Regulation of *Otx2* expression and its functions in mouse epiblast and anterior neuroectoderm

Daisuke Kurokawa[1], Nobuyoshi Takasaki[1], Hiroshi Kiyonari[2], Rika Nakayama[2], Chiharu Kimura-Yoshida[1], Isao Matsuo[1] and Shinichi Aizawa[1,2,*]

[1]Laboratory for Vertebrate Body Plan, Center for Developmental Biology (CDB), RIKEN Kobe, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0046, Japan
[2]Laboratory for Animal Resources and Genetic Engineering, Center for Developmental Biology (CDB), RIKEN Kobe, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0046, Japan
*Author for correspondence (e-mail: saizawa@cdb.riken.jp)

Accepted 8 April 2004

Development 131, 3307-3317
Published by The Company of Biologists 2004
doi:10.1242/dev.01219

Summary

We have identified cis-regulatory sequences acting on *Otx2* expression in epiblast (EP) and anterior neuroectoderm (AN) at about 90 kb 5′ upstream. The activity of the EP enhancer is found in the inner cell mass at E3.5 and the entire epiblast at E5.5. The AN enhancer activity is detected initially at E7.0 and ceases by E8.5; it is found later in the dorsomedial aspect of the telencephalon at E10.5. The EP enhancer includes multiple required domains over 2.3 kb, and the AN enhancer is an essential component of the EP enhancer. Mutants lacking the AN enhancer have demonstrated that these cis-sequences indeed regulate *Otx2* expression in EP and AN. At the same time, our analysis indicates that another EP and AN enhancer must exist outside of the –170 kb to +120 kb range. In *Otx2*$^{\Delta AN/-}$ mutants, in which one *Otx2* allele lacks the AN enhancer and the other allele is null, anteroposterior axis forms normally and anterior neuroectoderm is normally induced. Subsequently, however, forebrain and midbrain are lost, indicating that *Otx2* expression under the AN enhancer functions to maintain anterior neuroectoderm once induced. Furthermore, *Otx2* under the AN enhancer cooperates with *Emx2* in diencephalon development. The AN enhancer region is conserved among mouse, human and *Xenopus*; moreover, the counterpart region in *Xenopus* exhibited an enhancer activity in mouse anterior neuroectoderm.

Key words: *Otx2*, *Emx2*, Enhancer, Epiblasts, Anterior neuroectoderm, Mouse

Introduction

The Otx family of paired-type homeobox genes comprises vertebrate homologs of *Drosophila* head gap gene *otd* (Simeone et al., 1992). Mammals possess two homologs, *Otx1* and *Otx2*, whereas *Xotx4* and *otx3* are present in the natural tetraploid genomes of *Xenopus* and zebrafish; *Xotx4* and *otx3* are divergent copies of *Otx1*. *Otx2* is expressed in each step and at each site of head development throughout the tetrapod lineage. In mouse, its expression is seen in the epiblast, distal/anterior visceral endoderm, anterior mesendoderm, anterior neuroectoderm, forebrain/midbrain and cephalic mesenchyme. By contrast, *Otx1* expression occurs later, in forebrain and midbrain (Simeone et al., 1992; Simeone et al., 1993; Li et al., 1994; Mori et al., 1994; Bally-Cuif et al., 1995; Mercier et al., 1995; Pannese et al., 1995; Kablar et al., 1996; Kimura et al., 1997; Kimura et al., 2000; Kimura et al., 2001). In zebrafish, however, the expression patterns of Otx homologs are divergent. *otx1* is expressed in blastoderm prior to the shield stage. In addition, *otx1* and *otx3*, but not *otx2*, are expressed in the shield, i.e. the fish organizer (Mori et al., 1994). All zebrafish Otx genes are expressed in anterior mesendoderm. *otx1* and *otx3* expression also precede *otx2* expression in anterior neuroectoderm.

Mutant studies in mouse have suggested that *Otx2* plays essential roles in head development (Acampora et al., 1995; Acampora et al., 1997; Acampora et al., 1998; Ang et al., 1996; Matsuo et al., 1995; Rhinn et al., 1998; Rhinn et al., 1999; Suda et al., 1996; Suda et al., 1997; Suda et al., 1999; Suda et al., 2001; Tian et al., 2002; Kimura et al., 1997; Kimura et al., 2000; Kimura et al., 2001). However, owing to its expression in multiple sites, it has not to date been possible to distinguish clearly the functions of *Otx2* in each site. In addition, information is scarce regarding the upstream factors that regulate *Otx2* expression at each developmental site. In light of the essential roles played by Otx genes in head development, it will be important to develop an understanding of how mechanisms governing the regulation of Otx expression in each vertebrate are related and altered.

Previously, we have identified mouse enhancers of *Otx2* expression in visceral endoderm (VE), definitive anterior mesendoderm (AME) and cephalic neural crest cells or mesenchyme (CM); the sites located proximal to the *Otx2*-coding region (Kimura et al., 1997; Kimura et al., 2000). However, no ectodermal enhancers were present in this proximal region. Enhancers of *Otx2* expression in epiblast, anterior neuroectoderm and forebrain/midbrain were then sought by BAC transgenesis, and indications were that they

existed within the –170 kb 5′ upstream (A of the ATG translation start codon is taken as +1 bp). In the present study, we have analyzed the enhancers of *Otx2* expression in epiblast (EP) and anterior neuroectoderm (AN); analysis of the enhancers of *Otx2* expression in forebrain and midbrain is described in an accompanying paper (Kurokawa et al., 2004). EP and AN enhancers were identified at about 90 kb 5′ upstream. The EP activity was found in the inner cell mass of the blastocyst stage embryos and the entire epiblast of egg cylinder stage embryos. AN enhancer activity was detected initially at E7.0 and had ceased by E8.5. The EP enhancer included multiple required domains over 2.3 kb, and the AN enhancer was an essential component of the EP enhancer. Mutants lacking the AN enhancer were generated to demonstrate that these cis-sequences do indeed regulate *Otx2* expression in EP and AN, but additional EP and AN enhancers must exist outside of the –170 kb to +120 kb range. *Otx2* expression under the AN enhancer was essential to maintaining the anterior neuroectoderm once induced. Furthermore, *Otx2* under the AN enhancer cooperated with *Emx2* in diencephalon development.

Materials and methods

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

A mouse *Otx2* genomic BAC clone 582F09 (hereafter referred to as BAC#1) was isolated from a C57BL/6 BAC library (Research Genetics). It was subdivided into about 10 to 15kb fragments by *Sau*3AI partial digestion; the fragments were subcloned into the *Bam*HI site of pBluescript SK(–). These clones were aligned as shown in Fig. 2A by walking. Lengths and primers used to isolate mouse genomic sequences containing the κ, λ, μ, ν, ξ, ο, φ and χ, domains (Fig. 7A) by PCR were as follows: 3.1 kb with 5′-GCAAAACATGT-TACCTGCTAAGCAAC-3′ and 5′-TGAATTCAGAGAATGCAGCG-CATGC-3′; 3.3 kb with 5′-TCCTATGCAGACTTGGCTGGCCTGG-3′ and 5′-TCCCTCTCTGGCACTCAGGCTGAGG-3′; 2.8 kb with 5′-CTAGCTTTTAGCTGCAATATAGAAAGG-3′ and 5′-GGACCCT-GAAGAACCGGGCAGAAAT-3′; 3.5 kb with 5′-GAGAGTAGT-GCTGCATTGAACATGG-3′ and 5′-CAAGGTTGTCAAGGAGT-GTTCAATG-3′; 1.6 kb with 5′-CATAAATGTGCAATGGCAAGT-GTCC-3′ and 5′-CATCAGCGAGCAATCCGTAGCCAGG-3′; 1.9 kb with 5′-GAGATGATCATGCAGCTCTCGG-3′ and 5′-CTCTGT-GAACCATGTAAGACTGG-3′; 0.7 kb with 5′-GAGAGAGAGGA-GATTTTGCTTTCTTGAAGC-3′ and 5′-GTTCCCTTACCCTATC-CACAATGCACTGTTCC-3′; and 0.7 kb with 5′-ACTATTTAACTG-GCTGCCCTTTTTCGCACTGG-3′ and 5′-GAGCAGGGAGG-GCATCAGATCCGCTGTACAAG-3′, respectively. The *Xenopus (Silurana) tropicalis* genomic sequence containing domain δ was 1.6 kb with 5′-TGGAGAAACACAATAAAAGAGC-3′ and 5′-AAC-CAAAGAGTCTCCATAGC-3′.

Generation of BAC transgenic mice

BAC #1 reporter transgenic mice were generated as described by Helms et al. (Helms et al., 1998). The 3′ terminal of BAC #1 was located at –30 kb 5′ upstream from the *Otx2* translation start site (Fig. 1A). Five kb DNA at –35 kb to –30 kb 5′ upstream was ligated to 1.8 K-*lacZ*, which led to the production of 5′-*lacZ*. BAC #1 and 5′-*lacZ* were separated from vector-derived sequences via digestion with *Not*I and *Sal*I, respectively (Yang et al., 1997), followed by co-injection into the pronucleus of fertilized CD-1 mouse eggs (concentration of each, 1-2 ng/μl). Transgenic mice harboring the correct recombinant were identified by PCR. Primers employed for this purpose were 5′-GCCAAACACAGAATCAGCAAGGAGGAGGCCATGC-3′ in BAC #1 and 5′-CCAGATTTACATTCAGAACACAAGTCTTATTTCCC-CACCC-3′ in the *lacZ* gene.

