Regulation of *Otx2* expression and its functions in mouse forebrain and midbrain

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Summary

***Otx2* expression in the forebrain and midbrain was found to be regulated by two distinct enhancers (FM and FM2) located at 75 kb 5′ upstream and 115 kb 3′ downstream. The activities of these two enhancers were absent in anterior neuroectoderm earlier than E8.0; however, at E9.5 their regions of activity spanned the entire mesencephalon and diencephalon with their caudal limits at the boundary with the metencephalon or isthmus. In telencephalon, activities were found only in the dorsomedial aspect. Potential binding sites of OTX and TCF were essential to FM activity, and TCF sites were also essential to FM2 activity. The FM2 enhancer appears to be unique to rodent; however, the FM enhancer region is deeply conserved in gnathostomes. Studies of mutants lacking FM or FM2 enhancer demonstrated that these enhancers indeed regulate *Otx2* expression in forebrain and midbrain. Development of mesencephalic and diencephalic regions was differentially regulated in a dose-dependent manner by the cooperation between *Otx1* and *Otx2* under FM and FM2 enhancers: the more caudal the structure the higher the OTX dose requirement. At E10.5 $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutants, in which *Otx2* expression under the FM2 enhancer remained, exhibited almost complete loss of the entire diencephalon and mesencephalon; the telencephalon did, however, develop.**

Key words: *Otx2*, *Otx1*, Enhancer, Forebrain, Midbrain, Anterior neuroectoderm, WNT signaling, Mouse

Introduction

The vertebrate brain is composed of telencephalon, diencephalon, mesencephalon and metencephalon. It is generally said that anterior neuroectoderm first develops into prosencephalic and mesencephalic vesicles; the mesencephalic vesicle comprises mesencephalon and metencephalon. Subsequently, the prosencephalon segregates into diencephalon and secondary prosencephalon, from which alar plates protrude to generate the telencephalon. However, it is still not certain how the rostral brain is organized ontogenically. Moreover, although it is widely accepted that the diencephalon is divided rostrocaudally into ventral thalamus, dorsal thalamus and pretectum, the rostrocaudal organization of the telencephalon is still a matter of dispute.

Otx2, *Six3* and *Rpx/Hesx1* are the genes expressed earliest in the anterior neuroectoderm induced by anterior visceral endoderm and anterior mesendoderm (Ang et al., 1994; Thomas and Beddington, 1996; Rhinn et al., 1998; Kimura et al., 2000). The initial morphological landmark in this anterior neural plate is the preotic sulcus, which corresponds to the future boundary between rhombomere 2 and 3; the sulcus becomes apparent around the one-somite stage. In the neural plate rostral to the sulcus, *Otx2* expression covers the entire future forebrain and midbrain, though its caudal limit is initially not distinct. Within the *Otx2*-positive region, *Otx1* expression begins at around the two-somite stage (Simeone et al., 1992; Simeone et al., 1993; Suda et al., 1997). *Gbx2* expression in the region rostrally adjacent to the preotic sulcus, which corresponds to the future metencephalon, becomes apparent at around the three- to four-somite stage (Bouillet et al., 1995; Wassarman et al., 1997). *Otx2* and *Gbx2* expression initially overlap, but they become segregated by the six-somite stage, when the boundary between midbrain and hindbrain or ithmus is formed (Broccoli et al., 1999; Millet et al., 1999). Also at around the three- to four-somite stage, *Emx2* and *Pax6* expression begins in laterocaudal forebrain primordium (Suda et al., 2001; Inoue et al., 2000). At this stage, their caudal limits nearly coincide, but later *Emx2* expression is not found in the dorsal thalamus or pretectum, where *Pax6* is expressed. *Pax6* expression initially overlaps with *Pax2* expression caudally, but at around the six- to eight-somite stage its caudal margin coincides with the boundary between the diencephalon and mesencephalon (Araki and Nakamura, 1999; Schwarz et al., 1999; Matsunaga et al., 2000). Initially at the three-somite stage, *Pax2* expression covers the future entire midbrain (Rowitch and McMahon, 1995; Suda et al., 2001); however, as development proceeds it retracts caudally and is eventually found in caudal mesencephalon and metencephalon. *Six3* and

Irx3 expressions also initially overlap, but by the six-somite stage they become segregated in avian. The anterior margin of the *Irx3* expression is delineated by zona limitans interthalamica (ZLTH) (Oliver et al., 1995; Bosse et al., 1997; Kobayashi et al., 2002). It has been suggested that the ZLTH divides the anterior neuroectoderm into rostral and caudal halves that differentially respond to FGF8 and SHH signaling (Crossley et al., 1996; Shimamura and Rubenstein, 1997; Martinez et al., 1999; Kobayashi et al., 2002). The isthmus and anterior neural ridge act as local organizers in midbrain and forebrain development, respectively. *Fgf8* expression is faint and broad at the three-somite stage, but it focuses at isthmus by the six-somite stage (Crossley and Martin, 1995; Suda et al., 1997). *Fgf8* expression also occurs in the anterior neural ridge at around the four-somite stage (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997; Tian et al., 2002). Thus, the initial regionalization of the brain occurs at around the three- to six-somite stage.

The results of analyses of *Otx1*$^{+/-}$*Otx2*$^{+/-}$ mutants suggested that *Otx2* and *Otx1* cooperate in the regionalization of the rostral brain (Acampora et al., 1997; Suda et al., 1996; Suda et al., 1997; Suda et al., 2001). Owing to the occurrence of earlier visceral defects in *Otx2*$^{-/-}$ mutants, however, this cooperation could not be examined in the *Otx2* homozygous mutant state. Furthermore, *Otx1*$^{+/-}$*Otx2*$^{+/-}$ mutants are postnatally lethal, and *Otx1*$^{-/-}$ *Otx2*$^{+/-}$ phenotype could also not be examined. *Otx1*$^{-/-}$ mutants do not exhibit marked defects in forebrain or midbrain development (Suda et al., 1996). These situations obscured the onset and the extent of cooperation between *Otx1* and *Otx2* in rostral brain development.

Kurokawa et al. (Kurokawa et al., 2004) analyzed the enhancers of *Otx2* expression in anterior neuroectoderm at –95 to –80 kb. The activity of the AN enhancer ceased by E8.5 in anterior neuroectoderm. In the present study, we analyzed enhancers of *Otx2* expression in forebrain and midbrain; two distinct enhancers were identified at 75 kb 5′ upstream and 115 kb 3′ downstream. Their activities were absent in the entire forebrain and midbrain at E8.0 (two- to four-somite stage), but were found at E8.5 (6 to 8 somite stage). Thus, the transition of the activities from the AN enhancer to FM enhancer occurs at a stage crucial to rostral brain regionalization.

Studies of mutants lacking each of these enhancers demonstrated that they, in fact, regulate *Otx2* expression in vivo. Moreover, analysis of these mutants revealed that *Otx2* expression under FM and FM2 enhancers cooperates with *Otx1* in the development of the mesencephalon and diencephalon. The affected regions developed at E8.0 and were subsequently lost later than E8.5. The results of this investigation also suggested several upstream factors that are involved in the regulation of *Otx2* expression in forebrain and midbrain. Finally, the phylogenetic significance of enhancer organization was discussed on the basis of available genomic information.

Materials and methods

See the accompanying paper (Kurokawa et al., 2004) for materials and methods not described here.

Genomic clones of the *Otx2* locus of mouse and other animals

A mouse *Otx2* genomic BAC clone 391F17 here referred to as BAC #2 was isolated from a C57BL/6 BAC library (Research Genetics). This was subdivided into fragments of about 10 to 15 kb in length by Sau3AI partial digestion and subcloned into *Bam*HI site of pBluescript SK (–). These clones were aligned as shown in Fig. 4A by walking. A 3 kb human genomic DNA in Fig. 5B, part c was amplified by PCR with 5′-ACTAGTTCTCAAAGTGTCCACTAAGCCGCT-3′ and 5′-CCATGCACCTGGGAAGCCCTAAAAAGATCA-3′ as primers from RPCI11-1085N6 (BACPAC resources) human *OTX2* BAC clone. A 0.6 kb zebrafish genomic DNA containing domain β in Fig. 8A, part b was amplified with 5′-GCTGGAATTGCTCTGGTCTTTTTC-3′ and 5′-CCAACTCTAAAATCTAACATCACG-3′ as primers from zebrafish genomic DNAs.

Generation of BAC #2 transgenic mice

In order to generate the BAC #2 reporter transgene, a *lacZ* gene with the SV40 poly (A) signal was inserted into the translation start site of the 4.3 kb *Otx2* genomic fragment spanning –1.8 kb to +2.5 kb (Fig. 4A). Subsequently, the reporter was obtained by the BAC modification method of Yang et al. (Yang et al., 1997). The integrity of the BAC transgene in the mice was determined by PCR; primers for the 5′ end were 1F (5′-TAACTATGCGGCATCAGAGC-3′) and 1R (5′-GCCTGCAGGTCGACTCTAGAG-3′) and those for the 3′ end were 2F (5′-AGTATTCTATAGTGTCACCT-3′) and 2R (5′-CGTTGGCCGATTCATTAATG-3′).

