

Cis-acting elements conserved between mouse and pufferfish *Otx2* genes govern the expression in mesencephalic neural crest cells

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SUMMARY

Previous studies suggested that the *Otx2* gene plays an essential role in the development of cranial skeletons and nerves of mesencephalic neural crest origin. To clarify this role, we have identified the *cis*-acting elements in mouse and pufferfish *Otx2* genes responsible for the expression in the crest cells using a transgenic approach with the *lacZ* reporter gene. In mouse, 49 bp sequences in the proximal 5' region upstream were essential and sufficient to direct the transgene expression in the cephalic mesenchyme. In pufferfish, the 1.1 kb distal region, located far downstream (from +14.4 to +15.5 kb), had almost identical activity. Between them, several DNA sequences were conserved, and mutational analyses indicated that motif A was critical for the transgene expression in the premandibular region while motif B was critical in both premandibular and mandibu-

lar regions. Motif B, CTAATTA, contains the core motif for binding of homeodomain proteins while motif A, TAAATCTG, does not match any known consensus binding sequences for transcriptional factors. The cephalic mesenchyme that expressed β -galactosidase under these *cis*-elements is most likely to correspond to mesencephalic crest cells. Thus the molecular machinery regulating *Otx2* expression in these cells appears to be conserved between mouse and fish, implying a crucial role of the *Otx2* gene in development of the neural-crest-derived structures of the gnathostome rostral head.

Key words: cephalic mesenchyme, neural crest cell, *cis*-acting element, *Otx2*, homeobox, pattern formation, transgenic analysis, pufferfish

INTRODUCTION

Otx1 and *Otx2* genes are the mouse cognates of the *Drosophila* head gap genes, *orthodenticle* (*otd*) (Finkelstein and Perrimon 1991; Simeone et al., 1992). The cognates have also been identified in human, chick, *Xenopus* and zebrafish (Simeone et al., 1993; Ang et al., 1994; Li et al., 1994; Bally-Cuif et al., 1995; Bliz and Cho, 1995; Pannese et al., 1995; Mercier et al., 1995). Common expression among these vertebrate cognates has suggested that (1) *Otx2* may function as a head organizer component at the primitive streak stage, and (2) at a subsequent neurula to pharyngula stage those genes may be involved in regional patterning of the forebrain and midbrain (Puelles and Rubenstein, 1993; Simeone et al., 1992). Indeed, *Otx2* homozygous mutants failed to develop a structure anterior to rhombomere 3 (Matsuo et al., 1995; Acampora et al., 1995; Ang et al., 1996), as do mutants of *Lim1*, another head organizer component (Shawlot and Behringer, 1995). At the same time, the *Otx2* mutation displayed a craniofacial defect, designated otocephaly by haplo-insufficiency. Affected structures correspond to the most anterior and posterior parts of *Otx2* expression at the pharyngula stage, where *Otx1* is not or only weakly expressed (Matsuo et al., 1995). Defects were subtle in the *Otx1* mutation, while those exhibited by the

Otx1/Otx2 double heterozygous mutant mice were marked through fore- and midbrains, where abnormalities were never found with a single mutation alone; *Otx1* and *Otx2* cooperate in the development of the rostral brain (Suda et al., 1996).

Otx2 mutant defects caused by haplo-insufficiency were the particular focus of the present study (Matsuo et al., 1995). The major affected structures are the mandible and pre-mandibular skull elements, and the ophthalmic branch of the trigeminal nerve and mesencephalic trigeminal neurons, all of which originate from mesencephalic neural crest and its closely related derivatives. These structures become highly modified with the transition from agnatha to gnathostome states, which is one of the most important innovations in the entire history of vertebrates, implying some evolutionary significance of the *Otx2* gene. Furthermore, the defects are intimately related to defects by *Hoxa-2* mutation and are consistent with the idea first presented by Huxley (1874; reviewed by Goodrich, 1930; de Beer, 1931, 1937) that the trabecula is a premandibular component of the viscerocranium (Matsuo et al., 1995; Kuratani et al., 1997). To elucidate the role of the *Otx2* gene in mesencephalic neural crest cells, we attempted to determine the factors that specify the *Otx2* expression in these cells. The expression of homeobox genes is generally regulated at the level of transcription (Krumlauf,

