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Otx2 expression in anterior neuroectoderm and forebrain/midbrain is directed by more than six enhancers

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ABSTRACT

Otx2 plays essential roles in each site at each step of head development. We previously identified the AN1 enhancer at 91 kb 5′ upstream for the *Otx2* expressions in anterior neuroectoderm (AN) at neural plate stage before E8.5, and the FM1 enhancer at 75 kb 5′ upstream and the FM2 enhancer at 122 kb 3′ downstream for the expression in forebrain/midbrain (FM) at brain vesicle stage after E8.5. The present study identified a second AN enhancer (AN2) at 88 kb 5′ upstream; the AN2 enhancer also recapitulates the endogenous *Otx2* expression in choroid plexus, cortical hem and choroidal roof. However, the enhancer mutants indicated the presence of another AN enhancer. The study also identified a third FM enhancer (FM3) at 153 kb 5′ upstream. Thus, the *Otx2* expressions in anterior neuroectoderm and forebrain/midbrain are regulated by more than six enhancers located far from the coding region. The enhancers identified are differentially conserved among vertebrates; none of the AN enhancers has activities in caudal forebrain and midbrain at brain vesicle stage after E8.5, nor do any of the FM enhancers in anterior neuroectoderm at neural plate stage before E8.5.

Introduction

A paired-type homeobox gene, *Otx2*, is expressed in each site at each step of mouse head development (Simeone et al., 1993; Kimura et al., 2000; Kurokawa et al., 2004a). It is first expressed in inner cell mass (ICM) at the blastocyst stage and then in epiblast (EP) at the egg cylinder stage. The expression is regulated by an EP enhancer located 92 kb 5′ upstream (Kurokawa et al., 2004a). Recently, *Otx2* has been suggested to regulate the stem cell state of ICM and epiblast (Acampora et al., 2013). With the formation of the distal visceral endoderm (DVE) at E5.5 which expresses a series of the head organizer genes such as *Hex* and *Cerl*, *Otx2* is also expressed at this site, being regulated by an enhancer located at the promoter region proximal to the translation start site (Kimura et al., 2000; Kurokawa et al., 2010). This *Otx2* expression is essential for the anterior movement of the DVE cells to generate anterior visceral endoderm (AVE) (Kimura-Yoshida et al., 2005). *Otx2* is also expressed in definitive anterior mesoendoderm (AME), being regulated by enhancers at the promoter region (Kimura et al., 2000; Kimura-Yoshida et al., 2007; Kurokawa et al., 2010). The *Otx2* expression in AVE and AME is essential to the development of anterior neuroectoderm, probably to suppress posteriorizing signals (Ang et al., 1994; Acampora et al., 1995; Kimura et al., 2000). The enhancers also direct the *Otx2* expression in cephalic neural crest cells at the migratory phase (Kimura et al., 1997; Kurokawa et al., 2010), and structures associated with the cells are uniquely affected in *Otx2* heterozygous mutants (Matsuo et al., 1995).

Otx2 is also one of the genes earliest expressed in anterior neuroectoderm (AN) that is induced by AVE and AME; the caudal limit of its expression is initially obscured in front of the otic sulcus. We have identified an AN1 enhancer at 91 kb 5′ upstream; it does not have an activity in epiblast. The mutant that lacks this enhancer demonstrated that the *Otx2* expression in anterior neuroectoderm is essential to the development of anterior neuroectoderm once induced by AVE and AME or to suppress the caudalization of the ectoderm into isthmus and rhombomere 1 or metencephalon (Kurokawa et al., 2004a); the *Otx2* expression in anterior neuroectoderm is not essential to the induction of the ectoderm itself (Kimura et al., 2000). However, the *Otx2* expression is reduced but

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not lost in the AN1 enhancer mutant ($Otx2^{\Delta AN1/\Delta AN1}$), and the mutant phenotype is variable. This suggests that there is another enhancer for the *Otx2* expression in anterior neuroectoderm.