Generation of enhancer mutant mice

A neomycin resistance gene with Pgk1 promoter and SV40 polyadenylation signal was flanked by loxP sequences. This cassette (Neo) was replaced with the *Apa*I/*Hin*dIII 1314 bp or *Bsm*I/*Bam*HI 995 bp region to delete the FM or FM2 enhancer, respectively. Homologous recombinant ES clones were isolated with TT2 ES cells, as described previously (Matsuo et al., 1995). These were identified by PCR with 5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′ in the *neo* gene as the 5′ side primer and the following 3′ side primers: 5′-CATAAGACCATGAGTTTAGTTCACAGC-3′ and 5′-CTGAACACACAATACTCCTCAGCTGG-3′ for the FM and FM2 targeting vectors, respectively. Recombinants were confirmed by Southern blot analyses as described (Matsuo et al., 1995). Two mutant mouse lines were generated from independent homologous recombinant ES clones for each enhancer mutation. The genotype of each mutant mouse or embryo was routinely determined by PCR using tail or yolk sac specimens. Sense primers employed to detect the wild-type allele were 5′-GAGTGGCTTCTGTCTTTCCATTCCAC-3′ (FM) and 5′-TTGTCAACCTCCTCTTTGAAGAGCC-3′ (FM2); 5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′ (Neo) served as the primer to detect the mutant allele. The antisense primers were 5′-GAGCATGCTGCATCTCTGAAATACAC-3′ (FM) and 5′-AAGACTCTGTCATTGGGTGTGTTGC-3′ (FM2). The deletion of the *neo* insert by Cre-mediated *loxp* recombination was accomplished by mating *Otx2*$^{+/\Delta AN}$ mice with *Lefty-Cre* mice (Yamamoto et al., 2001).

RNA in situ hybridization

The probes used in this study were: *Emx2* (Yoshida et al., 1997), *Otx2* (Matsuo et al., 1995), *Fgf8* (Crossley and Martin, 1995) and *Pax6* (Walther and Gruss, 1991).

Results

5′ forebrain/midbrain enhancer

In a study described by Kurokawa et al. (Kurokawa et al., 2004), the enhancer of *Otx2* expression was mapped in forebrain and midbrain to a fragment (#13) located in the –80 kb to –70 kb 5′ upstream region (Fig. 1A). This region exhibited no activity up to E8.0 in the anterior neuroectoderm (Fig. 1B, parts a,b), while activity was detected in the anterior neural plate at E8.5 (Fig. 1B, part c). After the closure of the neural tube, β-gal expression was observed in the

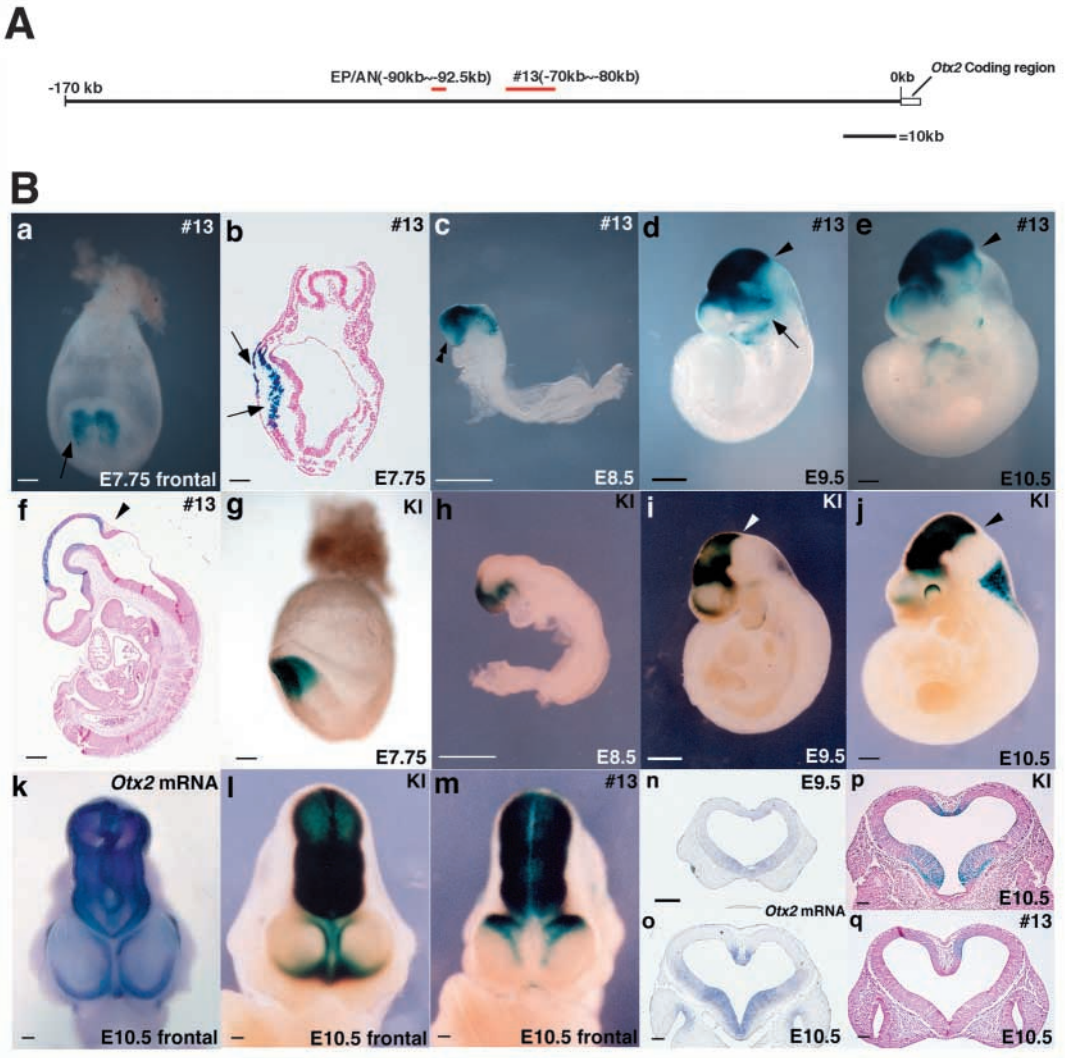


Fig. 1. Enhancer of *Otx2* expression in forebrain and midbrain at 5′ upstream. (A) The location of the #13 fragment that exhibited the enhancer activity in forebrain midbrain. EP/AN indicates the position of the EP and AN enhancer at –92.5 to –90 kb. (B) β-Gal expression driven by #13 (a-f,m,q) and by endogenous enhancers in $Otx2^{+/\lambda\alpha\chi Z}$ knock-in embryos (g-j,l,p), at the stages indicated. Endogenous *Otx2* mRNA expression is presented in k,n,o. (a,k-m) frontal views; (b-j) lateral views (anterior is towards the left); (n-q) frontal sections at the telencephalic level. Arrows and arrowheads indicate expression by the 1.8 kb promoter and the position of the isthmus, respectively. Double arrowheads in c indicate the absence of the β-gal expression in the most anterior neural plate, which may correspond to future telencephalon. Scale bars: 100 μm in a,b,g; 400 μm in c-f,h-q.

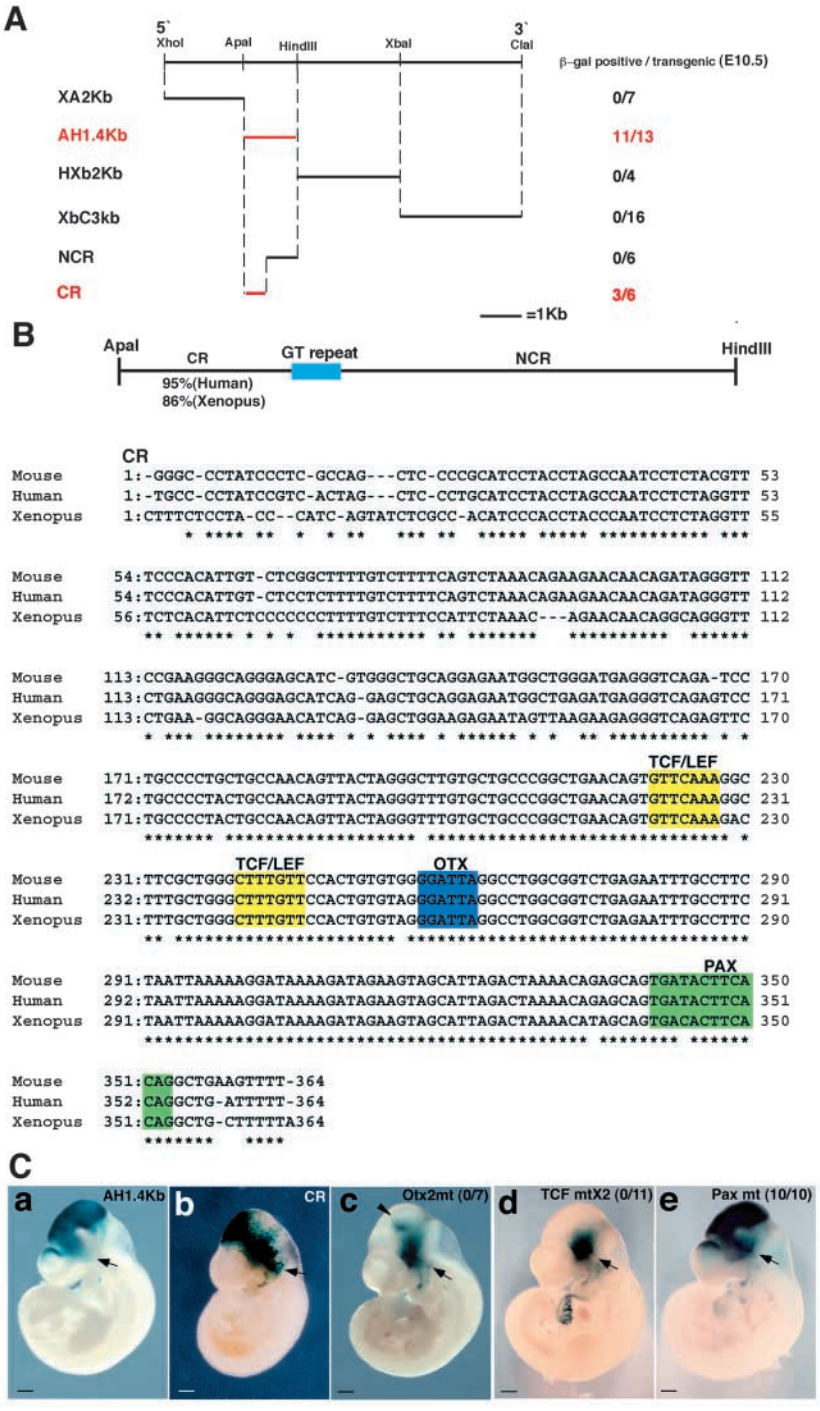
mesencephalon, diencephalon and the dorsomediocaudal region of the telencephalon corresponding to the archencephalon (Fig. 1B, parts d-f,m). The caudal limit of β-gal expression coincided with the isthmus at E9.5 (Fig. 1B, parts d-f); β-gal expression persisted in these regions at E10.5. We named the enhancer identified in this region the FM (forebrain and midbrain) enhancer.