Production and genotyping of transgenic mice

The 1.8 kb *lacZ* reporter is a modified version of the VEcis-*lacZ* vector of Kimura et al. (Kimura et al., 1997). The *Not*I-*Pma*CI-*Sac*II linker was inserted into the *Sal*I/*Bgl*II site of the 5′ polylinker in the vector. Each DNA fragment was inserted between these *Not*I and *Sac*II sites. Permanent transgenic mice and transient transgenic embryos were generated and β-gal expression was determined as described previously (Kimura et al., 1997; Kimura et al., 2000).

PCR mutagenesis

Utilizing the PCR-based overlap extension method (Kucharczuk et al., 1999), each 15 bp block of EB165 bp AN core enhancer (#1-#11 in Fig. 4B) was replaced with the linker sequence 5′-AGACTG-GATCCTGTG-3′. Putative transcription factor binding sites in each enhancer were also mutated by the transverse substitution of nucleotides using this approach.

Sequence analysis

All genomic DNAs isolated or mutagenized by PCR were sequenced to verify the absence of spurious mutations. Comparison of the mouse *Otx2* genomic sequences with other species was conducted with the Berkeley Genome Pipeline and Genome VISTA (Couronne et al., 2003) programs. Additionally, sequence alignment was confirmed with the BLAST program (Mayor et al., 2000). Putative transcription binding sites were predicted with the TFSEARCH program (Heinemeyer et al., 1998).

Generation of AN enhancer knockout mice

A neomycin resistance gene with Pgk1 promoter and SV40 polyadenylation signal was flanked by loxP sequences. This cassette (Neo) was replaced with the *Spe*I/*Bgl*II 559 bp region, which harbors the AN enhancer. Lengths of the homologous regions were 8.0 kb and 4.0 kb at the 5′ and 3′ sides of the *neo* cassette, respectively. Diphtheria toxin A fragment gene with MC1 promoter was used for the negative selection of homologous recombinants as described (Yagi et al., 1993b). The targeting vector was linearized with *Sal*I and electroporated into TT2 ES cells (Yagi et al., 1993a). Homologous recombinants were identified as those that generate 4.0 kb products by PCR with 5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′ in the *neo* gene as the 5′ side primer and 5′-GATCCTCCTGCCTCTGCG-TCAATAGC-3′ as the 3′ side primer; the 3′ primer is located outside of the 3′ side 4kb homologous region in *Otx2* locus. The recombinants were confirmed by Southern blot analyses as described (Matsuo et al., 1995). Two mutant mouse strains were generated from independent homologous recombinant ES clones. The genotype of mutant mouse or embryo was routinely determined by PCR using tail or yolk sac specimens. Wild-type allele was detected with the sense primer of the 5′-CTGCAGATGCTTGGGCTTTCTGCAGC-3′ and the antisense primer of the 5′-GGGGTCTTCATGAGTTTCTGTTGCAC-3′. The mutant allele was identified with the sense primer of 5′-ATCG-CCTTCTTGACGAGTTCTTCTG-3′ in the *neo* gene and the antisense primer. 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).

Histological analysis

Mouse embryos were fixed overnight with Bouin's fixative solution at room temperature. Specimens were subsequently dehydrated and embedded in paraplast. Serial sections (10 μm) were prepared and stained with Hematoxylin and Eosin or with 0.1% Cresyl Violet.

RNA in situ hybridization

Whole-mount and section in situ hybridization was performed using digoxigenin probes, as described by Suda et al. (Suda et al., 2001).

The probes used in this study were for the genes: brachyury/*T* (Herrmann, 1991), *Fgf8* (Crossley and Martin, 1995), *Cer1* (Belo et al., 1997), *Dlx1* (Bulfone et al., 1993) *Emx2* (Yoshida et al., 1997), *Gbx2* (Bulfone et al., 1993), *Lhx1/Lim1* (Fujii et al., 1994), *Otx2* (Matsuo et al., 1995), *Pax6* (Walther and Gruss, 1991), *Six3* (Oliver et al., 1995) and *Tcf4* (Korinek et al., 1998).

Quantitative RT-PCR

RNA isolation and reverse transcription PCR was performed according to Kimura et al. (Kimura et al., 2001). Primer sets used were as follows: *Otx2*, 5′-TCTTATCTAAAGCAACCGCCTTACGCAG-TC-3′ and 5′-GCACCCTGGATTCTGGCAAGTTGATTTTCA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGTCAT-CAACGGGAAGCCCA-3′ and 5′-TTGTCATGGATGACCTTGGC-3′.

Animal housing

Animals were housed in environmentally controlled rooms in accordance with the Institute of Physical and Chemical Research (RIKEN) guidelines for animal experiments.

Results

Otx2 expression in ectoderm is regulated by enhancers located remote from the coding region

We had previously searched the mouse genomic region from –50 kb 5′ upstream to +10 kb 3′ downstream of the *Otx2* translation start site for sequences involved in regulation of *Otx2* expression (Kimura et al., 1997; Kimura et al., 2000) (C.K.-Y., I.M. and S.A., unpublished). The major transcription start site of *Otx2* expression exists at –207 bp 5′ upstream of the translational start site (Guazzi et al., 1998; Courtois et al., 2003). The –1.8 kb 5′ region adjacent to the translational start site is capable of directing the expression in VE, AME and CM (Kimura et al., 1997; Kimura et al., 2000); the construct in which the 1.8 kb genome was combined with the *lacZ* gene was designated as 1.8k-*lacZ*. However, no sequences were detected which directed *Otx2* expression in epiblast, anterior neuroectoderm or forebrain/midbrain. In the present study, we generated transgenic mice harboring a genomic DNA spanning –170 kb to –30 kb 5′ upstream (BAC #1) with 1.8k-*lacZ* (Fig. 1A) in order to identify these sequences. The 5′ *BAC#1/lacZ* transgene was generated by homologous recombination in mouse zygotes (Helms and Johnson, 1998) by co-injecting BAC#1 and a *lacZ* reporter; the reporter had the 5kb 3′ end of BAC#1 ligated to the 1.8k-*lacZ*.

The *5′BAC#1/lacZ* transgenic mouse line captured β-gal expression in E6.5 epiblast, E7.75 anterior neuroectoderm and E9.5 forebrain and anterior midbrain (Fig. 1B, parts a-c). β-Gal expression was not detected in posterior midbrain (Fig. 1B, part c). Subsequently, the *Otx2* genomic region encompassing –170 kb to –30 kb was systematically dissected into 15 fragments with partial overlaps at both ends to determine the locations of regulatory elements (Fig. 2A). All enhancer analyses described below were conducted using the mouse *Otx2* 1.8 kb promoter. Expression by this promoter (Fig. 1B, part d) is indicated by arrows in each figure. For several DNA fragments, enhancer activities were also assayed using *Fugu Otx2* promoter (Kimura et al., 1997) or *hsp68* promoter. For example, an *Eco*RV/*Bgl*II 2.3kb fragment capable of driving *lacZ* expression in epiblast with the 1.8 kb promoter (Fig. 3B, parts b-d) exhibited the activity, though weak, using the *hsp68* promoter (Fig. 3B, parts e,f). An enhancer in the #13 fragment (Fig. 2A) that had the transcriptional activity in forebrain and midbrain with the 1.8 kb promoter showed a similar activity with the *Fugu* promoter. By contrast, a *Spe*I 6.2 kb fragment (Fig. 3A) that exhibited activity in the anterior neuroectoderm did not show the same activity with the *Fugu* promoter, and exhibited low activity in the ectoderm and ectopic activity in extra-embryonic ectoderm with the *hsp68* promoter (data not shown). Moreover, a *Eco*RV/*Bgl*II 2.3 kb fragment (Fig. 3A) exhibited similar activities in the anterior neuroectoderm with the *hsp68* promoter and mouse 1.8 kb promoter (Fig. 3B, parts g,h). Thus, the enhancer activity of several DNA fragments described below is promoter dependent. The details of the promoter dependence, however, remain a topic for future studies. Note that, in the present paper, the term ‘enhancer’ is used in the broad sense of ‘cis-regulatory sequence’.