1994). In this study, therefore, we identified *cis*-regulatory elements that direct *Otx2* expression in the crest cells using a transgenic approach with mouse and pufferfish *Otx2* genomes. The tetraodontoid fish, *Fugu rubripes* (*Fugu*), has a compact genome of approximately 400 Mb, nine times smaller than the mouse genome, although the two genomes have a similar number of genes (Brenner et al., 1993). This is due to smaller intergenic and intronic sequences and fewer repetitive DNA sequences in the *Fugu* genome. Utilizing this fact, the transcriptional *cis*-elements of *Hoxb-1* and *Hoxb-4* genes were identified in intergenic and intronic regions as sequences highly conserved between the mouse and *Fugu* genomes (Marshall et al., 1994; Aparicio et al., 1995; Pöpperl et al., 1995). A similar comparative analysis of the *Otx2* gene in this study also defined the genomic regions in both species that were capable of directing the expressions in mesencephalic crest cells. Among several DNA sequences highly conserved between them we have identified two DNA motifs that are essential for driving the expression.

MATERIALS AND METHODS

Transgene construction for mouse *Otx2* regulatory elements

All constructs used in this study were generated by standard molecular cloning techniques (Sambrook et al., 1989). The genomic fragments containing the mouse *Otx2* locus were isolated from the mouse TT2 ES cell genomic library (Matsuo et al., 1995). The pCH β GAL vector was constructed by inserting a 2.5 kb *SacI*-*Bam*HI fragment of the plasmid pFRT β GAL (Stratagene) into *SacI* and *Bam*HI sites of the plasmid pCH110 (Pharmacia). The pBSlacZ vector was constructed by inserting a 3.0 kb *Hind*III-*Bam*HI fragment of the plasmid pBS0CAT (Matsuo et al., 1991) into *Hind*III and *Bam*HI sites of the pCH β GAL. To construct the pBSlacZII vector, a *Bgl*III linker was inserted into the *Hind*III site of the multicloning sites located in the 5' end of the *lacZ* gene of the plasmid pBSlacZ and a *Sal*I linker into the *Apa*I site located in the 3' end of the *lacZ* gene. Construct 1 was made by inserting a 5' flanking region from the *Bgl*III site to the translational start site of mouse *Otx2* gene into the *Bgl*III site and the translational start site of the *lacZ* gene in the pBSlacZII, using polymerase chain reaction (PCR)-based mutagenesis (detailed procedures for in vitro mutagenesis are available upon request). Construct 2 was generated by removing the *Bgl*III-*Hind*III fragment from construct 1. Constructs 3, 4 and 5 were generated by deleting a 5' flanking region of *Otx2* gene in construct 2 using exonuclease III. The 5' endpoints of these deletion plasmids were determined by DNA sequencing. Constructs 21 and 22 have three tandem repeats of 49 and 84 bp fragments, which correspond to the 5' endpoint of construct 4-5 and the presumptive TATA box, respectively (Fig. 2A). These fragments were obtained by PCR; the primers used were 5'-AAAAGATCTTAAATCTGTTACTTACTTCGA-3' (the sense strand primer of construct 21 and 22), 5'-TCAGGATCCCCTAATTAGTAGTCTGGATAA-3' (the antisense primer of construct 21) and 5'-CACGGATCCCCACATTTGAAGTCATTTCC-3' (the antisense primer of construct 22). The 49 bp and 84 bp PCR fragments were digested with *Bgl*III and *Bam*HI, and the three respective tandem repeats were inserted into the *Bam*HI site of pBluescript (KS-). The repeats were confirmed by DNA sequencing. An *EcoRV*-*NotI* fragment containing the 49 bp or 84 bp tandem repeats was then inserted into the *NotI* and *Sma*I sites of construct 11, respectively. Constructs 23, 24, 25 and 26 were similarly generated using four kinds of mutant 84 bp fragments in place of the wild-type fragment. The mutated nucleotide sequences were CTAGCGG in place of ATCTGTT for two motifs, *snail* and

the first A (construct 23), ATCAC and CCCG in place of TAAAT and AAAT for two motif A sites (construct 24), GCTAGC and ATCAGT in place of TAATTA and TAATTA for two motif B sites (construct 25) and AGCTC in place of CCAGA for motif C (construct 26), respectively (cf. Fig. 8A,B).