With BAC #1, which covered –170 kb to –30 kb 5′ upstream, and the #12 fragment located at –90 to –92.5 kb, β-gal expression was found in the entire anterior neuroectoderm at E7.75, as described by Kurokawa et al. (Kurokawa et al., 2004). At E9.5, however, with either BAC#1 or the #13 fragment, β-gal expression was not detected in telencephalon with the exception of the dorsomedial region. At E8.5, β-gal expression was also absent in the most anterior neuroectoderm, which may correspond to the future telencephalon (double arrowheads in Fig. 1B, part c). To examine how the FM enhancer activity represents the endogenous *Otx2* expression in telencephalon, we re-evaluated the *Otx2* expression. At E9.5 and E10.5, mRNA expression was absent in the dorsal telencephalon except for the most dorsomedial aspect, while it was apparent in the ventral telencephalon (Fig. 1B, parts k,n,o). β-gal expression was also not found in the dorsal telencephalon of $Otx2^{+/LacZ}$ embryos (Fig. 1, parts i,j,l,p), but was present in ventral telencephalon (Fig. 1B, part p). Thus, the anterior part of the neuroectoderm corresponding the future dorsal telencephalon initially exhibits *Otx2* expression under the control of the AN enhancer, but not under the FM enhancer beyond E8.5 when telencephalon develops. The enhancer of *Otx2* expression in the ventral telencephalon remains to be identified; neither the #13 fragment nor the #12 fragment (analyzed by Kurokawa et al., 2004) nor the #29 fragment described below exhibited any activity in ventral telencephalon at E10.5 (Fig. 1B, part q, data not shown).

To define the FM enhancer in the #13 fragment at –70 kb to –80 kb 5′ upstream, the 10 kb region was divided into *Xho*I/*Apa*I 2.0 kb (XA2kb), *Apa*I/*Hin*dIII 1.4 kb (AH1.4kb), *Hin*dIII/*Xba*I 2.0 kb (HXb2kb) and *Xba*I/*Cla*I 3.0 kb (XbC3kb) fragments (Fig. 2A). The *Apa*I/*Hin*dIII 1.4 kb fragment exhibited full FM activity in a transient transgenic assay at E10.5. This finding was confirmed by the generation of permanent transgenic lines (Fig. 2C, part a). β-gal expression in the forebrain and midbrain was detected at E8.5 with this fragment. The caudal limit of β-gal expression occurred at the boundary between the midbrain and the hindbrain.

The 1.4 kb fragment is divided by a GT repeat into 5′ 364

bp and 3′ 904 bp sequences (Fig. 2B). A search of the human genome revealed a region that was 95% identical to the 5′ 364 bp sequence at 82 kb 5′ upstream of the human *OTX2* gene (Kurokawa et al., 2004). A homologous region (86% identical) was also discovered 36 kb 5′ upstream of the *Xenopus Otx2* locus. By contrast, no region homologous to the 3′ 904 bp was identified. The highly conserved 364 bp (CR) fragment drove β-gal expression in forebrain, whereas the 884 bp (NCR) fragment did not (Fig. 2A). CR activity was, however, limited to the diencephalon; the fragment could not drive β-gal expression in the mesencephalon (Fig. 2C, part b). Thus, sequences that direct β-gal expression in mesencephalon in cooperation with the 364 bp sequences must be present in the remaining region of the *Apa*I/*Hin*dIII 1.4 kb fragment. Putative binding sites for *Otx* and *Tcf/Lef* gene products (Mao et al., 1994; Brannon et al., 1997) (Fig. 2B) occur in the 364 bp fragment, and these binding sites are conserved in both human and *Xenopus* counterparts. To test their activity, transverse mutations were introduced to these sites in the *Apa*I/*Hin*dIII 1.4 kb fragment. The fragment containing the mutation at the OTX binding site exhibited only residual activity in midbrain (arrowhead in Fig. 2C, part c). Moreover, a mutation in the two TCF/LEF binding sites completely abolished all activity necessary to drive β-gal expression in forebrain and midbrain (Fig. 2C, part d). The 364 bp sequence also contains a putative binding site for PAX (Czerny and Busslinger, 1995), which is conserved in the human locus, and which exhibits one base substitution in the *Xenopus* locus. A transverse mutation in this site did not affect the transcriptional activity of the *Apa*I/*Hin*dIII 1.4 kb fragment (Fig. 2C, part e).



Targeted disruption of *Otx2* FM enhancer activity

In order to confirm that *Otx2* expression in forebrain and midbrain is governed by the FM enhancer after E8.5, a mutant, $Otx2^{\Delta FM/\Delta FM}$, in which the *Apa*I/*Hin*dIII 1.4 kb region was replaced with a cassette encoding a neomycin-resistant gene, was generated (Fig. 3A). In the homozygous enhancer mutant, *Otx2* expression was present, but diminished, at E9.5 (data not shown). Forebrain and midbrain development in this mutant also appeared normal (3B, parts a-d), suggesting that another enhancer of *Otx2* expression in forebrain/midbrain most probably exists.

Subsequently, a mutant in which one *Otx2* allele displayed deletion of the FM enhancer and in which the other allele was null, $Otx2^{\Delta FM/-}$, was generated. The mutant exhibited defects in midbrain and forebrain development. This finding demonstrated that the FM enhancer indeed functions as an enhancer of *Otx2* expression in forebrain/midbrain in vivo. Histologically, the telencephalon appeared normal and the ventral

Fig. 2. Deletion analysis of FM enhancer. (A) Fine mapping of the FM enhancer. (B) Schematic organization of the AH 1.4 kb subfragment and sequence alignment of the CR region in mouse, human and *Xenopus*. (C) β-Gal expression at E10.5 driven by the AH 1.4 kb fragment (a), by the CR domain of the fragment (b) and by the AH 1.4 kb fragment exhibiting a mutation in the OTX binding site (c), in both TCF-binding sites (d) and in the PAX-binding site (e). Expression in midbrain is lost in b; furthermore, only residual expression exists in midbrain (arrowhead). Arrows indicate β-gal expression in cephalic mesenchyme driven by the 1.8 kb promoter. Scale bars: 400 μm.