Transgenic lines were established for each of the 15 fragments as indicated in the parenthetical statement in Fig. 2A. Analyses of these lines mapped the enhancer of *Otx2* expression in E6.5 epiblast and E7.75 anterior neuroectoderm to fragment #12, which was located about –80 kb to –95 kb 5′ upstream. The enhancer of *Otx2* expression in E9.5 forebrain and midbrain was mapped to fragment #13, which was located about –70 kb to –80 kb 5′ upstream. In this mapping, heterozygous *Otx2*$^{+/lacZ}$ embryos (Kimura et al., 2000) were stained in parallel to identify β-gal expression from the endogenous *Otx2* locus (Fig. 2B, parts g-k). When necessary, whole-mount RNA

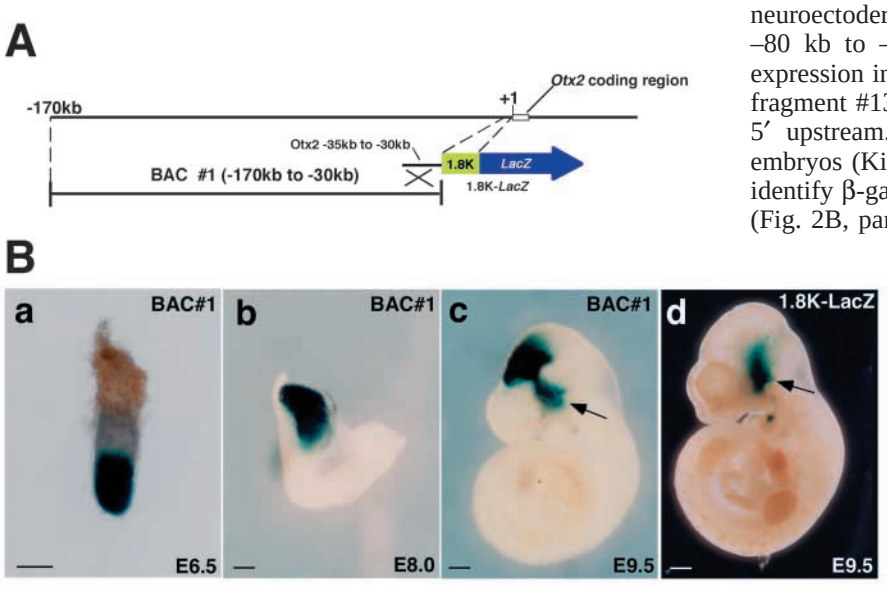

Fig. 1. Search for enhancers of *Otx2* expression in ectoderm at 5′ upstream by BAC transgenesis. (A) *BAC #1/lacZ* reporter gene. A of the ATG translation start codon is taken as +1 bp. (B) β-Gal expression in ectoderm at each stage by BAC #1 (a-c) and expression in cephalic mesenchyme (d) by the 1.8 kb promoter. Arrows indicate expression by the 1.8 kb promoter. Scale bars: 100 μm in a,b; 400 μm in c,d.

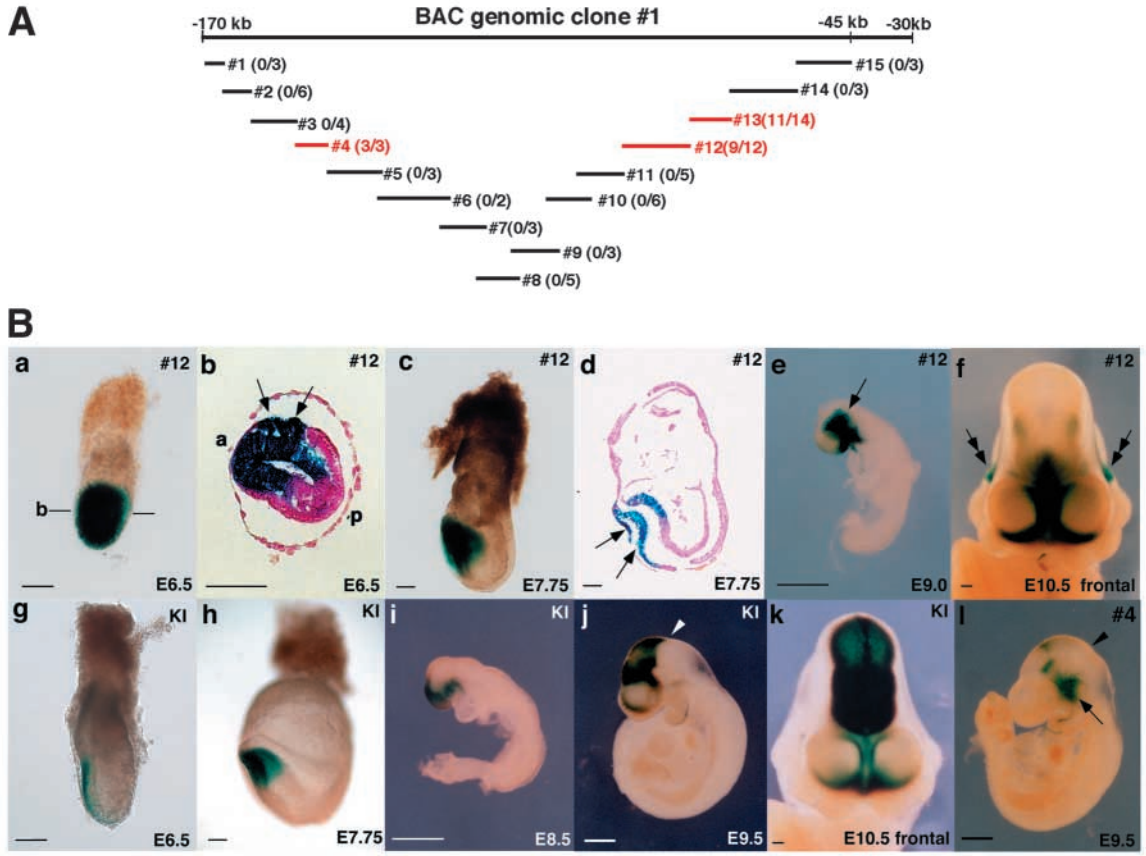

Fig. 2. Dissection of ectodermal enhancers at 5′ upstream. (A) Schematic diagram depicting the 15 fragments assayed for enhancer activity. Number of lines exhibiting β-gal expression among permanent transgenic lines established is indicated in parenthesis. Fragments displaying enhancer activity are indicated in red. (B) β-Gal expression driven by #12 (a-f), by endogenous enhancers in *Otx2*$^{+/lacZ}$ knock-in embryos (g-k) and by #4 (l). The β-gal expression indicated by arrows represents the expression in anterior visceral endoderm (b), anterior mesendoderm (d) and cephalic mesenchyme (e,l) by the 1.8 kb promoter (Kimura et al., 1997). The expression in e is solely due to the activity of the 1.8 kb promoter (compare with Fig. 1B, part d). Compare the expression in d with the 1.8 kb promoter with that in Fig. 3B, part h with the *hsp68* promoter. The #4 fragment exhibited β-gal expression in a region of the ventral diencephalon and dorsal mesencephalon. In lateral views (a,c-e,g-j,l), anterior is leftwards; (b) cross-section in the plane shown in a; (f,k) frontal views. Arrowheads and double arrows indicate the position of the isthmus and the expression in the eyes, respectively. a, anterior; p, posterior. Scale bars: 100 μm in a-d,g,h; 400 μm in e,f,i-l.

in situ hybridization analysis was performed in transgenic embryos to compare endogenous *Otx2* and transgenic *lacZ* expression. These analyses unequivocally indicated that fragments #12 and #13 contain sequences necessary to direct *Otx2* expression in ectoderm at each developmental stage. This study focuses on the enhancer activity in the #12 fragment; an analysis of the activity of the #13 fragment is reported (Kurokawa et al., 2004). In addition to #12 and #13 fragments, the #4 fragment drove β-gal expression in a region of the ventral diencephalon and dorsal mesencephalon, the significance of which will be addressed in future studies (Fig. 2B, part l). Some fragments exhibited β-gal expression in structures where *Otx2* is not expressed endogenously; no further analysis was made of these activities.

The activity of the #12 fragment was initially noted in entire epiblast; at E6.5 it was absent in the posterior region (Fig. 2B, parts a,b). At E7.75, β-gal expression was restricted to the anterior neuroectoderm, which corresponds to the presumptive forebrain and midbrain (Fig. 2B, parts c,d); this activity was lost by E8.5 (Fig. 2B, part e). After E9.0, the fragment exhibited activity in the most dorsomedial aspect of the telencephalon and in the eyes (Fig. 2B, part f). From this activity profile, the enhancer in the #12 fragment was designated as the EP/AN (epiblast/anterior neuroectoderm) enhancer.

The expression of β-gal in the epiblast at the *Otx2* locus is noteworthy. Endogenous *Otx2* is expressed in epiblast. However, β-gal expression is not observed in the epiblast of *Otx2*$^{+/lacZ}$ embryos (Fig. 2B, part g) (Boyl et al., 2001; Courtois et al., 2003). By contrast, β-gal expression was evident in epiblast with the BAC #1/*lacZ* transgene (Fig. 1B, part a) and with the *lacZ* transgene under the #12 fragment (Fig. 2B, parts a,b), respectively. The #12 fragment also drove β-gal expression in epiblast under the *Otx2*$^{+/-}$ background (data not shown); the discrepancy is not due to the *Otx2* dose effect. Translational control has been suggested in the expression of transgenes knocked-in into *Otx2* locus (Boyl et al., 2001). However, in both the *lacZ* knock-in mutation into the *Otx2* locus and the random *lacZ* transgenesis in the enhancer analysis, the *lacZ* gene was fused to the *Otx2* 5′ untranslated region and *SV40* 3′UTR in exactly the same manner. The cause of these variations of β-gal expression in epiblast is unclear.

