or immunostained for Hnf3β (C,D). Ectopic expression of *Nkx2.1*, *Nkx6.1* and Hnf3β, and the repression of *Nkx2.1*, are indicated by arrowheads (B,D,G,H) and an arrow (E), respectively. The sites of transgene expression were detected by GFP fluorescence (B′,D′,E′,G′,H′). Bars, 0.5 mm.

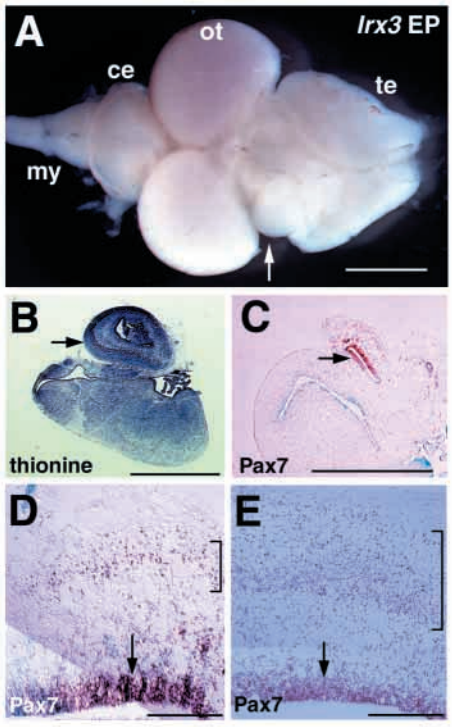

Fig. 5. Misexpression of *Irx3* resulted in an ectopic tectum formation in the forebrain. (A) Dorsal view of a dissected brain of a HH39 embryo that had been electroporated with *Irx3* in the forebrain region at HH11. A small bulge is visible on the right side (electroporated) of the diencephalic region (arrow in A). (B) Thionine staining and (C-E) immunostaining for Pax7 of coronal sections of the ectopic bulge (B-D) and the normal tectum (E). (D,E) High-power views of the sections, in which the ventricle is located at the bottom of the panels. The ectopic bulges exhibit layered organization (arrow in B), and expression of Pax7 in both the ventricular zone (arrow in C,D) and some upper layers of cells (bracket in D), which resembles the optic tectum (arrow and bracket in E). Bars, 0.5 mm. ce, cerebellum; my, myelencephalon; ot, optic tectum; te, telencephalon.

(Amaya et al., 1991) (Fig. 2H). This dominant-negative FGFR completely abolished ectopic expression of En2, which would have been induced by *Irx3* alone (Fig. 2I; *n*=19).

To examine the specificity of this phenomenon, we compared it with the case of *Pax2*, which was also shown to regulate mid-hindbrain genes including En2 (Araki and Nakamura, 1999; Okafuji et al., 1999). First, when *Pax2* was introduced into the anterior forebrain, it induced En2 ectopically as reported previously (Fig. 2J; *n*=11). Notably, *Pax2* induced En2 not only in the regions anterior to the ZLI but also away from the *Fgf8*-expressing sites, unlike *Irx3* (Fig. 2J). Secondly, this ectopic induction of En2 was not completely blocked, but was reduced to some extent by the dominant-negative *Fgfr3* (Fig. 2K; *n*=7). Finally, we found that *Pax2*, but not *Irx3*, induced *Fgf8* expression ectopically (Fig. 2F, arrowheads in L; *n*=4/4, 0/11, respectively). Therefore, we conclude that *Pax2* induces En2 by both Fgf8-dependent and -independent mechanisms, whereas *Irx3* induces En2 by an Fgf8-dependent mechanism.

We then asked what regulates the anterior competence for Fgf8, namely expression of *Bf1*. To reveal the competent region to express *Bf1* in response to *Fgf8*, *Fgf8* was overexpressed in a broad domain in the fore-midbrain. As a result, the *Bf1*-expressing domain expanded to include the anterior hypothalamus and optic stalk, but the posterior prosencephalon, such as the dorsal thalamus and pretectum, was always excluded (Fig. 3B). A homeodomain transcription factor Six3 is expressed in the presumptive prosencephalic region from HH5 (Bovolenta et al., 1998) (Fig. 6A). After HH8, the *Six3*-expressing domain is progressively restricted to subregions of the forebrain, such as the optic vesicles, optic stalks, anterior hypothalamus, and basal telencephalon as reported previously (Oliver et al., 1995; Bovolenta et al., 1998) (Fig. 3C). These domains were well correlated with the sites of the ectopic *Bf1* induction, raising the possibility that Six3 may be involved in this phenomenon. We thus examined *Bf1* induction by misexpression of *Six3*. First, we modified the New Culture technique of a whole embryo culture system for electroporation to overcome