Transgene construction for *Fugu Otx2* regulatory elements

A *Fugu* genomic phage library in the λ FIXII vector (Stratagene) was constructed from *Mbo*I partially-digested *Fugu* genomic DNAs as described (Sambrook et al., 1989). Isolation of the *Fugu rubripes Otx2* (*Fotx2*) gene will be described elsewhere. The pBSlacZIII was constructed by inserting the *Sac*II-*Hind*III fragment of a modified pBluescript (KS-), having the *Bgl*III site in place of the *Bam*HI site, into the *Sac*II and *Hind*III sites of the plasmid pBSlacZII. Construct 11 was made by inserting a 2.4 kb 5' flanking region from the *Nsp*V site to the translational start site of the *Fotx2* gene into the *EcoRV* site and the translational start site of the *lacZ* gene in the pBSlacZIII using PCR-based mutagenesis (detailed procedures for in vitro mutagenesis are available upon request). Construct 12 was generated by cloning a 3' *Xba*I-*Bam*HI genomic fragment into the *Bgl*III and *Xba*I sites of construct 11. Construct 13 was generated by inserting a 3' *Xba*I-*Bgl*III genomic fragment into *Bgl*III and *Xba*I sites of construct 11. Construct 14 was generated by inserting a *Bam*HI-*Bgl*III genomic fragment into the *Bgl*III site of construct 11. Constructs 15, 16 and 17 were generated by inserting the *Bgl*III-*Hind*III, *Hind*III-*Ban*II and *Ban*II-*Bam*HI fragments, each blunted by T4 DNA polymerase, into the *Sma*I site of construct 11.

Production and genotyping of transgenic mice

Transgenic mice were generated by microinjection of DNAs into fertilized eggs from ICR mice as described (Hogan et al., 1994). Transgenic embryos were identified by PCR analyses of yolk sac DNAs. Briefly, yolk sacs were carefully dissected, avoiding cross contamination between littermates, then washed once in cold phosphate-buffered saline (PBS). After 2 hours incubation at 55°C in 50 μ l Proteinase K solution, 1 μ l of the heat-inactivated digest was subjected to PCR analysis specific to *lacZ* and SV40 *t* intron. The oligonucleotides, 5'-CATTACCAGTTGGTCTGG and 5'-TTATGTTTCAGGTTTCAGG, amplify a 740 bp transgene-specific product. The mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center of Kumamoto University School of Medicine under University guidelines for animal and recombinant DNA experiments.

DNA sequencing

DNA sequences were determined on both strands of DNAs using the dideoxy chain termination method (Sambrook et al., 1989).

X-gal staining and histological analysis of embryos

Transgenic embryos at 7.5 to 12.5 days post coitum (dpc) were fixed in 1% formaldehyde, 0.8% glutaraldehyde and 0.02% NP-40 in PBS for 10 minutes, followed by three washes with PBS for 10 minutes at room temperature. Staining was carried out overnight at 37°C in PBS containing 1 mg/ml X-Gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 2 mM MgCl₂ in PBS. Stained embryos were washed twice with PBS and immediately stored in 10% formaldehyde in PBS. For histological analysis, embryos were embedded in Paraplast. Serial sections (10 μ m thick) were stained with 1% eosin.

In situ hybridization

Whole-mount in situ hybridizations were performed with digoxigenin probes by the protocol of Wilkinson (1993). Probes for the *Otx2* gene (Matsuo et al., 1995) were prepared with an RNA labeling kit (Boehringer Mannheim). Anti-digoxigenin antibodies conjugated with alkaline phosphatase were purchased from Boehringer Mannheim. The samples were sectioned using a D.S.K. microslicer (100 μ m thick).