Epiblast enhancer

The 15 kb #12 fragment was subdivided by *Spe*I digestion (Fig. 3A) in order to define the EP/AN enhancer. The enhancer activity of each subfragment was determined by transient transgenic assay at E6.5 and E7.75. However, none of these *Spe*I fragments drove β-gal expression in epiblast at E6.5. Subsequently, the #12 fragment was re-subdivided into five fragments so that each *Spe*I site was preserved (Fig. 3A). In

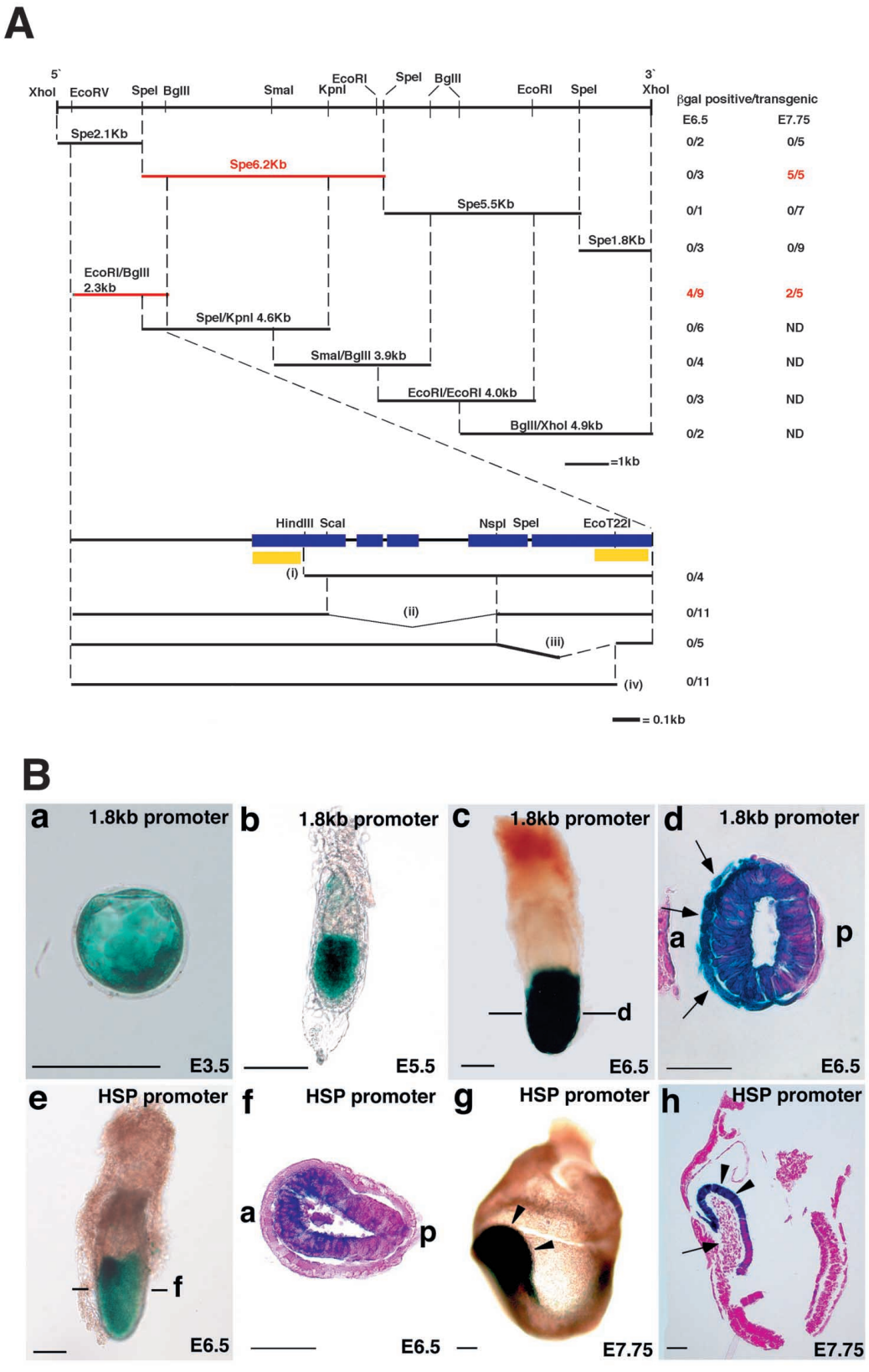

Fig. 3. Deletion analysis of EP enhancer. (A) Dissection of the #12 genomic fragment for EP and AN enhancers. The number of β-gal-positive embryos among transient transgenic embryos obtained is indicated on the right (ND, not determined). Blue boxes indicate regions demonstrating in excess of 80% identity over more than 100 bp between mouse and human; yellow boxes indicate the regions demonstrating in excess of 80% identity over more than 100 bp between mouse and *Xenopus*. (B) β-Gal expression driven by the *Eco*RV/*Bgl*II 2.3 kb fragment with 1.8 kb promoter (a-d) and with *hsp68* promoter (e-h) at E3.5 (a), E5.5 (b), E6.5 (c-f) and E7.75 (g,h). In lateral views (c,e,g), anterior is leftwards; (d,f) cross-sections at the level indicated in b,d, respectively; (h) a sagittal section. Arrows in d indicate β-gal expression in anterior visceral endoderm by the 1.8 kb mouse *Otx2* promoter; this expression is absent with *hsp68* promoter (f). The expression is absent in definitive anterior mesendoderm with *hsp68* promoter (an arrow in h, compare with Fig. 2B, part d). Scale bars: 100 μm.

this subdivision, β-gal expression in epiblast was driven by the *Eco*RV/*Bgl*II 2.3 kb fragment (Fig. 3A), which was confirmed by generation of permanent transgenic lines. Activity of the *Eco*RV/*Bgl*II 2.3 kb fragment was initially found in the inner cell mass of blastocysts at E3.5 (Fig. 3B, part a) and in the entire epiblast at E5.5 (Fig. 3B, part b), and gradually declined in the posterior region with the progress of gastrulation in a manner similar to endogenous *Otx2* expression (Fig. 3B, parts c-f). At the headfold stage the fragment exhibited the activity in anterior neuroectoderm (Fig. 3B, parts g,h).

Unexpectedly, however, when the *Eco*RV/*Bgl*II 2.3 kb fragment was subdivided further, fragments that lacked any of the following sequences lost their activity: (1) *Eco*RV/*Hin*dIII 0.95 kb, (2) *Sca*I/*Nsp*I 0.6 kb, (3) *Nsp*I/*Eco*T22I 0.57 kb or (4) *Eco*T22I/*Bgl*II 165 bp (Fig. 3A). Thus, all of these domains were essential for enhancer activity in epiblast. Regions homologous to the *Eco*RV/*Bgl*II 2.3 kb fragment exist at similar positions relative to the *Otx2* coding region in the rat, human and *Xenopus* (*Silurana*) *tropicalis* (hereafter, with regard to the genome information, '*Xenopus*' refers to this species) genomes (see Fig. 7). Regions displaying greater than 80% sequence identity over more than 100 bp are present throughout the *Eco*RV/*Bgl*II 2.3 kb fragment (Fig. 3A).

Anterior neuroectoderm enhancer

In contrast to the epiblast enhancer, the regulatory sequences of *Otx2* expression in anterior neuroectoderm were present in the *Spe*I 6.2 kb fragment (Fig. 3A). This fragment was successively dissected as shown in Fig. 4A, and the transient transgenic assay at E7.75 revealed the existence of activity exclusively in the *Spe*I/*Bgl*II 559 bp. With this fragment β-gal expression in the anterior neuroectoderm was detected initially at E7.0 and ceased by E8.5. The caudal boundary of β-gal expression was not distinct near the preotic sulcus (Fig. 4C, part a; data not shown).

The *Spe*I/*Bgl*II 559 bp fragment was further dissected, and finally the *Eco*T22I/*Bgl*II 165 bp fragment was found to retain the enhancer activity; this was confirmed by generation of permanent transgenic lines (Fig. 4C, part a). A region exhibiting 92% identity to this 165 bp fragment is found about 5′ 97 kb upstream in the human *Otx2* locus (Fig. 4B; see Fig. 7A). The 5′ part of the 165 bp fragment includes the sequences TGGCGACTGAC and TGGGCGCTGGC, which correspond to each other with the exception of two bases. Furthermore, these sequences are conserved in the human counterpart. Five tandem repeats of these sequences, however, did not direct β-gal expression in anterior neuroectoderm (Fig. 4A). In the 165 bp fragment, potential OCT/HOX (Herr and Cleary, 1995) and SOX (van Beest et al., 2000) binding sites exist; however, AN activity was unaffected by mutations in these sites.

A linker-scanner approach was then employed to define the core elements. Each 15 bp sequence of the EcoT22I/*Bgl*II 165 bp fragment was replaced with the *Bam*HI linker (Fig. 4B) (Kucharczuk et al., 1999). Replacement of any of the six 5′ side 15 bp blocks (#6-#11) affected β-gal expression in the anterior neuroectoderm at E7.75. Such replacements in three of these blocks (mt#6, mt#7 and mt#10) essentially abolished the activity (Fig. 4C, part b), whereas mutations in #8, #9 and #11 demonstrated faint β-gal expression (Fig. 4C, part c) at the frequencies indicated in the parentheses of Fig. 4B (asterisks). In addition, when 75 bp sequences corresponding to #11-7 were duplicated in tandem and combined with 1.8 k-*lacZ*, the transgene exhibited significant β-gal expression in E7.75 anterior neuroectoderm (Fig. 4C, part d). A region homologous to EcoT22I/*Bgl*II 165 bp is present about 5′ 44 kb upstream in the *Xenopus Otx2* locus (Fig. 7, Fig. 4B), displaying sequence identity of 77%. Identity is 87% in the #11-#6 90 bp sequences; however, no homologous region is present in the zebrafish or pufferfish genomes. A 1.6 kb *Xenopus* DNA region covering this domain was then isolated by PCR, and its enhancer activity was tested using the mouse 1.8 kb promoter. The 1.6 kb region clearly directed β-gal expression in mouse anterior neuroectoderm (Fig. 4C, part e)

Targeted disruption of *Otx2* epiblast enhancer activity

The analysis described above indicated that the *Spe*I/*Bgl*II 559 bp region was essential for expression in epiblast, though not, by itself, sufficient. This fragment is located ~90 kb away from the coding region, which raised a question regarding its involvement in the regulation of *Otx2* expression in vivo. In order to examine this point, mutant mice ($Otx2^{\Delta AN/\Delta AN}$) were generated in which the *Spe*I/*Bgl*II 559 bp was replaced with a cassette encoding a neomycin-resistant gene (Fig. 5A).