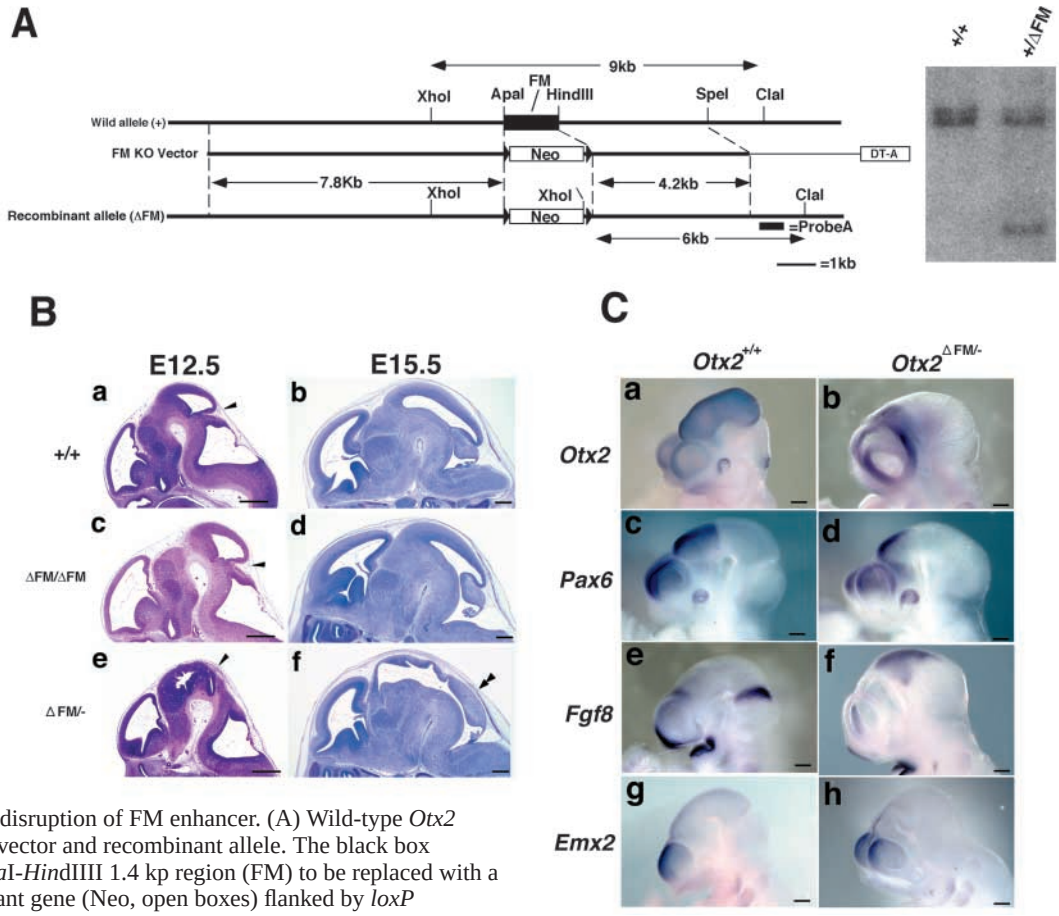


Fig. 3. Targeted disruption of FM enhancer. (A) Wild-type *Otx2* allele, targeting vector and recombinant allele. The black box indicates the *Apa*I-*Hin*dIIII 1.4 kp region (FM) to be replaced with a neomycin-resistant gene (Neo, open boxes) flanked by *loxP* sequences (black triangles). DT-A is the diphtheria toxin A fragment gene with MC1 promoter, which is used for the negative selection of homologous recombinants (Matsuo et al., 1995). Thick and thin lines indicate genomic and vector-derived sequences, respectively. Probe A is the Southern blotting probe employed for identification of homologous recombinant ES cells displayed in the right panel. (B) FM enhancer mutant phenotype. Wild-type (a,b), $Otx2^{\Delta FM/\Delta FM}$ (c,d) and $Otx2^{\Delta FM/-}$ (e,f) embryos at E12.5 (a,c,e) and E15.5 (b,d,f). A double arrowhead in f indicates the expanded cerebellum primordium. The phenotypes were examined with both the $Otx2^{\Delta FM}$ mutant in which the neo insert remained and the $Otx2^{\Delta FM}$ mutant in which the insert was deleted by Cre recombination. No differences were found, and the following marker analyses were performed with the mutant that retained the *neo* insert. (C) Marker analysis of $Otx2^{\Delta FM/-}$ phenotype. *Otx2* (a,b), *Pax6* (c,d), *Fgf8* (e,f) and *Emx2* (g,h) expression in E10.5 wild-type (a,c,e,g) and $Otx2^{\Delta FM/-}$ (b,d,f,h) embryos. Scale bars: 400 μm.

thalamus developed in $Otx2^{\Delta FM/-}$ mutants (Fig. 3B, parts e,f). However, the development of the region posterior to the zona limitans, especially the midbrain, was poor, and additionally, the isthmus was shifted rostrally. In molecular terms (Fig. 3C), the *Otx2*-positive, *Pax6*-negative midbrain was nearly absent. The *Emx2*-positive forebrain was normal, whereas the *Pax6*-positive, *Emx2*-negative forebrain was reduced. The *Fgf8*-positive isthmic stripe was concomitantly shifted rostrally, and, moreover, was expanded. At E15.5, the cerebellar primordium was also expanded in the $Otx2^{\Delta FM/-}$ mutants (Fig. 3B, part f). The loss of mesencephalon and diencephalon in the mutants appears to be the consequence of a transformation into anterior hindbrain (Suda et al., 1997; Suda et al., 2001; Acampora et al., 1997).

BAC #1 displayed no transcriptional activity in posterior mesencephalon at E9.5 (see Fig. 1B, part c in the preceding paper), while the #13 fragment did exhibit this activity. As a result, the presence of a silencer in the BAC #1 was initially hypothesized. However, we could establish only one *BAC #1/lacZ* transgenic line. The fact that the mesencephalon was affected in $Otx2^{\Delta FM/-}$ mutants demonstrates rather that the FM enhancer in the *Apa*I/*Hin*dIII 1.4 kb fragment is active in midbrain in vivo.

Search for the second enhancer in the 3′ side of the coding region

Enhancer mutants indicated the presence of another enhancer of *Otx2* expression in forebrain/midbrain. To investigate this, a search was conducted for the second enhancer in the 3′ region of the *Otx2* gene. A BAC transgenic mouse, which harbored a genomic DNA sequence that spanned –50 kb to +120 kb 3′ downstream, was generated (BAC #2; Fig. 4A). The 3′ *BAC#2/lacZ* transgene was prepared by homologous recombination in *E. coli* (Yang et al., 1997) between BAC#2 and a *lacZ* construct in which the *lacZ* gene was placed in-frame at the translational start site and flanked by 1.8 kb 5′ and 2.5 kb 3′ sequences. Two *3′BAC#2/lacZ* transgenic lines were produced, neither of which displayed activity in epiblast or

anterior neuroectoderm. At a later point, these lines exhibited activities in forebrain, midbrain, eyes and nose (Fig. 4B, part a).

The *Otx2* 3′ genomic region was then divided into 14 fragments, and the enhancer activity of each fragment was assayed via generation of permanent transgenic lines, as indicated in the parentheses in Fig. 4A. No activity was detected in epiblast or anterior neuroectoderm with any of these fragments. The #17 and #20 fragments showed activity in nasal placode (Fig. 4B, parts b,c). However, the enhancer of expression in E9.5 forebrain and midbrain was mapped to the #29 fragment, which locates at +110 kb to +120 kb 3′ downstream. This fragment displayed faint activity at E7.75 in the future mid/hindbrain boundary region (Fig. 4B, part d). Furthermore, activity was evident in future dorsal areas of the prospective mid/hindbrain boundary region at E8.5 (Fig. 4B, part e). At E9.5, β-gal expression directed by #29 covered the entire mesencephalon (Fig. 4B, parts f,g). β-gal expression in mesencephalon and diencephalon declined at E10.5 (Fig. 4B, parts h,i). However, intensive β-gal expression continued in the dorsocaudal telencephalon. With the exception of this aspect, the #29 fragment did not exhibit activity in telencephalon, as was true of the FM enhancer. The enhancer in the #29 fragment was designated as the FM2 enhancer.

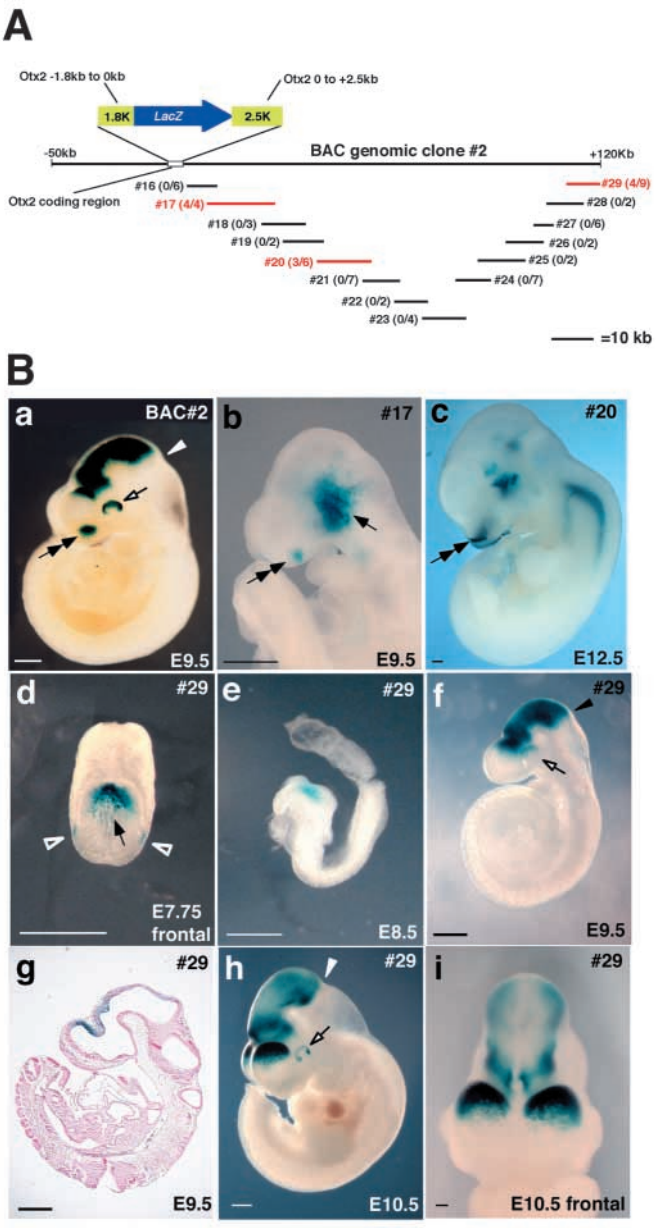


Fig. 4. Search for enhancers of *Otx2* expression in ectoderm at 3′ downstream. (A) The *3′BAC #2/lacZ* transgene and the 14 fragments assayed for enhancer activity. (B) β-Gal expression driven by the *3′ BAC #2/lacZ* transgene (a), by #17 in nasal placode (b), by #20 in nasal cavity (c) and by #29 in forebrain and midbrain (d-i) at indicated stages. Open arrows, β-gal expression in eyes; double arrows, β-gal expression in nasal placode or cavity; arrowheads, the position of the isthmus. The activity of the 1.8 kb promoter in cephalic mesenchyme (indicated by an arrow in b) is suppressed by the *3′BAC #2* (a) and the #29 fragment (f,h); by contrast, promoter activity in anterior mesendoderm is not (an arrow in d). Open arrowheads in d indicate weak β-gal expression in the future mid/hindbrain boundary region. With the exception of the nasal cavity, expression in c is ectopic. In lateral views (a-c,e-h), anterior is towards the left; (d,i), frontal views. Scale bars: 400 μm.

After E9.5 the endogenous *Otx2* expression in the dorsal telencephalon is found in its medial aspect (Fig. 1B, parts k,l,n-p). At E10.5, the AN, FM and FM2 enhancers all had differential activities in this region, the significance of which remains for future studies (Fig. 1B, parts m,q; Fig. 4B, part i) (Kurokawa et al., 2004).