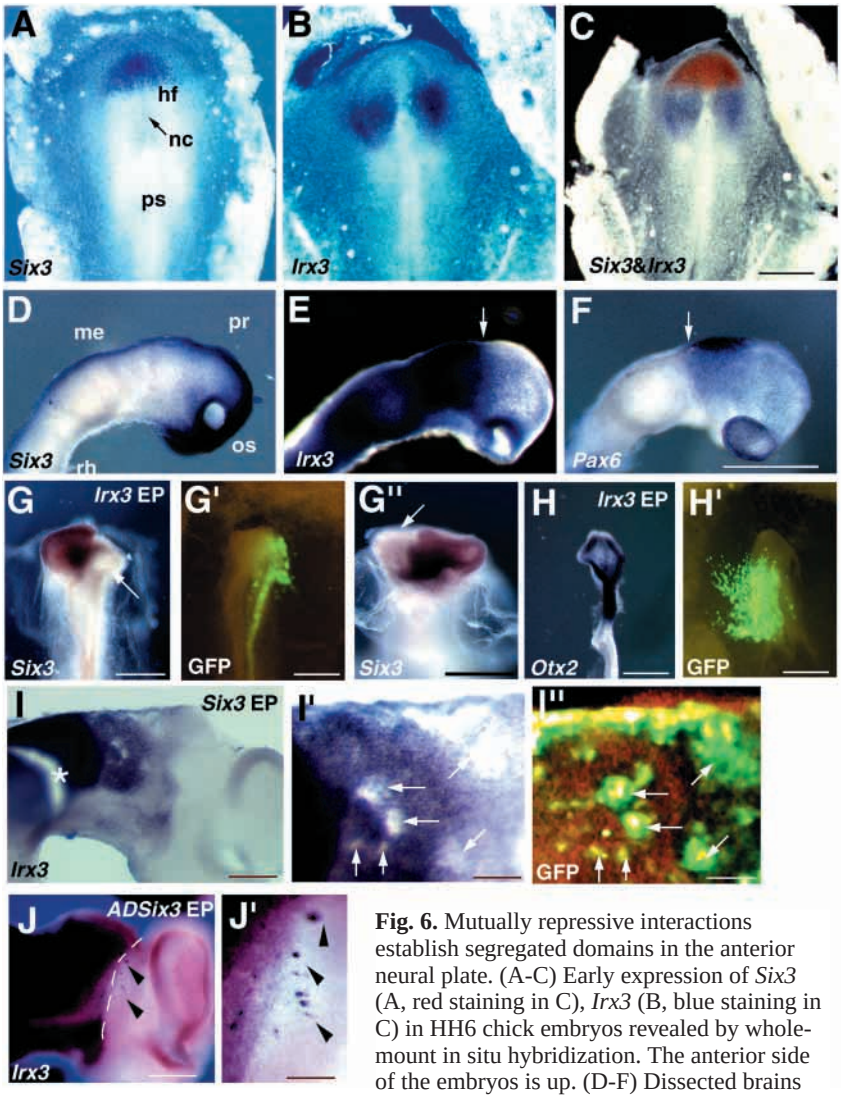

Fig. 6. Mutually repressive interactions establish segregated domains in the anterior neural plate. (A-C) Early expression of *Six3* (A, red staining in C), *Irx3* (B, blue staining in C) in HH6 chick embryos revealed by whole-mount in situ hybridization. The anterior side of the embryos is up. (D-F) Dissected brains from HH13 embryos in situ hybridized for *Six3* (D), *Irx3* (E), and *Pax6* (F). Note that the anterior border of *Irx3* expression (arrow in E) is located anterior to the posterior boundary of the *Pax6*-expressing domain (arrow in F). (G-J) *Six3* (G,G″) or *Otx2* (H) expression in presumptive HH10 embryos that had been electroporated with *Irx3* at HH4. HH21 dissected brains that had been electroporated with *Six3* (I,I′) and *ADSix3* (J,J′) at HH8 stained for *Irx3*. Dorsal (G,G′,H,H′), frontal (G″), and lateral views (I-J′) of the specimens. Arrows indicate sites where *Six3* or *Irx3* expression was suppressed (G,G″,I′,I″), and arrowheads mark ectopic expression of *Irx3* in the ventral thalamus (J,J′). Asterisk in I indicates a crack in the specimen. The ZLI is represented by dashed lines in J. The expression of transgenes is monitored by GFP fluorescence (G′,H′,I″). Bars, 0.5 mm (A-H), 0.25 mm (I′,I″,J′). hf, head fold; nc, notochord; ps, primitive streak.

difficulties in placing the electrodes in ovo at the early stages. The onset of transgene expression was detected within 3 hours after electroporation, as in the case of in ovo operation. When *Six3* was introduced in a broad domain including the midbrain and hindbrain, ectopic expression of *Bf1* in the mid-hindbrain junction was detected (Fig. 3E; *n*=6/7), which corresponds to where *Fgf8* is expressed (Fig. 3F). *Six3* did not induce *Fgf8* (data not shown; *n*=6). To confirm the involvement of FGF signaling, we examined the effects of Six3 and the constitutively active FGFR in embryos at HH8-10, as the introduction of the constitutively active *Fgfr*s at the early stages often caused severe deformation of the embryos, which compromised topological assessment. In those embryos, ectopic induction of *Bf1* was no longer obvious by *Six3* alone (data not shown). When *Six3* and $Fgfr1^{TDI}$ were co-introduced, however, several patches of cells expressing *Bf1* were observed at ectopic locations away from the *Fgf8*-expressing sites including the ventral thalamus, midbrain, and anterior hindbrain (r1 and 2) (Fig. 3G; *n*=5/16). The same finding was obtained with *Six3* and $Fgfr3^{TDI}$, again confirming the equivalent abilities of these FGFR in this system (*n*=3/8; data not shown). In addition, under these experimental conditions, ectopic expression of *Emx2* was observed in the anterior hindbrain (Fig. 3H; *n*=3/11). Neither of the active *Fgfr*s alone induced *Bf1* in these ectopic locations (data not shown).

The regionally distinct competence for Shh

Distinct competence is also evident for Shh, another signaling molecule that is essential for specification of the ventral properties of the entire CNS. For instance, a homeodomain transcription factor Nkx2.1, required for development of the hypothalamus (Kimura et al., 1996) is induced by Shh in the prosencephalic neural plate, whereas Nkx6.1, essential for somatic motor neurons (Sander et al., 2000) is induced in the more posterior neural plate (Ericson et al., 1995; Dale et al., 1997; Shimamura and Rubenstein, 1997; Qiu et al., 1998). As the border of these responses approximated to the p2/3 boundary (Shimamura and Rubenstein, 1997; Qiu et al., 1998), we asked whether *Irx3* and *Six3* can also regulate this property. When the anterior forebrain was forced to express *Irx3*, many patches of cells that express *Nkx6.1* emerged anterior to the p2/3 boundary (Fig. 4B; *n*=6/18). In addition, Hnf3β, another posterior marker by HH20 (Dale et al., 1997) (Fig. 4C), was also expressed anteriorly (Fig. 4D; *n*=3/5), whereas *Nkx2.1* was downregulated in some small patches of cells upon misexpression of *Irx3* (Fig. 4E; *n*=6/18).

Conversely, when *Six3* was introduced into the posterior diencephalon, the domain of *Nkx2.1* expression expanded posteriorly beyond the p2/3 boundary (Fig. 4G; *n*=4/6). In similarly electroporated specimens, *Nkx6.1* expression in the posterior diencephalon was not significantly suppressed by exogenous *Six3* (data not shown; *n*=5/5). This was also the case for specimens electroporated at earlier stages (HH4; data not shown; *n*=4), suggesting together that *Six3* was not able to perturb the onset or maintenance of *Nkx6.1* expression.