The epiblast develops in *Otx2*-null mutant ($Otx2^{-/-}$) embryos (Kimura et al., 2000), and it also developed in $Otx2^{\Delta AN/\Delta AN}$ embryos. In this epiblast, the *Otx2* expression was diminished but not abolished (Fig. 5B, parts a,b). Quantitative RT-PCR confirmed a reduction in *Otx2* expression (Fig. 5B, part c), indicating that the *Spe*I/*Bgl*II 559 bp region functions as an epiblast enhancer, but also that another enhancer of *Otx2* expression in epiblast must be present.

In mammals, the anteroposterior axis is first generated along the distoproximal axis. Prior to primitive streak formation, distal visceral endoderm cells move to the future anterior and proximal epiblast cells to the future posterior. This axis rotation does not occur in $Otx2^{-/-}$ mutants (Kimura et al., 2000). However, axis rotation proceeded normally in $Otx2^{\Delta AN/\Delta AN}$ mutants as well as in $Otx2^{\Delta AN/-}$ embryos, in which one *Otx2* allele lacks the *Spe*I/*Bgl*II 500 bp and the other allele is null, as indicated by *cerberus-like* expression in the anterior visceral endoderm (Fig. 5C, part a) and *T* expression in the primitive streak (Fig. 5C, part b) at E6.5. This observation is consistent with our previous conclusion that *Otx2*-positive distal visceral endoderm cells, but not *Otx2*-positive epiblast, are responsible for the axis rotation defect in $Otx2^{-/-}$ mutants (Kimura et al., 2000).

Targeted disruption of *Otx2* anterior neuroectoderm enhancer activity

The *Spe*I/*Bgl*II 559 bp was essential and sufficient for driving *Otx2* expression in anterior neuroectoderm. In $Otx2^{\Delta AN/\Delta AN}$ mutants, however, no defects were found in the forebrain or midbrain. *Otx2* expression was reduced but not eliminated in E7.5 $Otx2^{\Delta AN/\Delta AN}$ mutants (data not shown). Subsequently, *Otx2* levels were reduced further via the generation of $Otx2^{\Delta AN/-}$ mutants. At E12.5 the $Otx2^{\Delta AN/-}$ mutant phenotype was variable. In milder cases forebrain was present though greatly deformed; in severe cases both forebrain and midbrain were lost (Fig. 6A, part b). At E7.5 *Six3*-positive anterior neuroectoderm was induced in all $Otx2^{\Delta AN/-}$ mutants examined

(Fig. 6B, part b). At E8.5, however, *Six3*-positive region was reduced (Fig. 6B, part d), and concomitantly *Fgf8*- or *Gbx2*-negative anterior neuroectoderm was reduced but still present at E8.5 (Fig. 6B, parts f,h). The phenotype became variable at E9.5, and in severe mutants *Gbx2*-negative forebrain/midbrain and *Emx2*-positive forebrain were entirely missing (Fig. 6B, parts j, l). Thus, the *Spe*I/*Bgl*II 559 bp located 90 kb 5′ upstream clearly regulates *Otx2* expression in anterior neuroectoderm; however, another enhancer must also exist.

In our previous analysis of $Otx2^{+/-}Emx2^{-/-}$ mutants, we reported that *Otx2* cooperates with *Emx2* in diencephalon development (Suda et al., 2001). *Emx2* expression occurs at the three-somite stage (E8.0) when the AN enhancer is active. To confirm that *Emx2* cooperates with *Otx2* under the AN enhancer, $Otx2^{\Delta AN/\Delta AN}Emx2^{-/-}$ mutants were generated. In these mutants, diencephalon was lost (Fig. 6A, part c); at E11.5 *Pax6*- and *Dlx1*-positive ventral thalamus and *Tcf4*- and *Gbx2*-positive dorsal thalamus were absent (Fig. 6C, parts a-h), while *Lhx1*- and *Tcf4*-positive commissural region of the pretectum developed (Fig. 6C, parts g-j). This phenotype was very similar to the $Otx2^{+/-}Emx2^{-/-}$ mutant phenotype.

Search for the second EP/AN enhancer

Enhancer mutants indicated the presence of another enhancer of *Otx2* expression in epiblast and anterior neuroectoderm. Consequently, a search was conducted for the second enhancer in the 3′ region of the *Otx2* gene. BAC#2 transgenic mice, which harbor a genomic DNA region spanning –50 kb to +120 kb 3′ downstream, were generated (Fig. 7) (Kurokawa et al., 2004). However, this region displayed no activity in epiblast or anterior neuroectoderm.

Mouse *Otx2* locates at chromosome14 19 cM; the nearest genes are encoded ~350 kb upstream on the 5′ side and ~150 kb downstream on the 3′ side (Fig. 7). More

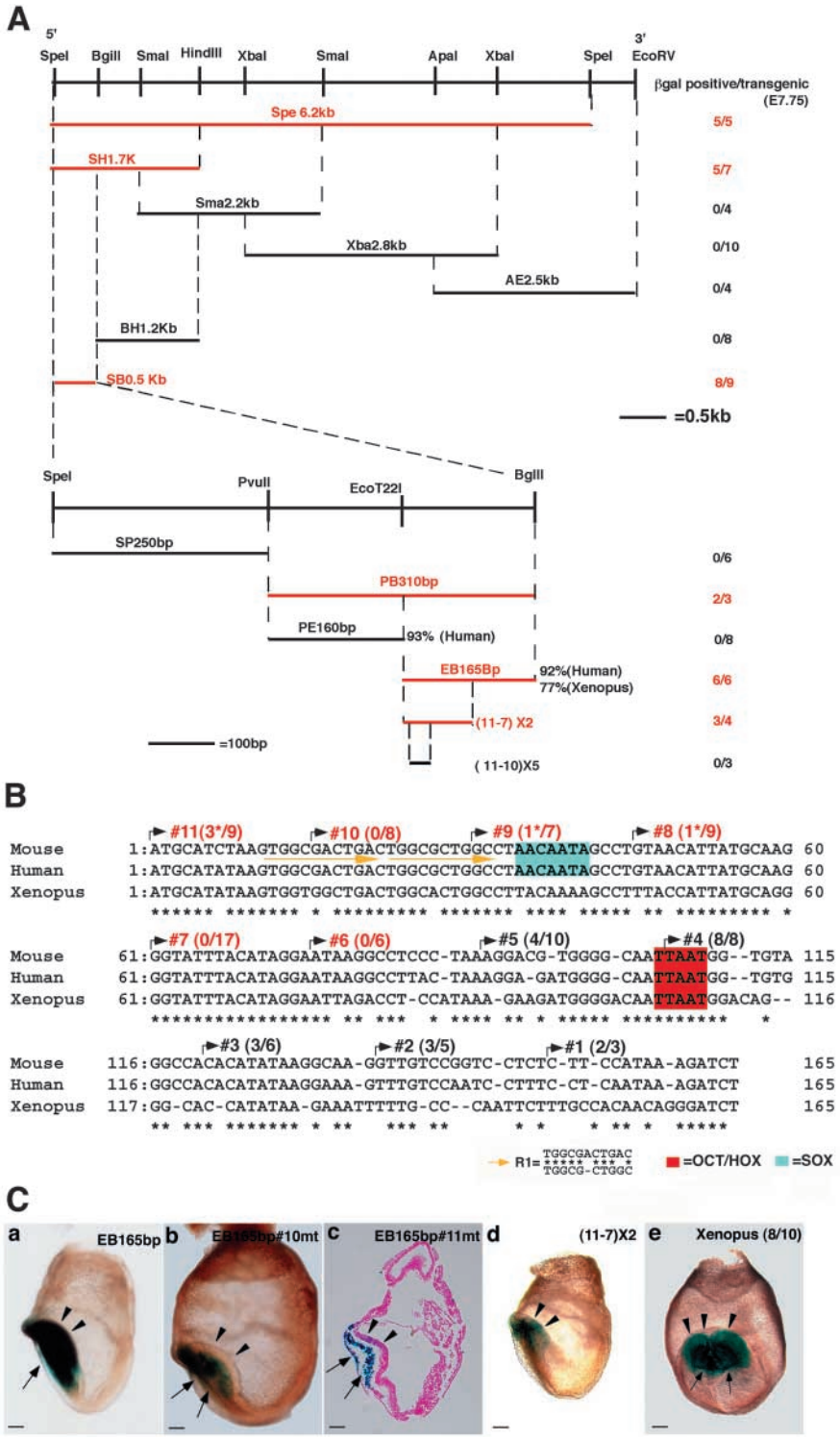

Fig. 4. Deletion analysis of AN enhancer. (A) Fine mapping of the AN enhancer. (11-7)×2 is a duplicate of the #11-#7 sequences (see B), whereas (11-10)×5 is a quintuplet of the #11-#10 sequences. Percentages indicate the sequence identity of the fragment with the counterpart region of human or *Xenopus*. (B) Nucleotide sequences of the mouse, human and *Xenopus* EB 165 bp region. Linker-scanner mutations were introduced such that each 15 bp block was replaced with a transcriptionally inert 15 bp linker. The number of β-gal-positive embryos among transient transgenic embryos in each block is provided in parenthesis; mutations affecting enhancer activity are indicated in red. Asterisks in #8, #9 and #11 represent residual expression as shown in C, part c. (C) β-gal expression driven by the EcoT22I/*Bgl*II(EB)165 bp subfragment (a), by the EB165 bp with the mutation at #10 (b), by the EB165 bp with the mutation at #11 (c), by the (11-7)×2 fragment (d) and by a 1.6 kb *Xotx2* region (e) at E7.75. (a-d) Lateral views, anterior is leftwards; (e) a frontal view. Note the loss of expression by the #10 mutation (b), residual expression by the #11 mutation (c) and significant expression with the (11-7)×2 fragment (d) in the anterior neuroectoderm (arrowheads). Arrows indicate the expression in anterior mesoderm and endoderm by the 1.8 kb promoter. Scale bars: 100 μm.