3′ forebrain/midbrain enhancer

The #29 10 kb region was divided into *Mlu*I/*Sal*I 2.5 kb (MS2.5kb), *Sal*I/*Spe*I 2.5 kb (SS2.5kb), *Spe*I/*Bam*HI 2.8 kb (SB2.8kb) and *Bam*HI/*Hin*dIII 1.2 kb (BH1.2kb) fragments to characterize the FM2 enhancer (Fig. 5A). FM2 enhancer activity was inherited by the *Spe*I/*Bam*HI 2.8 kb fragment (Fig. 5B, part a). The *Bam*HI/*Hin*dIII fragment exhibited activity in the cortical area (Fig. 5B, part b); however, this activity was not observed with the BAC #2 or the #29 fragment. Moreover, no endogenous *Otx2* expression was detected in the cortex (Fig. 1Bi-l, n-p). Based on these, no further analysis was conducted on the BamHI/HindIII fragment.

In the *Spe*I/*Bam*HI 2.8 kb fragment, the 5′ 700 bp region is well conserved between mouse and human with sequence identity of 81%; however, no region homologous to the 3′ 2.1 kb region (NCR) exists in the human or *Xenopus* genome. Sequence identity of the NCR region between mouse and rat is 83%. Unexpectedly, FM2 enhancer activity was present in this NCR region (Fig. 5A). Subsequently, the NCR was divided into *Hin*cII 1.3 kb and *Bsm*I/*Bam*HI 1 kb (BB1.0kb) fragments. The BB 1.0 kb subfragment retained enhancer activity of expression in forebrain and midbrain. Moreover, its sequence identity with the rat counterpart region was 83%. This fragment, however, exhibited intense ectopic activity in the trunk dorsal root ganglion (DRG). This finding was confirmed via generation of permanent transgenic lines (Fig. 5B, part d). Expression in DRG was faint with the SB 2.8 kb fragment (arrowhead in Fig. 5B, part a) and moderately increased with NCR. β-Gal expression was observed at E8.5 in midbrain with the BB 1.0 kb fragment to an extent similar to that of the #29 fragment (Fig. 4B, part e). Expression persisted until E10.5, after which it gradually decreased.

Further deletion of the *Bsm*I/*Hin*cII 127 bp region at the 5′ end of the BB 1.0 kb fragment drastically reduced enhancer

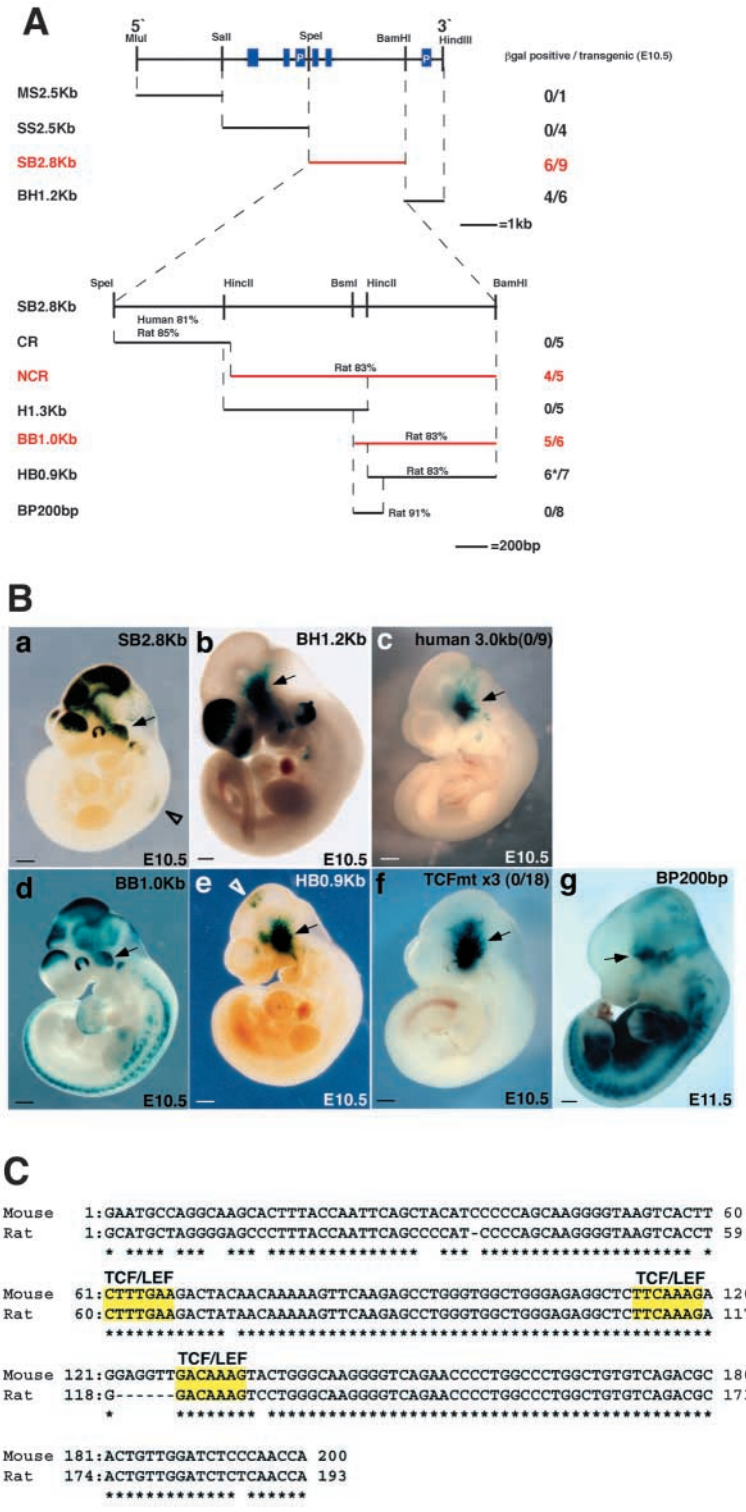


Fig. 5. Deletion analysis of FM2 enhancer. (A) Fine mapping of the FM2 enhancer. Blue boxes indicate the domains conserved between human and mouse characterized by sequence identity exceeding 80% over more than 100 bp. Sequences in the boxes denoted by P were used as primers to isolate the counterpart 3.0 kb region in human genome by PCR. Asterisk in HB 0.9 kb indicates residual expression shown in (Be). Percentages on each bar provide the sequence identity of each domain with human or rat. (B) β-Gal expression driven by the SB 2.8 kb fragment (a), by the BH 1.2 kb fragment (b), by the human 3.0 kb counterpart of the SB 2.8 kb region (c), by the BB 1.0 kb fragment (d), by the HB 0.9 kb fragment (e), by the BB 1.0 kb fragment with mutations in three TCF binding sites (f) and by the BP 200 bp fragment (g) at E10.5 (a-f) and at E11.5 (g). Arrowheads in a and e indicate faint ectopic expression in DRG and residual expression in dorsal midbrain, respectively. Arrows indicate β-gal expression in cephalic mesenchyme by the 1.8 kb promoter. Scale bars: 400 μm. (C) Alignment of nucleotide sequences of BP 200 bp between mouse and rat.

activity (HB 0.9 kb; Fig. 5A and 5B, part e), although residual activity remained in midbrain. Thus, the 5′ region is essential for FM2 activity. This region displays 91% sequence identity between mouse and rat and includes three TCF/LEF binding sites, all of which are conserved in the rat *Otx2* locus (Fig. 5C). Mutations in all three TCF/LEF binding sites nearly abolished the enhancer activity of the mouse BB 1.0 kb fragment (Fig. 5B, part f). However, the BP 200 bp fragment (Fig. 5A) containing *Bsm*I/*Hin*cII 127 bp failed to capture FM2 activity, but directed disorganized expression (Fig. 5B, part g).

It has been shown that enhancer activity can be conserved in spite of the high divergence of the sequences overall if the core sequences for transcription are conserved (Flint et al., 2001; Müller et al., 2002). NCR demonstrating FM2 activity occurs between the domains conserved in the human genome (blue boxes in Fig. 5A). This region in the human genome (human 3.0 kb) was isolated by PCR employing conserved sequences (indicated by P in blue boxes of Fig. 5C) and was then tested for enhancer activity. No consensus sequences for TCF binding sites exist in the human 3.0 kb, nor was any FM activity detected with human 3.0 kb (Fig. 5B, part c).

Targeted disruption of FM2 enhancer activity

In order to confirm that *Otx2* expression in forebrain and midbrain is also governed by the FM2 enhancer later than E8.5, a mutant was generated in which the *Bsm*I/*Bam*H1 1.0 kb region was replaced with a cassette encoding a neomycin-resistant gene (Fig. 6A). The homozygous mutant, $Otx2^{\Delta FM2/\Delta FM2}$, demonstrated normal forebrain and midbrain development (Fig. 6B, parts a,b). However, the mutant in which one *Otx2* allele displayed deletion of the FM2 enhancer and in which the other allele was null, $Otx2^{\Delta FM2/-}$, exhibited defects in midbrain and forebrain development (Fig. 6B, parts c,d) similar to those observed in $Otx2^{\Delta FM/-}$ (Fig. 3B). Marker analyses indicated that the defects in the $Otx2^{\Delta FM2/-}$ mutant were milder (Fig. 6C) than those in the $Otx2^{\Delta FM/-}$ (Fig. 3C). Reductions of the *Emx2*-negative, *Pax6*-positive region and *Pax6*-negative, *Otx2*-positive regions were minimal, and the anterior shift and expansion of the *Fgf8*-positive isthmus was less pronounced.