Importantly, in both cases, the ectopic expression of the target genes was restricted to within the basal plate where Shh acts, despite the widespread expression of exogenous *Six3* and *Irx3*, consistent with the idea that those genes do not induce

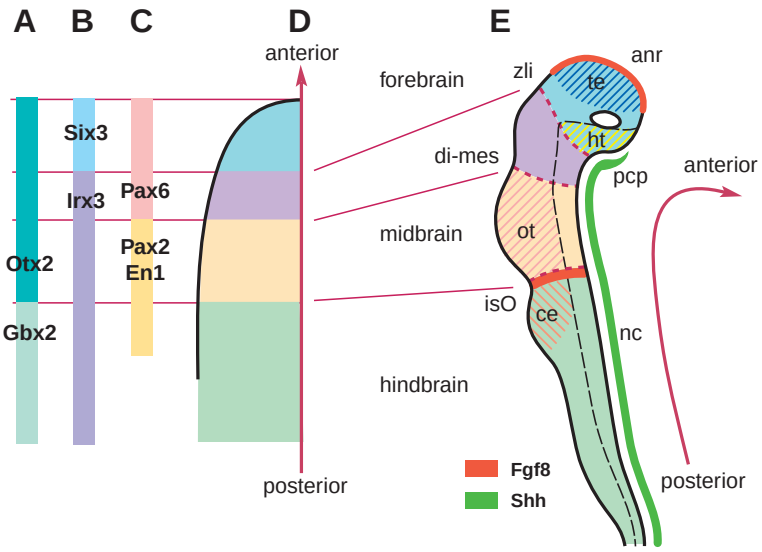

Fig. 7. A model for the early regionalization of the chick anterior neural plate. During HH4-6, a system that involves Otx2 and Gbx2 sets up domains and a boundary corresponding to the prospective mid-hindbrain boundary where the isthmic organizer (isO) develops (A) (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). At around the same time, mutual repression between Irx3 and Six3 positions a boundary within the prospective forebrain, which later coincides with the ZLI (B). Slightly later, mutual repression between Pax6 and Pax2/En1 defines an intermediate boundary between the above two that corresponds to the dien-mesencephalic boundary (C) (Matsunaga et al., 2000). As these systems function independently, the early anterior neural plate accordingly acquires three boundaries at different anterior-posterior levels, being subdivided into four discrete domains potentially by a combinatorial code of transcription factors (D). Subsequently, localized signals produced from various signaling centers (e.g. anr, isO, pcp, nc) create fields of distinct histogenetic properties (e.g. te, ht, ot, ce; E). The longitudinal axes of the neural plate and tube are indicated by red arrows. anr, anterior neural ridge; ce, cerebellum; di-mes, dien-mesencephalic boundary; ht, hypothalamus; isO, isthmic organizer; nc, notochord; ot, optic tectum; pcp, prechordal plate; te, telencephalon; zli, zona limitans intrathalamica.

the marker genes directly, but alter the way cells respond to Shh. In fact, co-electroporation of *Shh* and *Six3* resulted in emergence of a large domain of *Nkx2.1* expression around the dien-mesencephalic boundary in the alar plate (Fig. 4H; *n*=2/2). Neither exogenous *Irx3* nor *Six3* affected the expression of endogenous *Shh* in the basal plate (data not shown).

Irx3 can transform the dorsal diencephalon into a tectum-like structure

Having established that Irx3 confers the posterior competence for Fgf8, we then asked whether this type of molecular alteration indeed leads to conversion of the tissue fates in the brain. We therefore analyzed the later phenotypes of the *Irx3*-electroporated specimens. Abnormal bulges were obvious in the gross morphology of embryos electroporated with *Irx3* at HH8 that survived until HH38-40 (Fig. 5A; *n*=6). Histological analysis revealed that these bulges exhibited cell organization characteristic to the optic tectum. Several layers of cells were recognized in the bulge (Fig. 5B). The choroidal tissue was sometimes seen to invade the lumen of the vesicle. In addition, we found that Pax7 was expressed in the ventricular layer of the structure, reminiscent of the optic tectum (Fig. 5C-E). Furthermore, there were Pax7-positive small cells aligned in upper layers of the ectopic vesicle, which resembled the normal tectum (Fig. 5D,E). Since Pax7 is not expressed in the ventricular layer of prosomere 2 or 3 (Matsunaga et al., 2000), we concluded that the progenitor cells in the anterior diencephalon had acquired molecular properties of the mesencephalon that led to the histogenesis characteristic of the optic tectum.