The *Otx2* expression is lost in the most rostral forebrain from E8.5, and at E9.5 the expression is not found in telencephalon except for the most dorsomedial region and most ventral telencephalon (Kurokawa et al., 2004b; Sakurai et al., 2010). The caudal limit of the *Otx2* expression at this stage is sharply delineated at the midbrain/hindbrain boundary (MHB); the expression covers caudal forebrain and midbrain. An *Otx2* paralogue, *Otx1*, starts to be expressed at E8.25 in forebrain and midbrain (FM). The AN1 enhancer loses its activity around E8.5, and we have identified the FM1 enhancer located 75 kb 5′ upstream and the FM2 enhancer located 122 kb 3′ downstream responsible for the subsequent *Otx2* expression in caudal forebrain and midbrain at brain vesicle stage (Kurokawa et al., 2004b). In mouse embryos, neither FM1 nor FM2 enhancer has activities in anterior neuroectoderm at the neural plate stage when the AN1 enhancer is active; the AN1, FM1 and FM2 activities overlap around 3–6 somite stages at E8.5. The caudal limit of the FM1 activity coincides with the MHB by the counteraction with *Gbx2* (Joyner et al., 2000; Katahira et al., 2000; Simeone, 2000; Inoue et al., 2012). Rostrally the enhancers do not have activities in most of the telencephalon. Enhancer mutants that lack both FM1 and FM2 enhancers together with *Otx1* ($Otx1^{-/-}Otx2^{\Delta FM1\Delta FM2/\Delta FM1\Delta FM2}$) indicated that the *Otx2* expression under these enhancers cooperates with *Otx1* for caudal forebrain and midbrain development or to suppress the caudalization of caudal forebrain and midbrain into anterior hindbrain or metencephalon (Suda et al., 1997; Kurokawa et al., 2004b; Sakurai et al., 2010). However, the *Otx2* expression remains in the FM1 and FM2 double enhancer mutants ($Otx2^{\Delta FM1\Delta FM2/\Delta FM1\Delta FM2}$) to suggest that there is another enhancer for the expression in forebrain and midbrain (Sakurai et al., 2010).

AN1, FM1 and FM2 enhancers were identified by examining enhancer activities of genomic DNA fragments from 174 kb 5′ upstream to 124 kb 3′ downstream encompassing the mouse *Otx2* locus (Fig. 5A; Kurokawa et al., 2004a, 2004b). We have reexamined enhancer activities of these fragments and here identified AN2 and FM3 enhancers at 88 kb and 153 kb 5′ upstream, respectively. However, the *Otx2* expression is not lost in the mutants that lack both AN1 and AN2 enhancers, suggesting that there is another enhancer for the *Otx2* expression in anterior neuroectoderm. Thus, the *Otx2* expression in anterior neuroectoderm and forebrain/midbrain is regulated by more than six enhancers located far apart from the coding region. The enhancers identified are differentially conserved among vertebrates, and none of the FM enhancers has activities in anterior neuroectoderm at neural plate stage, nor do any of the AN enhancers in caudal forebrain and midbrain at brain vesicle stage.

Materials and methods

Genome information

A homology search was performed with the VISTA program (http://genome.lbl.gov/vista/index.shtml). Parameters were set as follows: calculation window, 100 bp; consensus width, 100 bp; and consensus identity, 70%. Genomic sequence of human, mouse, *Xenopus*, coelacanth, fugu, medaka, zebrafish and skate were obtained by UCSC genome browser (http://genome.ucsc.edu/).

Enhancer analysis and βGal staining

The C–E fragments in Fig. 2C were obtained by polymerase chain reaction (PCR) using a XbaI/SmaI 1077 bp DNA fragment, which had been subcloned previously into pBluescript II SK(+), as the template (Kurokawa et al., 2004a). Primers and lengths of amplified DNAs are, the C fragment, 736 bp with M13 Reverse (5′-GGAAACAGCTATGACCATG-3′) and XS-R (5′-AGATTTAAAACAACTCCCTG-3′); the D fragment, 477 bp with XS-F (5′-CCCTAAGGACAAGAGATGCC-3′) and M13 Forward (5′-GTAAAACGACGGCCAGT-3′); and the E fragment, 204 bp with b motif-F (5′-TTTTCTTTCTTAAATTGTATTTTCTGTG-3′) and b motif-R (5′-CCCGGGGCAACAAGTGCGTCTGTAATT-3′). The DNA fragments containing κ and σ domains were obtained by PCR using C57BL/6 mouse genomic DNA as template. Primers and lengths of amplified products are, κ, 1516 bp with Fp1 (5′-TCCGAAAGGTCAACTCCCCAAAGCACATGC-3′) and Rp1 (5′-AGCACCTATATGGAACATGTAGCTCTCAGT-3′); and σ, 1188 bp with Fp2 (5′-CAATTCTTCATTTAAATAAAGCTAGACTAG-3′) and Rp2 (5′-ACAGTTCGAGGGACAAGAAGCACGTTCCAT-3′).