RESULTS

***Otx2* expression in cephalic mesenchyme**

Our previous studies suggested the role of *Otx2* in mesencephalic neural crest cells (Matsuo et al., 1995). Little is known, however, about *Otx2* expression in these cells, although expression in the neural tube has been examined in detail (Simeone et al., 1992, 1993; Ang et al., 1994). In order to identify the *cis*-elements responsible for *Otx2* expression in the crest cells, we first confirmed the expression in the cephalic mesenchyme of 9.5 dpc mouse embryos. Although in whole-mount embryos *Otx2* expression in cephalic mesenchyme could not be discerned on top of the strong expression in the rostral neural tube, optic vesicles and otic vesicles (Fig. 1A), in sections *Otx2* expression was found throughout the mesenchymal cells at the level of the dorsal mesencephalon (Fig. 1B). It was also found in the mesenchyme of the ventral region (Fig. 1C,D) and of the mandibular arch but not of the hyoid arch (Fig. 1D). Thus, the *Otx2* gene was indeed expressed in

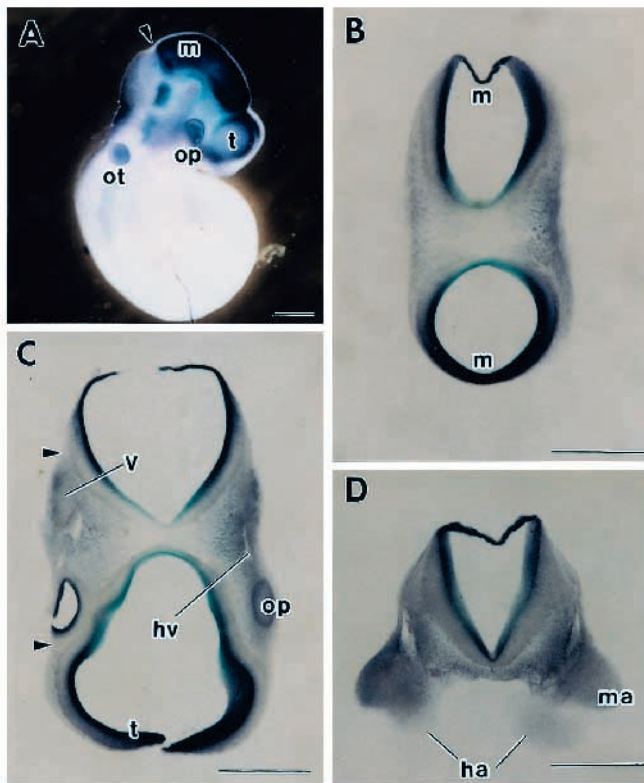


Fig. 1. In situ hybridization analyses of *Otx2* mRNA expression in 9.5 dpc mouse embryos. (A) Whole-mount in situ RNA hybridization showing a lateral view of the *Otx2* expression in the rostral brain with the posterior boundary at mesen/metencephalic junction (arrowhead). (B–D) Transverse sections of an embryo stained by whole-mount in situ RNA hybridization (100 μ m thickness). (B) *Otx2* is expressed in the entire cephalic mesenchyme at the level of the mesencephalon (m). (C) The expression is also seen in the cephalic mesenchyme near head veins (hv), but is not found in the most rostral mesenchyme nor in the mesenchyme caudal to mesencephalon (arrowheads). (D) *Otx2* is also expressed in the mandibular arch (ma), but not in the hyoid arch (ha). Abbreviations: op, optic vesicle; ot, otic vesicle; t, telencephalon; V, trigeminal ganglion. Scale bars, 400 μ m.

cephalic mesenchyme. The expression appeared to distribute caudally to optic vesicles; it was not apparent in the mesenchyme at the levels of telencephalon and rhombencephalon, nor in the trigeminal ganglion (Fig. 1C), nor was it found in the surface ectoderm. It was difficult, however, to determine the precise extent of *Otx2* expression in cephalic mesenchyme, since the signal was not strong enough.

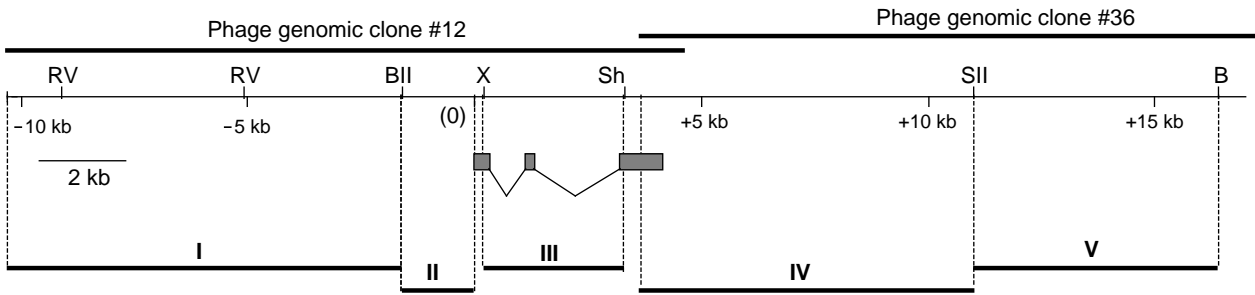
Mouse *Otx2 cis*-element directing expression in cephalic mesenchyme