Since the expression of the transgenes is transient, identification of the precise origins of the transformed tissues was not possible. Nonetheless, through careful inspection of the serial sections of those specimens, it appeared that the dorsal parts of prosomere 2, 3 and possibly 4, where *Irx3* is not normally expressed, were transformed into the ectopic tectum-like structure. For instance, the medial habenullar region was clearly absent (data not shown), providing evidence that the fate of the dorsoanterior part of prosomere 2 was altered.

Irx3 and *Six3* define mutually exclusive domains in the early neural plate

Although *Six3* and *Irx3* start to be expressed in domains that slightly overlap, as development proceeds they become segregated (Fig. 6A-E). *Six3* expression is subsequently localized in subregions of the anterior forebrain (Fig. 6D; also see Fig. 3C), whereas *Irx3* expression appears to stay constant, bordered by a boundary distinct from the posterior border of the *Pax6*-expressing domain (Fig. 6E,F). This early expression profile suggested that there may be a regulatory relationship between these transcription factors. When *Irx3* was misexpressed anteriorly at the onset of *Six3* expression (HH4), *Six3* expression was severely reduced, whereas *Otx2*, another anterior marker, was unaffected (Fig. 6G,H; *n*=6/6 and 4/4, respectively). Likewise, forced expression of *Six3* in the *Irx3*-expressing domain at both HH4 and HH8 resulted in emergence of patches of cells devoid of *Irx3* expression (data not shown; Fig. 6I; *n*=14/14 and 6/15, respectively), consistent with the recent finding that Six3 acts as a transcriptional repressor in the eye and forebrain development in zebrafish (Kobayashi et al., 2001). Moreover, electroporation of a dominant transcriptional activator form of *Six3*, which was shown to cause the opposite phenotype to *Six3* in zebrafish (ADSix3) (Kobayashi et al., 2001), into the anterior forebrain led to ectopic expression of *Irx3* anterior to the ZLI (Fig. 6J; *n*=4/18).

It was shown that the early neural plate is subdivided into adjacent domains by the mutually repressive interactions of transcription factors. For instance, *Otx2* and *Gbx2* repress each other's expression (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000), and similar interactions were demonstrated between *Pax6* and *Pax2* or *En1* (Araki and Nakamura, 1999; Matsunaga et al., 2000). The overlapping zones among *Otx2*/*Gbx2*, *Pax6*/*Pax2*, and *Six3*/*Irx3* systems

(for instance, see Fig. 6D-F) suggest that each functions independently from the others. In fact, when *Six3* was misexpressed in the hindbrain neural plate at HH5, the pattern of *Gbx2* expression was indistinguishable from the controls up to 24 hours (approx. HH12) after electroporation (data not shown; *n*=9/9). Conversely, exogenous *Gbx2* did not affect *Six3* expression in the anterior neural plate under the same experimental conditions (data not shown; *n*=8/8), whereas it repressed *Otx2* expression, as reported previously (data not shown; *n*=2/2) (Katahira et al., 2000). Likewise, when *Pax2* was misexpressed in the prosencephalic neural plate, *Six3* expression was unchanged up to 24 hours after electroporation (data not shown; *n*=5/5).

DISCUSSION

During the last decade, secreted signaling molecules have received much attention in many areas of biological sciences. These signaling molecules are thought to mediate activities of the local organizing centers in many aspects of animal development. The same signaling system induces a particular set of molecules and leads to specific biological responses, depending on the cellular and developmental contexts.