than 100 highly conserved domains (sequence identity greater than 80% over more than 100 bp) exist in this 500 kb span among mouse, rat and human. Of these domains, 22 are also conserved in the *Xenopus* genome. Fig. 7 depicts the locations of these domains in the mouse, human and *Xenopus Otx2* loci. The organization is identical in mouse and human. The contiguous mapping is partial in the *Xenopus Otx2* locus; however, domains α-ι, ρ and σ locate in identical order in a more compact fashion.

The EP/AN enhancer region identified above was one of the 22 domains conserved, δ. We next sought a second EP/AN enhancer among domains κ-ο, φ and χ, which exist outside of the regions analyzed with BAC#1 and #2. Depending on the sizes of the conserved regions, the lengths of genome DNAs isolated by PCR were 3.1, 3.3, 2.8, 3.5, 1.6, 1.9, 0.7 and 0.7 kb for κ, λ, μ, ν, ξ, ο, φ and χ domains, respectively. Subsequently, sequences were confirmed and enhancer activity was determined with the 1.8 kb promoter by transient transgenic assay. None of these domains, however, exhibited enhancer activity in E6.5 epiblast or E7.75 anterior neuroectoderm.

Discussion

We previously identified 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). In the present study, we have identified enhancers of *Otx2* expression in epiblast (EP) and anterior neuroectoderm (AN); Kurokawa et al. (Kurokawa et al., 2004) report enhancers of *Otx2* expression in forebrain/midbrain (FM). DVE/AVE, AME and CM enhancers located near the *Otx2*-coding region; by contrast, all ectodermal enhancers occurred at great distances from the coding region.

Otx2 epiblast and anterior neuroectoderm enhancers

Otx2 expression is differentially regulated in each germ layer. The regulation of these expressions by different enhancers could be reasonably expected; the existence of distinct VE, AME, CM and ectoderm enhancers was anticipated. In mouse, *Otx2* expression in epiblast and anterior neuroectoderm is continuous. However, *Otx2* expression in chick and in *Xenopus* is consistent with discrete EP and ANE enhancers. In avian, following the disappearance of *Otx2* expression in epiblast, expression occurs transiently in Hensen's node and in the anterior primitive streak exclusively and appears in the anterior neuroectoderm subsequently (Bally-Cuif et al., 1995; Kablar et al., 1996). AN enhancer activity begins around E7.0 and ceases by E8.5. Of note is that the EP enhancer also directed the expression in inner cell mass of blastocysts.

Generally, the identification of enhancers is conducted solely by random transgenesis. This study unequivocally demonstrated the necessity of mutating the enhancer in vivo to determine whether and how the enhancer is responsible for endogenous gene expression. The EP/AN enhancer mutants indicated that another enhancer exists for *Otx2* expression in epiblast and anterior neuroectoderm. However, the second enhancer was not present within the 3′ 120 kb region covered by BAC #2. The overall sequences in EP and AN enhancer region are well conserved in mouse, human and *Xenopus*. Then

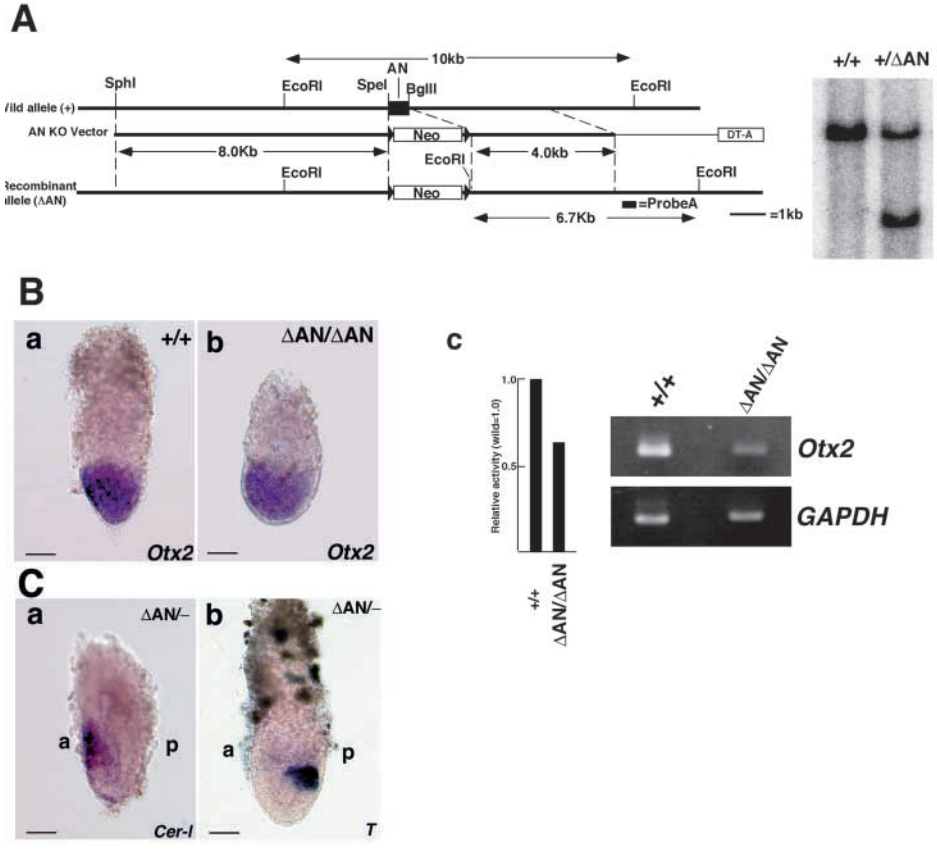

Fig. 5. Targeted disruption of the EP/AN enhancer. (A) Wild-type *Otx2* allele, targeting vector and recombinant allele. The black box indicates the *Spe*I-*Bgl*II 559 bp region (AN) that is replaced with a neomycin-resistant gene (Neo, white boxes) flanked by *loxP* sequences (black triangles). DT-A is the diphtheria toxin-A fragment gene with MCI promoter, which is used for negative selection of homologous recombinants (Yagi et al., 1993b). Thick and thin lines indicate genomic and vector-derived sequences, respectively. Probe A is the Southern blotting probe used for identification of homologous recombinant ES cells displayed in the right panel. (B) *Otx2* expression at E6.5 by whole-mount in situ hybridization (a,b) and by quantitative RT-PCR (c). +/+ and ΔAN/ΔAN denote wild-type embryos and homozygous mutants lacking the *Spe*I/*Bgl*II region, respectively. (C) *Otx2*$^{\Delta AN/-}$ mutant phenotype at E6.5. Normal expression of *cerberus-like* in anterior visceral endoderm (a) and of *T* in primitive streak (b). Scale bars: 100 μm.