The genomic region of mouse *Otx2* gene encompassing -10.2 to 16.4 kb (0 is the translation start site) was surveyed for enhancer activity by generating transgenic mouse embryos with the reporter *Escherichia coli lacZ* gene (Fig. 2A). Screening was made in transgenic embryos at 10.5 dpc immediately after the emigration of mesencephalic neural crest cells had been completed. It identified the enhancer activity in the 5' flanking region encompassing -1.8 to 0 kb (fragment II in Fig. 2A; construct 1 in Fig. 2C); no activity for the expression in cephalic mesenchyme was found in other regions (data not shown). The 10.5 dpc transgenic embryos harboring construct 1 exhibited β -gal expression in the cephalic mesenchyme at the levels of diencephalon, mesencephalon and mandibular arch (Fig. 3E,F). Weak expression was also found in the heart (Fig. 3E). The expression with construct 1 was then examined in detail to determine whether and how these β -gal-positive cells are related to mesencephalic crest cells. No β -gal expression was detected in the cephalic mesenchyme of transgenic embryos at 7.5 dpc (data not shown). The expression was first found in the cephalic mesenchyme at 8.5 dpc (Fig. 3A,B). It was never detected in the most anterior region of cephalic mesenchyme nor in the neural plate (Fig. 3A,B). The β -gal expression in the cephalic mesenchyme was restricted to the levels of diencephalon, mesencephalon, metencephalon and mandibular arch at 9.5 dpc (Fig. 3C,D). Later, at 10.5 dpc the expression persisted in the ventral or distal parts of these regions (Fig. 3E,F). The transgene expression was greatly decreased at 11.5 dpc, even in ventral cephalic mesenchyme (Fig. 3G). Still later at 12.5 dpc, β -gal expression was not found at all in the cephalic mesenchyme (Fig. 3H).

β -gal expression was further analyzed in the sections. In the 8.5 dpc embryo the transgene began to be expressed in the cephalic mesenchyme but not in the neural plate, neural crest or the surface ectoderm (Fig. 4A). At 9.5 dpc, β -gal-positive cells were distributed throughout the entire dorsal mesenchyme at the level of the mesencephalon (Fig. 4B); however, most of the trigeminal ganglion cells did not express β -gal (Fig. 4C). The anterior limit of the positive cells was located caudal to the optic vesicles, and no positive cells were found at the telencephalic level (Fig. 4C). The β -gal expression was also found in the mandibular arch (Fig. 4D). Besides mesenchymal cells, the expression was found in the ventral diencephalon (Fig. 4C, D).

At 10.5 dpc, β -gal-positive cells were distributed, as at younger stages, in the cephalic mesenchyme at the level of diencephalon and mesencephalon and were absent rostrally to the optic vesicles (Fig. 4E–G). The β -gal expression, however, was decreased towards the dorsal side (Fig. 4E,F). β -gal-positive cells were rarely seen in the trigeminal ganglia, but were found in their most rostral portion (Fig. 4F); this expression was lost by 11.5 dpc (data not shown). β -gal-

A)



B)