Our results showed that the activities of Fgf8 in brain patterning can be recapitulated at least in part by the downstream machinery of FGFR, although which is the endogenous agent among these receptors is still unclear. We do not know, furthermore, what exactly happens downstream of FGFR that leads to differential gene expression. One possibility could be that Six3 and Irx3 somehow switch different signal transduction pathways downstream of FGFR. Riou and colleagues have recently reported that PLCγ-dependent and Raf-dependent signaling pathways downstream of FGFR are both involved in the distinct aspects of the CNS patterning (Umbhauer et al., 2000). However, the fact that *Six3* and *Irx3* were also capable of altering the response to Shh signaling, which uses a signal transduction pathway distinct from FGF, rather favors the idea that the switching occurs beyond the signaling pathways. It also could be that Six3 and Irx3 regulate other extracellular signals or cofactors that function in combination with FGF or Shh signaling. Although the in vivo electroporation method often creates unevenly distributed transfectants in the target fields, this mosaicism is not completely reliable due to the unstable nature of transgene expression. It is thus not suitable for precise assessment of cell autonomy. Nevertheless, the situation does appear to discount the possibility that they induced extracellular cofactors acting non-cell-autonomously, as the ectopic expression of the target genes (i.e. *Nkx2.1*, *Bf1*) was strictly within the cells that expressed transgenes (i.e. *Six3*, *Irx3*, *GFP*). Perhaps a more plausible possibility is that such differences are determined within the nucleus. Recent analysis in *Drosophila* has shown that a combinatorial code, which includes the actions of signaling and transcription factors, determines a specific cell fate by regulating the distinct enhancer elements (Halfon et al., 2000). Possible functions of Six3 and Irx3 are discussed below.

Regionally distinct competence defined by homeodomain transcription factors

In *Drosophila*, it was shown that *iroquois* complex (*iro-C*) genes confer the dorsal identity of the eye and head cuticle by suppressing the ventral fate, thus functioning as a compartment selector gene (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi et al., 1999; Pichaud and Casares, 2000). Recent analysis in the spinal cord has also revealed the context-dependent patterning activities that involve *Irx3* (Briscoe et al., 2000): in the presence of Irx3, a homeodomain transcription factor Nkx6.1 assembled expression of molecular markers indicative of V2 interneurons, whereas it induced the motor neuron (MN) phenotype in the absence of Irx3 (Briscoe et al., 2000). Since *Nkx6.1* and both MN and V2 characteristics are induced by Shh in the spinal cord (Ericson et al., 1997; Qiu et al., 1998; Briscoe et al., 2000), this phenomenon can be regarded as another example of *Irx3* controlling the cellular context for the inductive actions of Shh.

While we showed that *Six3* and *Irx3* did alter competence for Fgf8 and Shh, the correlation between their expression and the induction of the target genes responsive to those signals was not entirely perfect, even in the presence of the signals. For instance, electroporation of *Six3* with *Shh* in the posterior diencephalon exhibited a rather small domain of ectopic *Nkx2.1* expression compared to the GFP-expressing domains (Fig. 4H). A similar tendency was also observed for *Irx3*, suggesting that neither of them acts as a direct ‘selector’ gene, such as has recently been shown in *Drosophila* (Guss et al., 2001). The fact that Six3 acts as a repressor in mediating the phenotypes described (Kobayashi et al., 2001) and so does Irx3 (D.K. and K.S., unpublished observation), would also support this idea. Yet, it is important to note that the mutual repression of *Six3* and *Irx3* is not necessarily linked to alteration of regional competence. For instance, *Six3* is no longer detectable even in the normal situation where exogenous *Irx3* induced En2 ectopically (see Fig. 2D, Fig. 3C), suggesting that Irx3 can provide the posterior competence without repressing *Six3*. On the other hand, *Six3* induced *Nkx2.1* without repressing *Nkx6.1* in the posterior diencephalon (Fig. 4G; data not shown). Although the induction of those *Nkx* genes by Shh does not appear to be a binary choice, as *Nkx2.1* and *Nkx6.1* are normally transiently coexpressed (Qiu et al., 1998), this may suggest that the full spectrum of regional identity was not switched by the ectopic *Six3*. It could be that those transcription factors, along with our methodology, are not sufficient to dominate other endogenous regulatory factors. While region-specific competence is undoubtedly a crucial part of regional identity, the extent to which the regional identity is defined by those transcription factors as well as hypothetical factors that act as a ‘selector’ remains to be determined.

Misexpression of *Irx3* was capable of transforming the anterior diencephalon into the optic tectum, although in a less frequent manner than just ectopic En2 induction. On the other hand, the fate of cells forced to express *Six3* was not explicitly determined. Given the fact that *Six3* induced the retinal hyperplasia and the transformation of the midbrain and anterior hindbrain tissues into the retina (Loosli et al., 1999; Bernier et al., 2000), cells that ectopically express *Bf1* upon misexpression of *Six3* could be cells in the optic vesicle. This would be consistent with the fact that *Bf1* is also expressed and plays a role in the retina (Tao et al., 1992; Xuan et al., 1995; Yuasa et al., 1996; Huh et al., 1999). However, *Six3* and the constitutively active *Fgfr3* induced *Emx2* ectopically (Fig. 3H),

a gene expressed in the telencephalon and diencephalon but not in the eye (Simeone et al., 1992). Since the overlap between *Bf1*- and *Emx2*-expressing domains is largely restricted to the telencephalon, it is more likely that these ectopic *Bf1*-positive cells acquired the telencephalic character.