The $Otx2^{\Delta FM/-}$ and $Otx2^{\Delta FM2/-}$ phenotypes were similar to those observed in $Otx1^{+/-}Otx2^{+/-}$ defects reported previously (Fig. 7A, part d) (Suda et al., 1997). A series of mutants were produced to assess the cooperation of *Otx1* with *Otx2*

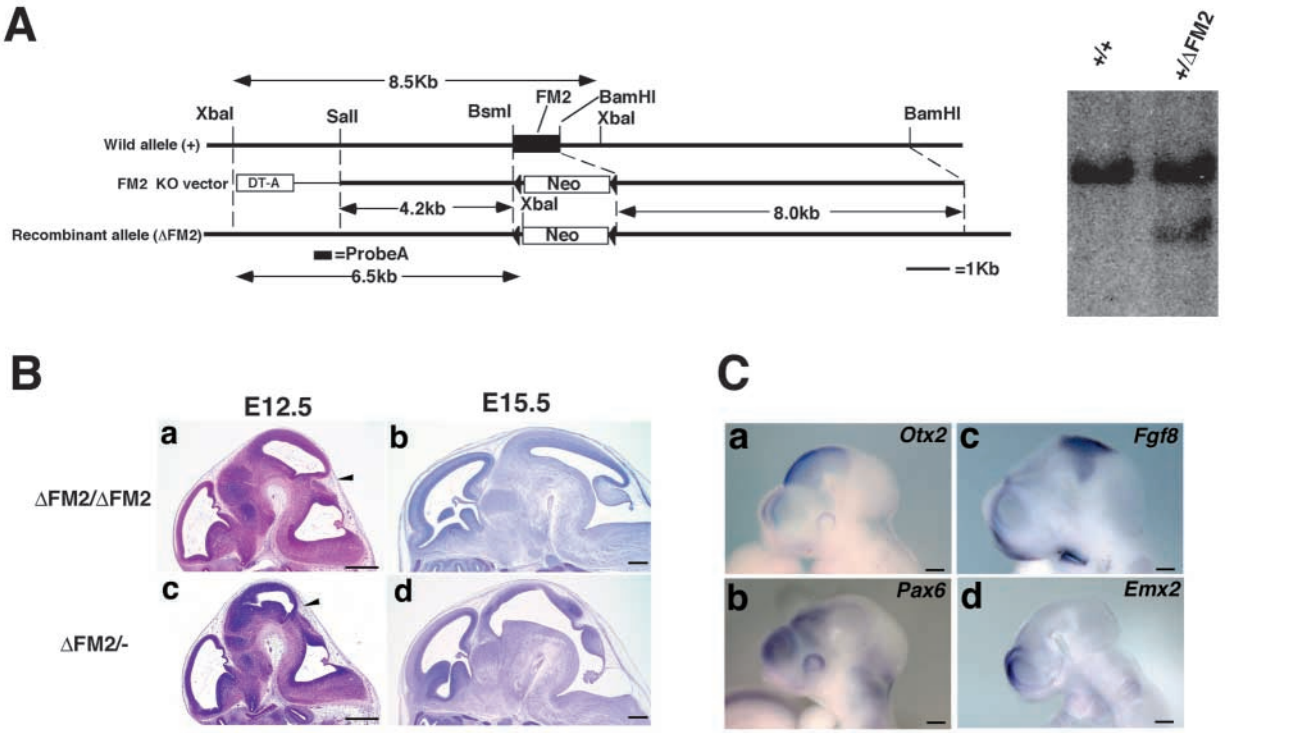


Fig. 6. Targeted disruption of FM2 enhancer. (A) Diagrammatic representation of wild-type *Otx2* allele, targeting vector and recombinant allele. The black box indicates the *Bsm*I-*Bam*HI 1.0 kb region (FM2) to be replaced with a neomycin-resistant gene (Neo) (see Fig. 3A for others). (B) $Otx2^{\Delta FM2/\Delta FM2}$ (a,b) and $Otx2^{\Delta FM2/-}$ (c,d) phenotype at E12.5 (a,c) and E15.5 (b,d). The phenotypes were examined with both the $Otx2^{\Delta FM2}$ mutant in which the neo insert remained and the $Otx2^{\Delta FM2}$ mutant in which the insert was deleted by Cre recombination. No differences were found, and the following, marker analyses were performed with the mutant that retained the *neo* insert. (C) Marker analysis of $Otx2^{\Delta FM2/-}$ phenotype at E10.5; *Otx2* (a), *Pax6* (b), *Fgf8* (c) and *Emx2* (d) expression (see Fig. 3C for the expression in wild-type embryos). Scale bars: 400 μm.

expression under the FM and FM2 enhancers (Fig. 7A). These mutants indicated that the development of mesencephalic and diencephalic regions is dependent on OTX dose. In the most severe $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutant, in which *Otx2* expression under the FM2 enhancer remains, the *Emx2*-negative, *Pax6*-positive domain and the *Emx2*-negative, *Otx2*-positive domain were residual at E10.5 (Fig. 7B). Thus, diencephalon and mesencephalon were almost entirely missing, but the *Emx2*-positive telencephalon developed normally (Fig. 7B, part d). This is consistent with a lack of FM activity in telencephalon. Moreover, the FM enhancer did not have the activity at E8.0, and *Otx2*-, *Emx2*- and *Pax2*-positive regions developed normally at the five-somite stage in $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutants as expected (data not shown). Furthermore, in a series of double mutants in Fig. 7A, the lower the *Otx* dose and the more severe the phenotype, the more expanded the *Fgf8*-positive isthmus (Fig. 3C, Fig. 6C and data not shown). Interestingly, however, *Fgf8*-positive isthmus was lost in the most severe $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutant (Fig. 7B, part c). This mutant also lacked the *Fgf8* expression in the anterior neural ridge.

FM enhancer is deeply conserved in gnathostome

The expression pattern of the Otx family of genes in zebrafish is divergent from that in tetrapods (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995). Among teleosts, genome information is available for pufferfish (*Fugu rubripes*) and zebrafish. The 22 domains conserved in tetrapod *Otx2* loci were assigned to the loci of all Otx homologues in these species. The FM2 enhancer, which was not conserved in human or *Xenopus*, was also not conserved in the genomes of these fish. The pufferfish possesses one *Otx1* and two *Otx2* homologues; at the moment, these homologues are denoted as *FrOtx1*, *FrOtx2a* (Ensembl Gene ID is SINFRUG00000151879) and *FrOtx2b* (Ensembl Gene ID is SINFRUG00000136398) (Fig. 8A). Eight domains (β, ζ, η, θ, ι, κ, ξ and ρ) are conserved in both pufferfish and zebrafish Otx genes. Domains α and γ are conserved exclusively in zebrafish and domain ο exclusively in pufferfish. In pufferfish, none of these domains is conserved in the *FrOtx1* locus; by contrast, one (θ) and two domains (γ, κ) are conserved in *Otx3* and *Otx1*, respectively, in zebrafish. Domain δ, which harbors the EP and AN enhancers in tetrapods, is not conserved in the pufferfish or the zebrafish genome. Among the eight domains conserved in mouse, human, *Xenopus*, pufferfish and zebrafish, β, θ and ρ demonstrated enhancer activity in the mouse, as described above. Domain β harbored the FM enhancer, whereas domain θ exhibited activity in regions of ventral diencephalon and dorsal mesencephalon. Domain ρ displayed activity in nasal placode. Regions homologous to domain β are present in *FrOtx2a*, *FrOtx2b* and zebrafish *Otx2* loci. These regions retained OTX and a single TCF/LEF binding site (Fig. 8B). Another TCF/LEF binding site was converted into a SOX-binding site in zebrafish and pufferfish via a one-base substitution (Mertin et al., 1999). The conservation of TCTAATTAAAAWGGATA (red box in Fig.