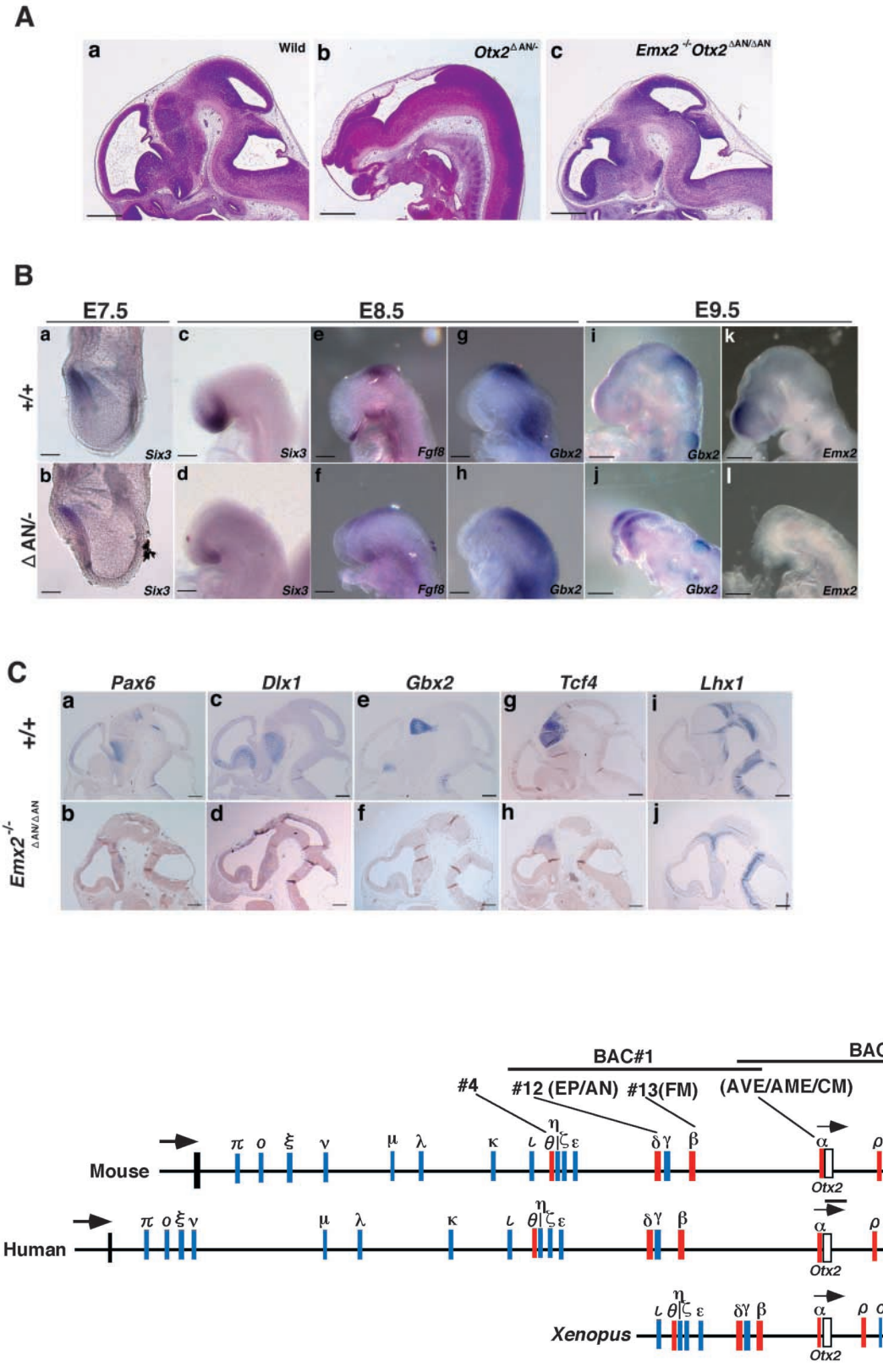

Fig. 6. AN enhancer mutant phenotype. (A) Histological features of forebrain defects in $Otx2^{\Delta AN/-}$ (b) and $Emx2^{-/-}Otx2^{\Delta AN/\Delta AN}$ (c) mutants at E12.5; (a) wild-type brain. These phenotypes were examined with both the $Otx2^{\Delta AN}$ mutant in which the neo insert remained and the $Otx2^{\Delta AN}$ 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. The E12.5 $Otx2^{\Delta AN/-}$ phenotype was variable, and a severe phenotype is shown in b. Scale bars: 400 μm. (B) Marker analyses of wild-type (a,c,e,g,i,k) and $Otx2^{\Delta AN/-}$ mutant (b,d,f,h,j,l) embryos at E7.5 (a,b), E8.5 (c-h) and E9.5 (i-l); expression of *Six3* (a-d), *Fgf8* (e,f), *Gbx2* (g-j) and *Emx2* (k,l). The E9.5 phenotype was variable; severe examples are shown. Scale bars: 100 μm in a,b; 150 μm in c-h; 400 μm in i-l. (C) Marker analyses of diencephalon in E11.5 wild-type (a,c,e,g,i) and $Emx2^{-/-}Otx2^{\Delta AN/\Delta AN}$ mutant (b,d,f,h,j) embryos; expression of *Pax6* (a,b), *Dlx1* (c,d), *Gbx2* (e,f), *Tcf4* (g,h) and *Lhx1* (i,j). Scale bars: 400 μm.

Fig. 7. Genome organization in tetrapod *Otx2* locus. Locations of the 22 domains in *Otx2* locus conserved among mouse, human and *Xenopus*. Black bars in mouse and human *Otx2* locus define the locations of the nearest genes at the 5′ and 3′ sides, respectively. Domains that exhibited enhancer activity are shown by red bars [see Kurokawa et al. (Kurokawa et al., 2004) for details of the analysis of the 3′ region]. The contiguous mapping is partial in the *Xenopus Otx2* locus; however, domains κ–π and τ–χ exist in the genome. White boxes indicate the *Otx2*-coding regions.

the second EP/AN enhancers were searched for among domains conserved in mouse, human and *Xenopus*, outside of the 290 kb region covered by BAC#1 and BAC#2. However, no EP/AN activity was detected in any of these domains. The second enhancer might exist much farther away over the adjacent genes. At the same time, the possibility remains that the second EP/AN enhancer exists uniquely in rodent outside of the 290 kb genomic region, as the second enhancer of *Otx2* expression in forebrain and midbrain, FM2, is not conserved in human or *Xenopus*, and is most probably unique to the rodent (Kurokawa et al., 2004).

The EP enhancer appears to consist of multiple domains, and the AN enhancer is a component of the EP enhancer, the significance of which remains to be addressed in future studies. EP and AN enhancer regions are conserved in human and *Xenopus*, a *Xotx2* 1.6 kb region covering the AN enhancer region exhibited the enhancer activity in mouse anterior neuroectoderm. The EP/AN region is, however, not conserved in zebrafish or pufferfish. In zebrafish blastoderm *Otx1*, but not *Otx2*, is expressed. Fish blastoderm may be a product independent of the amniote epiblast. However, the lack of conservation of the AN enhancer region in fish genome was unexpected. *Otx2* alone in tetrapod and all Otx genes in zebrafish are expressed in the anterior neuroectoderm. It is possible that the ancient AN enhancer widely diverged between the lineages to the extant teleost and tetrapod to cope with the divergence in anterior neuroectoderm development, and so the molecular machinery involved in its development might be extremely varied between teleost and tetrapod (Kurokawa et al., 2004). Discussion of the phylogenetic significance of enhancer organization obviously requires the identification of the second EP/AN enhancer. In addition, a comprehensive enhancer analysis must be performed in fish Otx genes (Kimura et al., 1997; Kimura-Yoshida et al., 2004).

Otx2 functions in anterior neuroectoderm

The generation of enhancer mutants to allow the dissection of *Otx2* functions in each site at each step of head development was a primary objective of enhancer analysis in the present investigation. The presence of two enhancers of *Otx2* expression in anterior neuroectoderm complicated the analysis. Nevertheless, $Otx2^{\Delta AN/-}$ mutants clearly demonstrated that *Otx2* expression under the AN enhancer functioned to maintain the anterior neuroectoderm once induced. This was originally suggested by analyses of *Otx1* knock-in mutants into *Otx2* locus ($Otx2^{Otx1/Otx1}$) and a hypomorphic *Otx2* mutant ($Otx2^{frt\text{-}neo/frt\text{-}neo}$) (Suda et al., 1999; Li and Joyner, 2001; Martinez-Barbera et al., 2001; Tian et al., 2002). However, in these mutants the role of *Otx2* expression in anterior mesendoderm in the loss of the anterior neuroectoderm could not be ruled out.

Studies of $Otx2^{+/-}Emx2^{-/-}$ mutants have previously suggested that *Otx2* cooperates with *Emx2* in the development of the diencephalic region (Suda et al., 2001). However, the onset of the defects was not clear. $Otx2^{\Delta AN/\Delta AN}Emx2^{-/-}$ phenotypes were quite similar to $Otx2^{+/-}Emx2^{-/-}$ phenotypes; at E12.5 ventral thalamus, dorsal thalamus and anterior part of the pretectum were lost, but the commissural region of the pretectum had developed. No marker analyses were made, but histologically archipallium was not apparent, while neopallium and ganglionic eminences developed. Mesencephalon was largely normal, while cerebellum was enlarged in the double mutants. *Emx2* expression takes place in the laterocaudal forebrain primordium around the three-somite stage (E8.0) when the AN enhancer is active. Furthermore, β-gal expression under the AN enhancer was not detected at E8.5; β-gal is stable and usually persists longer than endogenous gene products. Thus, we propose that the diencephalic region may be induced around the three-somite stage by cooperation between *Emx2* and *Otx2* under the AN enhancer. With loss of this cooperation, the structures that derive from the *Emx2*-positive region at the three-somite stage may be lost.

We thank Ms S. Nagayoshi, T. Ohmura and Mr. N. Takeda for their technical assistance. We are indebted to Drs E. M. De Robertis, M. A. Frohman, N. Heintz, B. G. Herrmann, P. Gruss and G. R. Martin, who generously provided us a number of plasmids, and to Dr H. Hamada, who kindly provided us *Lefty-Cre* mice. We are also grateful to the Laboratory for Animal Resource and Genetic Engineering for the housing of mice. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A. and Brulet, P. (1995). Forebrain and midbrain regions are deleted in $Otx2^{-/-}$ mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279-3290.

Acampora, D., Avantaggiato, V., Tuorto, F. and Simeone, A. (1997). Genetic control of brain morphogenesis through Otx gene dosage requirement. *Development* **124**, 3639-3650.

Acampora, D., Avantaggiato, V., Tuorto, F., Briata, P., Corte, G. and Simeone, A. (1998). Visceral endoderm-restricted translation of Otx1 mediates recovery of Otx2 requirements for specification of anterior neural plate and normal gastrulation. *Development* **125**, 5091-5104.