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AGATCTTGGGAAGAAGGAACACAGAATATAAAGTGTTCCTCGCAAAAGTAAGTACTAGTTTCACTCGTTAACAAATCCAGTTCTCTTAGAAGGACATTTATAAAAAATCACATTTTAGGGTGGGGA 120
#1 BglII
AATAAGACTTGTGTTCTGAATGTAAATCTGGAAGAAAACACAGCTGTGAGTTTGTGTTGTGAAGCTTTGCCTTTGTCTCTTAAGCAAGGTAATATGAAAACCTAAGGCTCTTAATTTTTT 240
#2 HindIII
TCATTAATGAACTTTATGAAGTGACAGAGAACTCAGTCTTGTATCCGCCATTTTGAGGAAGTTTCTCAGAGTGTCAAAGTTTCAAAGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG 360
AGAGAGCTTTAAGTGCCTTATTTTTGGAAAAATAAAGCCAGCCTTACACACATTGCCTGCCTGTATTAACATCGATGGGGGAGGGGTTTCAGTAAAGGAACACAGATGACTTTTCTTTTT 480
CTCCCCCACCAGCTCCTTTTCTTACAAAAATTGAGGAAATTTAAAAAGCAGAGAGAAGTGCAGTTAGTTCCATTGCTTTGGATGTTTATTGACTTCAAACCTAAATATATGGGAAAGG 600
TGGCTCCCGTTGCAGCCCCCTCTCCCCCAACCCAAAATCCAAGAAAAAGTGTTCCTCCGCTGGATCTGTAGCCTCCTGACATAAAAAACATGGAAGCGCTTGCTGTAGGAATGCACCCC 720
TCTGGACTTGCAAAATGTGGGGCAGCAGTTTATCTGACTTTACTTCAGTTAGGATTCTAACTTAAGCATTCTGTCAAGCCCTGTGCTAGTCTTGAAGAAAAGACTTTTTTTTCCCTCT 840
CAGTATGTTTAAATTAATATTTGATGGGTGTCGCTTAAAAGTTAAATAGTTGTTTGTGTTAGCTATAGTTCTTTAGACACATGTAATCAAACCTCCTCCAAAACCTAATAAATCTGTTAC 960
TTACTTCGAAATCTAATTATCCAGACTACTAATTAGGTGAAAAATGATTACTGGAAATGACTTCAAATGTGGGATAATAGTGATTGGGCAGTTTTCACAAGTTTGTGTTTCAGCAAGTTGG 1080
#3
#4
#5
primer 1
primer 2
TGTTTTAAATGTTGAATGCGTTTACATTTTAGGTTACACAGCAGTTGAAAGTGGTAATAATTTAAAAAATAAATCAGCTAAGATAATTAACCTCTGCCAGGAAAGGCAACAGTCTG 1200
GGAAGAGGTAGATTCTTGAATGTTTCTTTGTTTCTCACCATGGGCTTTGTTATCAGCATTATTTATTTAGCCAAAGAGTTCTTTGTCTCTGCAAATGGCCCAATCAAGTTTGTGTT 1320
GAGACAATTAGCTAGTCCAGCCAATGAGTCTATGCAAAATGTTGGGTTAGGAATGTAGAGCGTGCAATTTGGAGCGTGGGGTTTTGTTCTTGGGGAAGGCAGATTGTAATTGCTTTTCCT 1440
CGGGTCGGTTTTCTTTCTTTCTTTCTTTTTTAAAGTTTAGGGTGGGGTGGGGAAGACAGGTTTATCTGGTCTCACTCCATCCCCTCTAGTTTTGGAGCTGCTGGGGGTGGGGGGACG 1560
GCGGGGTGGGGGACGCATCTGCAACTCCTTTAAAAGCCTGTGCCAGCGTCTCCCGGTTCTTTTTAGTTAGTGTGGAACGTGGAGGAAGCTGCTCCCTCCGAAGCAGTAAACCAGCA 1680
TTTCTGTTTGTGTTGTTGTTTGGCCCTTAGTTCCTGCTCACTCCAATCTACCACCAAGGACCCTGACCCTGTCCACTCCAGGCAATCGAGACCCTCCGGCTGGGTCCCCCAATTTGGG 1800
CCGACTTTGCGCCTCCAAAACCTTAGCATG 1832
primer 3 M
    
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C)