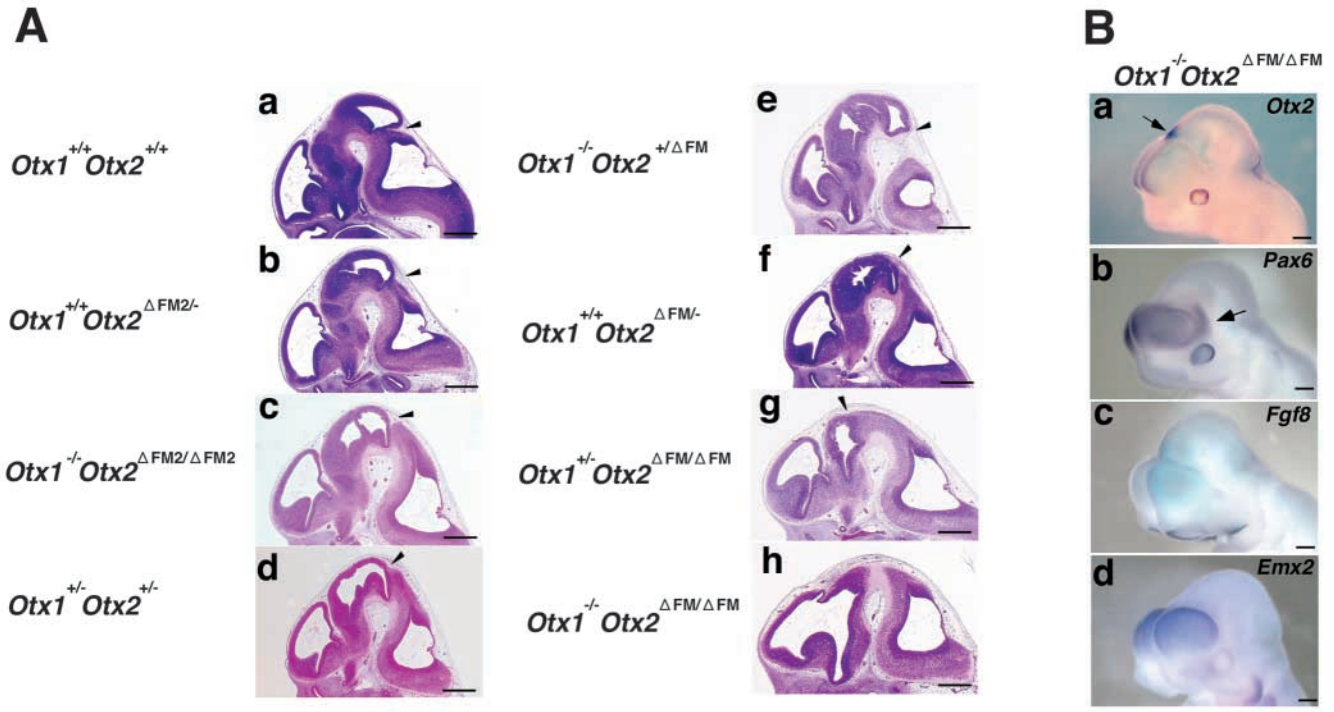


Fig. 7. Cooperation between *Otx1* and *Otx2* under FM and FM2 enhancers. (A) Histological features of a series of double mutants at E12.5; the genotype of each mutant is indicated at the left. Among mutants not shown, defects were either absent or subtle in $Otx2^{\Delta FM2/\Delta FM2}$, $Otx1^{+/-}Otx2^{+/\Delta FM2}$, $Otx1^{-/-}$, $Otx2^{+/-}$, $Otx2^{\Delta FM/\Delta FM2}$, $Otx1^{+/-}Otx2^{+/\Delta FM}$, $Otx2^{\Delta FM/\Delta FM}$, $Otx1^{+/-}Otx2^{\Delta FM2/\Delta FM2}$ and $Otx1^{-/-}Otx2^{+/\Delta FM2}$ mutants. $Otx1^{+/-}Otx2^{\Delta FM2/-}$ was affected to the same extent as $Otx2^{\Delta FM/-}$ (f); furthermore, defects in $Otx1^{+/-}Otx2^{\Delta FM/-}$ were as severe as those in $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ (h). Arrowheads indicate the position of the isthmus. Scale bars: 400 μm. (B) Marker analysis of the defects in $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ at E10.5; *Otx2* (a), *Pax6* (b), *Fgf8* (c) and *Emx2* (d) expression. See Fig. 3C for the expression in wild-type embryos. Arrows in a and b indicate residual *Otx2*- and *Pax6*-positive domains, respectively. Scale bars: 400 μm.

8B) sequence is noteworthy, although its significance remains for future studies to investigate.

Based on these comparative genomic analyses, the existence of enhancer activity in domain β of zebrafish *Otx2* was determined. A 0.6 kb zebrafish *Otx2* region covering the 170 bp (Fig. 8B) in the middle was isolated by PCR. Using the 1.8 kb promoter, this 0.6 kb region clearly directed β-gal expression in mouse forebrain and midbrain (Fig. 8A, part b).

Discussion

Kurokawa et al. (Kurokawa et al., 2004) reported the identification of enhancers of *Otx2* expression in epiblast (EP) and anterior neuroectoderm (AN). The present report identifies enhancers of *Otx2* expression in forebrain and midbrain. In contrast to the enhancers of *Otx2* expression in distal/anterior visceral endoderm (DVE/AVE), anterior mesendoderm (AME) and cephalic mesenchyme (CM) (Kimura et al., 1997; Kimura et al., 2000), all of these ectodermal enhancers were found at considerable distances from the coding region. Furthermore, at least two distinct enhancers of *Otx2* expression exist in each ectoderm at each step. Fundamental questions remain as to how essential such enhancer organization is to *Otx2* gene functions and how it was established phylogenetically.

Enhancers of *Otx2* expression in forebrain and midbrain

The separation of the enhancer of *Otx2* expression in forebrain and midbrain later than E8.5 from the AN enhancer was unexpected. *Otx2* expression initially covers the entire anterior neuroectoderm, but is subsequently lost in the most anterior region corresponding to the dorsal telencephalon. This change can be explained readily by the transition from *Otx2* expression under the AN enhancer to that under the FM and FM2 enhancers. β-gal expression under the AN enhancer is evident at E8.0, but not at E8.5. FM and FM2 enhancer activities are not found at E8.0 over the entire anterior neuroectoderm, but become apparent at E8.5. β-Gal expression is sensitive and stable, usually persisting longer than the expression of endogenous gene products. However, the AN enhancer must retain the activity at the stage when the *Emx2* expression takes place (three-somite stage) as demonstrated by $Otx2^{\Delta AN/\Delta AN}Emx2^{-/-}$ mutants (Kurokawa et al., 2004). The timing of the switch from the AN enhancer to FM/FM2 enhancers corresponds to the stage critical for the regionalization of the rostral brain (see Introduction).

More unexpected was the presence of two distinct enhancers, FM and FM2, of *Otx2* expression in forebrain and midbrain. Not only are the overall sequences not conserved between FM and FM2 enhancers, there are no apparent conserved sequences between them except for TCF/LEF-binding sites. The overall sequences in the FM enhancer region are conserved in mouse, human and *Xenopus*. The FM2 enhancer, which is not conserved in human or *Xenopus*, is most probably unique to the rodent. This raises questions for future studies regarding whether human *OTX2* functions without the

second FM2 enhancer or whether the human *OTX2* gene acquired the second FM enhancer independently. Does the second FM enhancer exist in avian or *Xenopus*? Moreover, it is possible that the putative second EP/AN enhancer that failed to be identified by Kurokawa et al. (Kurokawa et al., 2004) is also unique to the rodent.

Organization of *Otx2* enhancers

Analysis of *Otx* homologues in lamprey suggests that the ancestral vertebrate originated with a single copy of the *Otx* gene and that divergence into the *Otx2* and *Otx1* lineages occurred in the gnathostome lineage (Ueki et al., 1998; Williams and Holland, 1998; Tomsa and Langeland, 1999; Germot et al., 2001). In the tetrapod lineage, *Otx2* is thought to have retained its ancestral roles. By contrast, *Otx1* appears to have been co-opted for more complex functions associated with vertebrate brains (Acampora et al., 1996; Acampora et al., 1999; Suda et al., 1999; Morsli et al., 1999; Mazan et al., 2000). However, the specialization of *Otx2* and *Otx1* functions appears quite divergent in the lineage leading to the extant teleosts (Li et al., 1994; Mori et al., 1994; Mercier et al., 1995). In light of the essential roles of *Otx* in head development, the question of how the organizations of *Otx* enhancers in each vertebrate are related is one of keen interest. The mouse *Otx1* locus has no regions homologous to the mouse *Otx2* enhancers identified.

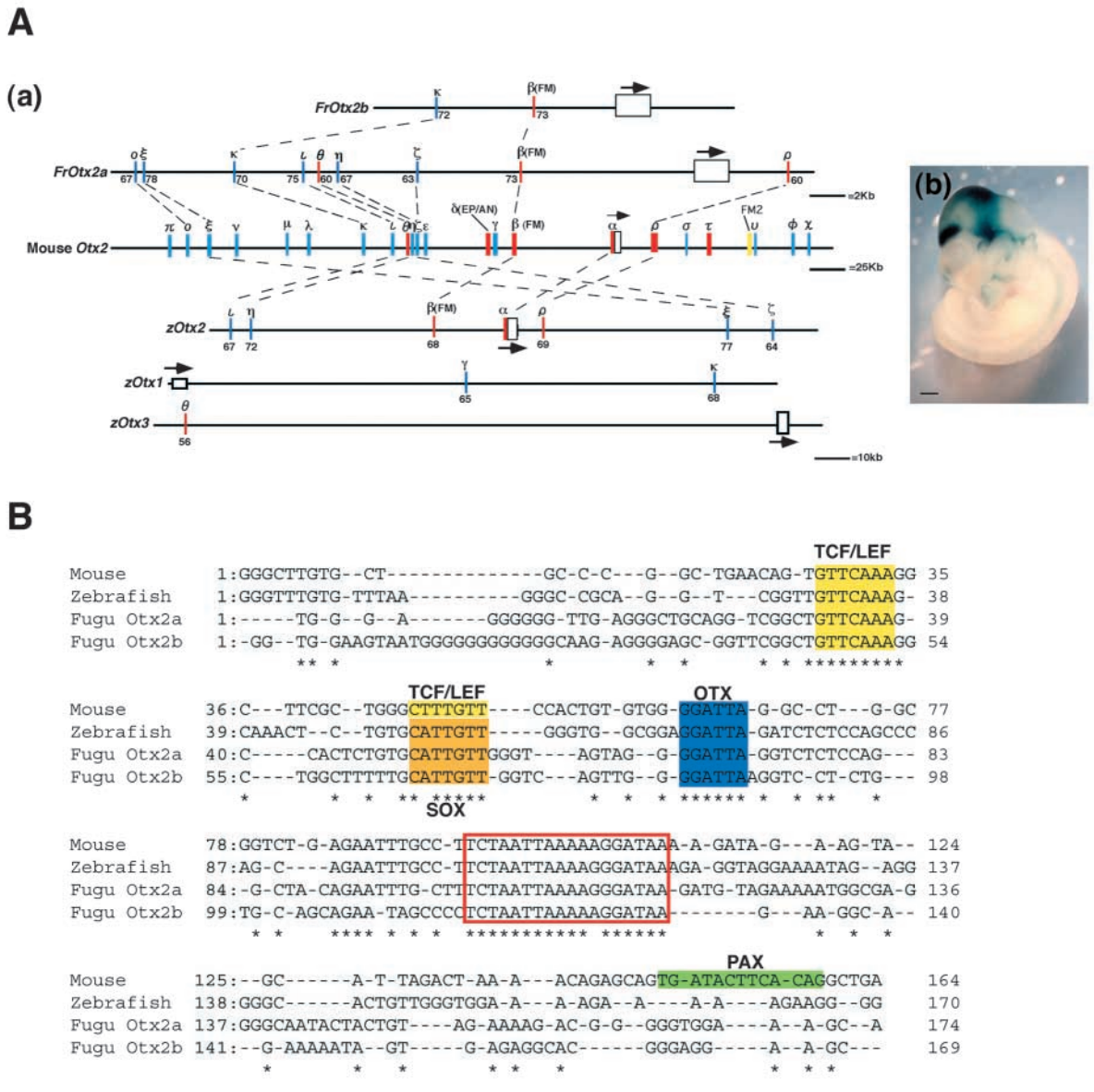


Fig. 8. Genome organization in fish Otx loci. (A) Locations of the 22 domains conserved among mouse, human and *Xenopus* in pufferfish and zebrafish *Otx* loci. No homologous region existed in the *FrOtx1* locus. Domains that exhibited enhancer activity are shown by red bars. Regions homologous to the mouse FM2 enhancer region (indicated by a yellow bar) were also not found in fish genomes. White boxes indicate the coding regions. The numbers below each bar represent sequence identity with the mouse counterpart in percentage terms. β-Gal staining in b yields expression directed by a 600 bp region covering domain β in zebrafish *Otx2*. (B) Sequence alignment of domain β in mouse, zebrafish and two pufferfish *Otx2* loci. Red square indicates a sequence conserved throughout gnathostome *Otx2* FM regions, the significance of which is currently unknown. Scale bars: 400 μm.