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L. and Rossant, J. A. (1996). Targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122**, 243-252.

Bally-Cuif, L., Gulisano, M., Broccoli, V. and Boncinelli, E. (1995). c-Otx2 is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryo. *Mech. Dev.* **49**, 49-63.

Belo, J. A., Bouwemeester, T., Leyns, L., Ketrtesz, N., Gallo, M., Follettie, M. and de Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68**, 45-57.

Boyl, P. P., Signore, M., Acampora, D., Martinez-Barbera, J. P., Ilengo, C., Annio, A., Corte, G. and Simeone, A. (2001). Forebrain and midbrain development requires epiblast-restricted Otx2 translational control mediated by its 3′ UTR. *Development* **128**, 2989-3000.

Bulfone, P., di Blas, E., Gulisano, M. H., Frohman, M. A., Martin, G. R. and Rubenstein, J. L. R. (1993). Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13**, 3155-3172.

Couronne, O., Poliakov, A., Bray, N., Ishkhanov, T., Ryaboy, D., Rubin, E., Pachter, L. and Dubchak, I. (2003). Strategies and tools for whole-genome alignments. *Genome Res.* **13**, 73-80.

Courtois, V., Chatelain, G., Han, Z.-Y., Novere, N. L., Brun, G. and Lamonerie, T. (2003). New Otx2 mRNA isoforms expressed in the mouse brain. *J. Neurochem.* **84**, 840-853.

Crossley, P. H. and Martin, G. R. (1995). The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.

Fujii, T., Pichel, J. G., Taira, M., Toyama, R. and Dawid, I. B. (1994). Expression patterns of the murine LIM classes homeobox gene lim1 in the developing brain and excretory system. *Dev. Dyn.* **199**, 73-83.

Guazzi, S., Pintonello, M. L., Vigano, A. and Boncinelli, E. (1998). Regulatory interactions between the human HOXB1, HOXB2, and HOXB3

proteins and the upstream sequence of the Otx2 gene in embryonal carcinoma cells. *J. Biol. Chem.* **273**, 11092-11099.

Helms, A. W. and Johnson, J. E. (1998). Progenitors of dorsal commissural interneurons are defined by MATH1 expression. *Development* **125**, 919-928.

Herr, W. and Cleary, M. A. (1995). The POU domain: versatility in transcriptional reguration by a flexible two-in-one DNA binding domain. *Genes Dev.* **9**, 1679-1693.

Herrmann, B. G. (1991). Expression pattern of the brachyury gene in whole-mount TWis/TWis mutant embryos. *Development* **113**, 913-917.

Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., Podkolodny, N. L. and Kolchanov, N. A. (1998). Databases on transcriptional regulation: TRANSFAC, TRRD, and COMPEL. *Nucleic Acids Res.* **26**, 364-370.

Kablar, B., Vignali, R., Menotti, L., Pannese, M., Andreazzoli, M., Polo, C., Giribaldi, M. G., Boncinelli, E. and Baracchi, G. (1996). Xotx genes in the developing brain of Xenopus laevis. *Mech. Dev.* **55**, 145-158.

Kimura, C., Takeda, N., Suzuki, M., Oshimura, M., Aizawa, S. and Matsuo, I. (1997). Cis-acting elements conserved between mouse and pufferfish Otx2 genes govern the expression in mesencephalic neural crest cells. *Development* **124**, 3929-3941.

Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S. and Matsuo, I. (2000). Visceral endoderm mediates forebrain development by suppression posteriorizing signals. *Dev. Biol.* **225**, 304-321.

Kimura, C., Shen, M. M., Takeda, N., Aizawa, S. and Matsuo, I. (2001). Complementary functions of Otx2 and Cripto in initial patterning of mouse epiblast. *Dev. Biol.* **235**, 12-32.

Kimura-Yoshida, C., Kitajima, K., Oda-Ishii, I., Tian, E., Suzuki, M., Yamamoto, M., Suzuki, T., Kobayashi, M., Aizawa, S. and Matsuo, I. (2004). Characterization of the pufferfish *Otx2* cis-regulators reveals evolutionarily conserved genetic mechanisms for the vertebrate head specification. *Development* **131**, 57-71.

Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. and Clevers, H. (1998). Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* **18**, 1248-1256.

Kucharczuk, K. L., Love, C. M., Dougherty, N. M. and Goldhamer, D. J. (1999). Fine-scale transgenic mapping of the MyoD core enhancer: MyoD is regulated by distinct but overlapping mechanisms in myotomal and non-myotomal muscle lineages. *Development* **126**, 1957-1965.

Kurokawa, D., Kiyonari, H., Nakayama, R., Kimura-Yoshida, C., Matsuo, I. and Aizawa, S. (2004). Regulation of *Otx2* expression and its functions in mouse forebrain and midbrain. *Development* **131**, 3319-3331.

Li, Y., Allende, M. L., Finkelstein, R. and Weinberg, E. S. (1994). Expression of two zebrafish orthodenticle-related genes in the embryonic brain. *Mech. Dev.* **48**, 229-244.

Li, J. Y. H. and Joyner, A. L. (2001). *Otx2* and *Gbx2* are required for refinement and not induction of mid-hindbrain gene expression. *Development* **128**, 4979-4991.

Martinez-Barbera, J. P., Signore, M., Boyl, P. P., Puelles, E., Acampora, D., Gogoi, R., Schubert, F., Lumsden, A. and Simeone, A. (2001). Regionalization of anterior neuroectoderm and its competence in responding to forebrain and midbrain inducing activities depend on mutual antagonism between OTX2 and GBX2. *Development* **128**, 4789-4800.

Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. and Aizawa, S. (1995). Mouse Otx2 functions in the formation and patterning of rostral head. *Genes Dev.* **9**, 2646-2658.

Mayor, C., Brudno, M., Schwartz, J. R., Poliakov, A., Rubin, E. M., Frazer, K. A., Pachter, L. S. and Dubchak, I. (2000). VISTA: Visualizing Global DNA Sequence Alignments of Arbitrary Length. *Bioinformatics* **16**, 1046-1047.

Mercier, P., Simeone, A., Cotelli, F. and Boncinelli, E. (1995). Expression pattern of two otx genes suggests a role in specifying anterior body structures in zebrafish. *Int. J. Dev. Biol.* **39**, 559-573.

Mori, H., Miyazaki, Y., Morita, T., Nitta, H. and Mishina, M. (1994). Different spatio-temporal expression of three otx homeoprotein transcripts during zebrafish embryogenesis. *Mol. Brain Res.* **27**, 221-231.

Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and in expressed during eye development. *Development* **121**, 4045-4055.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The *Xenopus* homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., le Meur, M. and Ang, S.-L. (1998). Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**, 845-856.

Rhinn, M., Dierich, A., le Meur, M. and Ang, S.-L. (1999). Cell autonomous and non-cell autonomous function of Otx2 in patterning the rostral brain. *Development* **126**, 4295-4304.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992). Nested expression domains of four homeobox genes in the developing rostral brain. *Nature* **358**, 687-690.

Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M. R., Nigro, V. and Boncinelli, E. (1993). A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* **12**, 2735-2747.

Suda, Y., Matsuo, I., Kuratani, S. and Aizawa, S. (1996). Otx1 function overlap with Otx2 in development of mouse forebrain and midbrain. *Genes Cells* **1**, 1031-1044.

Suda, Y., Matsuo, I. and Aizawa, S. (1997). Cooperative between Otx1 and Otx2 genes in developmental patterning of rostral brain. *Mech. Dev.* **69**, 125-141.

Suda, Y., Nakabayashi, J., Matsuo, I. and Aizawa, S. (1999). Functional equivalency between Otx2 and Otx1 in development of the rostral head. *Development* **126**, 743-757.

Suda, Y., Hossain, Z. M., Kobayashi, C., Hatano, O., Yoshida, M., Matsuo, I. and Aizawa, S. (2001). Emx2 directs the development of diencephalon in cooperation with Otx2. *Development* **128**, 2433-2450.

Tian, E., Kimura, C., Takeda, N., Aizawa, S. and Matsuo, I. (2002). Otx2 is required to respond to signals from anterior neural ridge for forebrain specification. *Dev. Biol.* **242**, 204-223.

van Beest, M., Dooijes, D., van de Wetering, M., Kjaerulff, S., Bonvin, A., Nielsen, O. and Clevers. H. (2000). Sequence-specific high mobility group box factors recognize 10-12-base pair minor groove motifs. *J. Biol. Chem.* **275**, 27266-27273.

Walther, C. and Gruss, P. (1991). Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y. and Aizawa, S. (1993a). A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* **214**, 70-76.

Yagi, T., Nada, S., Watanabe, N., Tamemoto, H., Kohmura, N., Ikawa, Y. and Aizawa, S. (1993b). A novel negative selection for homologous recombinants using diphtheria toxin A fragment gene. *Anal. Biochem.* **214**, 77-86.

Yamamoto, M., Meno, C., Sakai, Y., Shiratori, H., Mochida, K., Ikawa, Y., Saijoh, Y. and Hamada, H. (2001). The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse. *Genes Dev.* **15**, 1242-1256.

Yang, X. W., Model, P. and Heintz, N. (1997). Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15**, 859-865.

Yoshida, M., Suda, Y., Matsuo, I., Miyamoto, N., Takeda, N., Kuratani, S. and Aizawa, S. (1997). Emx1 and Emx2 functions in development of dorsal telencephalon. *Development* **124**, 101-111.