Subdivision of the early neural plate by independent systems

In the classical view, the brain primordium is subdivided into three domains: the prosencephalon, mesencephalon and rhombencephalon. Subsequently, the prosencephalon or forebrain is further subdivided into the telencephalon and diencephalon, and the metencephalon and myelencephalon develop from the rhombencephalon.

We have demonstrated that expression of *Six3* dictates competence specific for the anterior prosencephalon, whereas *Irx3* provides competence for the posterior prosencephalon and the mesencephalon for the two distinct organizing signals, Fgf8 and Shh. These findings confirm at the molecular level the previous assumption that the ZLI or p2/3 boundary is the border defining the competence for Fgf8 and Shh (Crossley et al., 1996; Shimamura and Rubenstein, 1997), as the *Irx3*-expressing domain is exactly delineated by this boundary. Furthermore, our results that *Bf1* and *Nkx2.1*, which are required for development of the telencephalon, retina and hypothalamus, respectively, are both induced and dependent on the same genetic context, supports an important aspect of the prosomeric model: that the telencephalon, eye and hypothalamus are derived from the same developmental unit, the secondary prosencephalon, whereas other authentic diencephalic derivatives such as the dorsal thalamus and pretectum are derived from the diencephalon proper (prosomere 1 to 3) (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Rubenstein et al., 1994). Although the boundary between the secondary prosencephalon and the diencephalon proper is not the ZLI, our present results, together with those of others, strongly argue that the ZLI is the boundary that delineates units of distinct developmental programs.

Accumulating evidence appears to support the scenario that initial patterns set primarily by diffusible signals are subsequently translated into expression of a distinct set of transcription factors. Those transcription factors regulate the expression of themselves and others to establish discrete domains of expression. Recently it has been shown that homeodomain transcription factors Otx2 and Gbx2 specify a boundary that corresponds to the mid-hindbrain boundary or the isthmus (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000). It was also demonstrated that Pax6 and Pax2 or En1 position the diencephalon-mesencephalon boundary (Araki and Nakamura, 1999; Schwartz et al., 1999; Matsunaga et al., 2000). The present study has revealed an analogous system in which Six3 and Irx3 define another boundary that is likely to be the p2/3 boundary or ZLI. Furthermore, these boundary-setting systems appear to act independently, such that each of the boundaries is defined without interference. As a result, the three boundaries demarcate the fore-midbrain region into three discrete domains, as if three transparent sheets were layered (Fig. 7).

This is reminiscent of what was shown recently for the ventral patterning of the spinal cord (Briscoe et al., 2000). In the spinal cord, graded actions of Shh primarily set up the distinct dorsoventral levels of the domains of transcription factors. Thus it is of particular interest whether or not the initial set-up of these distinct boundary systems in the fore-midbrain region is achieved by the actions of a single consecutive patterning mechanism. Fragmental evidence has been obtained concerning this issue. In the mouse, induction and/or maintenance of *Otx2* expression involves actions from the anterior visceral endoderm and the anterior mesendoderm (Ang et al., 1994; Rhinn et al., 1998; Waldrip et al., 1998; Shawlot et al., 1999; Kimura et al., 2000). Induction of *Gbx2* requires the activity of *Fgf8* in the primitive streak or the mesoderm migrating from it (Sun et al., 1999). The mechanism that induces *Pax6* in the prosencephalic neural plate is currently unknown, while *Pax2* and *En1* are induced by the notochord, presumably via a transient activity of Fgf4 (Shamin et al., 1999). Although we are currently investigating mechanisms that define the expression domains of *Six3* and *Irx3*, it may be that these three boundary systems are initially set by distinct mechanisms. It is also possible that temporally or quantitatively differential activities of the same inductive substance direct those boundary systems. The molecular nature of the inductive signals, as well as their precise ways of operation, will clarify this issue.

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