In the genomic region surrounding *Otx2*-coding region, 22 domains are conserved in mouse, human and *Xenopus*. The organization of these domains is also conserved among these animals (Kurokawa et al., 2004), and probably throughout all tetrapods. Furthermore, nine and five of these domains are conserved in *FrOtx2*a and zebrafish *Otx2*, respectively, in the same array (Fig. 8A). To consider these *Otx2*-cis elements in evolutional terms, *Otx2* genome information is desired for coelacanth/lungfish, shark and lamprey. With the exception of the FM2 enhancer, all *Otx2* enhancers identified correspond to one of these 22 domains; enhancer activities were confirmed in six domains in mouse in the present and accompanying study (Kurokawa et al., 2004). Many other domains may also exhibit enhancer activities at later stages.

Visceral endoderm is unique to mammals; the neural crest cells responsible for generation of cephalic mesenchyme arose with vertebrate evolution. Moreover, mesendoderm is common to chordates (Satou et al., 2001). In *Xenopus Otx2* and zebrafish *Otx2*, the regions homologous to the mouse non-ectodermal enhancers (domain α) also exist near the coding region; it remains for future studies to determine whether the domains retain these non-ectodermal enhancer activities. By contrast, non-ectodermal enhancers occur at about 3′ 15 kb downstream in *FrOtx2a*, and overall sequences are not conserved (Kimura et al., 1997) (C.K.-Y., I.M. and S.A., unpublished).

EP and AN enhancer regions are not conserved in either zebrafish or pufferfish (see Kurokawa et al., 2004). The lack of conservation of the AN enhancer region in these fish genomes was particularly unexpected. *Otx2* alone in tetrapod and all *Otx* genes in zebrafish are expressed in the anterior neuroectoderm. By contrast, the FM enhancer region (domain β) is deeply conserved in gnathostome *Otx2*. Obviously, discussion of the phylogenetic significance of the non-conservation of the EP/AN enhancer region and the conservation of the FM region requires the identification of the second mouse EP/AN enhancer. Nevertheless, the differences in *Otx2* expression in anterior neuroectoderm in teleost and tetrapod is notable. In tetrapods, *Otx2* is initially expressed in the entire neuroectoderm (under AN enhancer) and subsequently the expression is lost in the anterior region, which corresponds to the dorsal telencephalon (under FM enhancer). In zebrafish, *Otx2* is not expressed in the most anterior part of the neuroectoderm, even at the earliest phase (Li et al., 1994; Mori et al., 1994). It is tempting to speculate that in fish the FM enhancer might function also as the AN enhancer. A comprehensive analysis of *Otx* enhancers in fish is awaited (Kimura et al., 1997; Kimura-Yoshida et al., 2004).

Otx2 functions in forebrain/midbrain and upstream regulators

After an analysis of $Otx1^{+/-}Otx2^{+/-}$ double mutants, we reported previously that *Otx2* cooperates with *Otx1* for midbrain development (Suda et al., 1997). The cooperation, however, could not be examined in $Otx1^{-/-}Otx2^{+/-}$, $Otx1^{+/-}Otx2^{-/-}$ or $Otx1^{-/-}Otx2^{-/-}$ mutants because of earlier visceral endoderm defects in $Otx2^{-/-}$ mutants and postnatal lethality of $Otx1^{+/-}Otx2^{+/-}$ mutants. This situation obscured the onset and the extent of the cooperation between *Otx1* and *Otx2* in rostral brain development (Acampora et al., 1997; Suda et al., 1997). With the AN and FM/FM2 enhancers and their mutants, we now propose that the initial brain regionalization occurs around the three-somite stage under the AN enhancer, at a stage when FM and FM2 enhancers are still inactive (see Kurokawa et al., 2004). Metencephalic, mesencephalic, diencephalic and telencephalic regions differentiate at this stage; however, these regions are not yet determined when *Otx2* expression under FM and FM2 enhancers takes place. In the most severe $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutants, which lacked most of mesencephalon and diencephalon at E10.5, their primordia or the *Otx2*-, *Emx2*- and *Pax2*-positive regions developed normally at the 5 somite stage.

Even in the most severe $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutants *Emx2*-positive telencephalon developed normally. Apparently, the cooperation between *Otx1* and *Otx2* under FM and FM2 enhancers does not participate in telencephalon development. This is consistent with the lack of *Otx2* expression under these enhancers in dorsal telencephalon and of *Otx1* expression in ventral telencephalon (Frantz et al., 1994). Moreover, an analysis of the mutants suggests that the future telencephalic region has already differentiated when *Otx1* cooperates with *Otx2* under FM and FM2 enhancers. However, the precise anterior limit of the regions affected by the loss of the cooperation remains to be determined.

In $Otx2^{\Delta FM/-}$ and $Otx2^{\Delta FM2/-}$ mutants, *Fgf8*-positive isthmus expanded at E10.5 and cerebellum was enlarged at E15.5 as in $Otx1^{+/-}$ $Otx2^{+/-}$ mutants (Suda et al., 1997). In a series of double mutants in Fig. 7A, the lower the *Otx* dose and the more severe the phenotype, the more expanded was the *Fgf8*-positive ithmus. Most probably, mesencephalic and diencephalic regions that initially differentiated at the three-somite stage were secondarily transformed into metencephalon in these mutants (Acampora et al., 1997; Suda et al., 1997). Interestingly, however, *Fgf8*-positive isthmus was lost in the most severe $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutant (Fig. 7B, part c). It has been reported that the induction of the *Fgf8* expression in isthmus is independent of *Otx2*; rather, that *Otx2*, along with *Gbx2*, refines the *Fgf8* expression (Li and Joyner, 2001; Martinez-Barbera et al., 2001; Ye et al., 2001). However, these are observations made in the presence of *Otx1* expression. Moreover, *Fgf8* expression is also lost in the anterior neural ridge of $Otx1^{-/-}Otx2^{\Delta FM/\Delta FM}$ mutant. The details of the double mutant defects warrant examination in future studies.

The double mutants in Fig. 7A also indicate that OTX dose dependence differs regionally, i.e. the posterior mesencephalon requires elevated OTX for development. The onset of FM and FM2 enhancer activities, at around E8.5, coincides with the stage when broad, faint *Fgf8* expression contracts and the boundary between the midbrain and the hindbrain or the isthmus is established. A higher OTX dose might be required in the more posterior portion of the forebrain/midbrain to suppress posteriorizing signals from isthmus and/or anterior hindbrain (Martinez et al., 1999). The caudal end of the activities of both FM and FM2 enhancers coincides with the midbrain/hindbrain boundary. An attempt to identify the sequences that delineate the expression at the boundary is currently under way.

The identification of direct upstream regulators of *Otx2* expression at each site and at each stage is also an objective of our enhancer analysis. Potential TCF/LEF binding sites are essential in both FM and FM2 enhancers, whereas a putative OTX-binding site is additionally required in the FM enhancer. These sites in the FM enhancer are conserved in mouse,

human, *Xenopus*, pufferfish and zebrafish; one TCF/LEF-binding site is converted into a potential SOX-binding site in the fish. It is probable that *Otx2* expression under the FM enhancer is initiated by OTX2 directed by the AN enhancer. Subsequently *Otx2* expression under the FM and FM2 enhancers may be regulated by the WNT and TCF cascade. *Wnt* and *Tcf/Lef* expressions appear in dorsal forebrain/midbrain following E8.5 and in the cortical hem following E10 (Parr et al., 1993; Galceran et al., 2000; Lee et al., 2000).

This investigation, however, presents only a starting point in terms of the elucidation of many unknown factors that directly regulate these enhancers. AN enhancer analysis by Kurokawa et al. (Kurokawa et al., 2004) has been most successful in narrowing the focus of the essential, sufficient region; however, this essential region is still 90 bp long. The limited size of mouse anterior neuroectoderm also limits the biochemical identification of factors that bind to the 90 bp sequence. Full FM activity is harbored in the *Apa*I/*Hin*dIII 1.4 kb fragment; a 364 bp region within this fragment was essential to the activity and could not be shortened further. The FM region is also present in zebrafish *Otx2*, *FrOtx2a* and *FrOtx2b*, where the TCTAATTAAAAWGGATA sequence is conserved, the significance of which remains for future studies. The *Bsm*I/*Bam*H1 1.0 kb region displayed full FM2 enhancer activity. Within this region, *Bsm*I/*Hin*cII127bp was essential for FM2 activity; however, the 127 bp fragment alone did not exhibit this activity, and neither did the 3.0 kb FM2 region in the human *OTX2* locus. Moreover, the EP enhancer required multiple domains exceeding 2.3 kb. Enhancers that regulate expression in vivo during development may commonly exhibit such complexity, meaning that novel strategies will be required to identify critical upstream regulators with such enhancers.

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