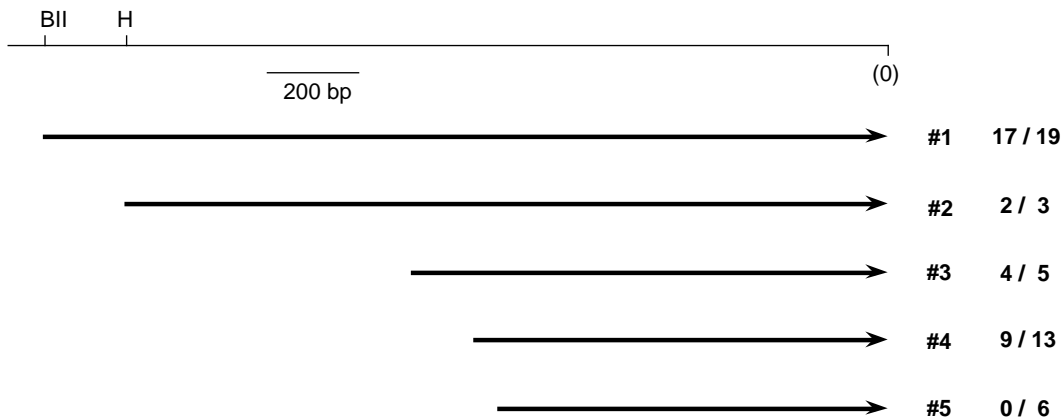


Fig. 2. Identification of the mouse *cis*-element critical for the transgene expression in the cephalic mesenchyme. (A) The restriction map of the mouse *Otx2* genomic locus surveyed in this study is shown along with the phage clones used on the top and coding regions (filled boxes) underneath. The translational start site is indicated by (O). Each fragment examined is identified by bars marked I-V. (B) Nucleotide sequences of 5'-flanking region of the mouse *Otx2* gene. Restriction enzyme sites, putative TATA box and three primers used for RT-PCR analyses are underlined. The 5' end of each deletion construct (2-5) is indicated by filled triangles with the number of constructs (see Fig. 2C). The translational start codon is indicated by M at the end of the nucleotide sequences. Gene bank accession no. U96488. (C) Schematic diagram of the deletion constructs used to identify the mouse *cis*-acting region within fragment II in A. The number of β -gal-positive embryos in the cephalic mesenchyme among transgenic embryos is indicated on the right. In all these embryos, the expression patterns were virtually the same although their levels were variable. The transgenic embryos were counted as β -gal negative, possibly because of the effects of integration sites and others, when the expression was not found in cephalic mesenchyme. Abbreviations of restriction enzyme sites: B, *Bam*HI; BII, *Bgl*III; H, *Hind*III; RV, *Eco*RV; S, *Sal*I; SII, *Sac*II; Sh, *Sph*I; X, *Xba*I.

positive cells also appeared to converge into and halt at the sites that may correspond to a future cartilaginous mesenchyme or peripheral nerves in the premandibular and mandibular regions (Fig. 4G-I; see Discussion). Actually, β -gal-positive cells were found in the cells migrating into the mandibular arch (Fig. 4I). At the same time, β -gal-positive cells were present more posteriorly and distally, in the mesenchymal cells of hyoid arch and the myocardium (Fig. 4J; data not shown). At this stage β -gal expression was found in the ventral diencephalon, and the expression shifted to the neurohypophyseal bud (Fig. 4K); it was not found in Rathke's pouch or in the oral ectoderm. Later, by 11.5 dpc, the transgene expression was limited to the ventral

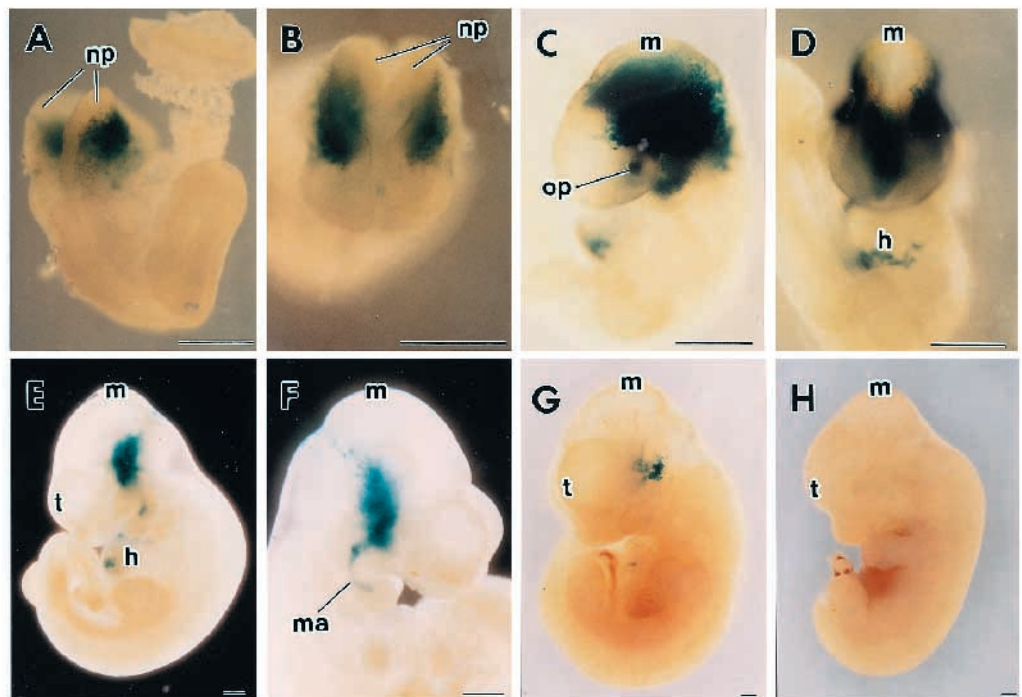
part of the cephalic mesenchyme, the floor of the diencephalon and neurohypophysis (Fig. 4L, data not shown). In summary, most of the expression under the control of the 1.8 kb DNA fragment appeared to correspond spatially and temporally with the distribution of mesencephalic neural crest cells, except for the ventral part of diencephalon (see Discussion).