Each genomic DNA fragment was verified by sequencing and conjugated with the 1.8 kb *Otx2* promoter proximal to the translation start site (Kimura et al., 1997; Kurokawa et al., 2004a) and *lacZ* gene. They were then injected into CD1 zygotes as described (Kurokawa et al., 2004a). Embryos were dissected in phosphate-buffered saline (PBS) and fixed for few minutes at room temperature in 0.2% glutaraldehyde and 2% paraformaldehyde in PBS. βGal expression was determined as described (Kimura et al., 1997, 2000).

Mutant mice

Otx1 null mutant mice (Acc. no. CDB0017K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html; Suda et al., 1997), *Otx2* null mutant mice (Acc. no. CDB0010K; Matsuo et al., 1995) and *Otx2* AN1 enhancer mutant mice (Acc. no. CDB0049K; Kurokawa et al., 2004a) were generated previously. To target the AN2 enhancer, the targeting vector was constructed by replacing the 2.2 kb SmaI fragment with the *Neo* cassette (Supplementary Fig. 1); the cassette consists of *Pgk1* promoter, neomycin resistant gene and SV40 polyadenylation signal, being flanked with *loxP* sequences (Kurokawa et al., 2004a). The lengths of the homologous regions were 6.5 kb and 3.9 kb at the 5′ and 3′ sides of the cassette, respectively. To target both AN1 and AN2 enhancers, the targeting vector was constructed by replacing the 3.5 kb SpeI/SmaI fragment with the *Neo* cassette (Supplementary Fig. 1). The lengths of the homologous regions were 5.2 kb and 3.9 kb at the 5′ and 3′ sides of the cassette, respectively. The construction of the targeting vectors, isolation of homologous recombinant ES clones and production of the AN2 enhancer mutant mice ($Otx2^{\Delta AN2}$; CDB0054K) or AN1/AN2 enhancer mutant mice ($Otx2^{\Delta AN1\Delta AN2}$; CDB0053K) were performed as described (Yagi et al., 1993a, 1993b); the details of which will be provided upon request. The genotypes of mutant mice were routinely detected by PCR using tail or yolk sac specimens. The primers used to identify the wild type AN2 allele were p1 (5′-TCAGCTGAAAAGCTCACACCTACAGC-3′) and p2 (5′-CAAAGTCACTGGCTAAAGCAACCAGG-3′); ΔAN2 allele were p2 and p3 (5′-ATCGCCTTCTTGACGAGTTCTTCTG-3′) in the *Neo* cassette; those to identify the wild type AN1/AN2 allele were p4 (5′-ATCTCTTCACACTGGTTCTGACTC-3′) and p5 (5′-GGCCTACACCATTAATTGCCCCACGTCC-3′); and those to identify the mutant ΔAN1ΔAN2 allele were p4 and p6 (5′-TGTCACGTCCTGCACGACGCGACT-3′) in the *Neo* cassette. The locations of these primers are indicated in Supplementary Fig. 1. The PCR yields 205 and 522 bp products for the wild type and mutant AN2 alleles, and 583 and 410 bp products for the wild type and mutant AN1/AN2 alleles, respectively. Mice were housed and experiments were performed under the CDB guidelines for animal and recombinant DNA experiments.

RNA in situ hybridization

Embryos were dissected in PBS and fixed overnight at 4 °C in 4% paraformaldehyde in PBS. Whole-mount and section in situ