Regulatory elements essential for the transgene expression

The nucleotide sequences of the 1.8 kb 5' flanking region were determined (Fig. 2B). The major transcription start site of the *Otx2* gene was preliminarily reported with cultured cells to locate at 3140 bp downstream of the 5' *Xba*I site (Simeone et al., 1995). We confirmed this by reverse-transcription and polymerase chain reaction (RT-PCR) experiments with 9.5 dpc mouse embryos. Primer 2 and primer 3 amplified the 713 bp fragment but primer 1 and primer 3 did not amplify the predicted 845 bp fragment (data not shown). This suggests that the first transcription start site locates between primer 1 and primer 2. The canonical TATA box sequence is present between these two primers (Fig. 2B).

To define the critical *cis*-element for the expression in the cephalic mesenchyme, a series of deletions was introduced into construct 1 (constructs 2, 3, 4 and 5; Fig. 2B,C). Transgenic embryos with the three deletion constructs 2, 3 and 4 showed almost the same pattern of β -gal expression in the cephalic mesenchyme (Fig. 5A-C). Analysis of sections confirmed that the β -gal-positive cells were distributed in the cephalic mesenchyme, ventral diencephalon and mandibular arch (Fig. 5E; data not shown). Finally, we made construct 5 that had a 49 bp deletion from the 5' end of construct 4 (Fig. 2B,C). It sent out no β -gal signals in the cephalic mesenchyme of transgenic embryos (Figs 2C, 5D). In contrast, the transgene expression in ventral diencephalon was preserved even after this 49 bp deletion (Fig. 5F). Thus it was concluded that the major regu-

Fig. 3. Developmental changes in β -gal expression (blue) with construct 1. Lateral (A) and frontal (B) views of an 8.5 dpc embryo showing the onset of expression. Lateral (C) and ventral (D) views of a 9.5 dpc embryo showing increase of the β -gal expression in the cephalic region and the mandibular arch. (E,F) Transgene expressions in 10.5 dpc embryos. (G) Lateral view of a 11.5 dpc embryo showing a great decrease in the transgene expression. (H) Lateral view of a 12.5 dpc embryo showing no β -gal expression. Abbreviations: m, mesencephalon; ma, mandibular arch; h, heart; np, neural plate; op, optic vesicle; t, telencephalon. Scale bars, 400 μ m.



latory element for the transgene expression in the cephalic mesenchyme was located in the 49 bp sequence between the 5' ends of constructs 4 and 5.

Pufferfish *cis*-element for expression in cephalic mesenchyme

We have speculated that the *cis*-element that controls *Otx2* expression in mesencephalic crest cells may be conserved among all gnathostomes (Matsuo et al., 1995). In parallel with the analysis in mouse, we thus have pursued characterization of the regulatory region in the Japanese pufferfish, *Fugu rubripes*. Among gnathostomes, the sequences of *Otx2* cognates are highly conserved, while those of *Otx1* cognates are relatively diverged. The deduced amino acid sequences of the *Fugu Otx* gene that we isolated showed 96% homology with that of zebrafish *zOtx2* and 92% with mouse *Otx2*; 60% homology with *zOtx1*, 57% with *zOtx3* and 57% with mouse *Otx1* genes. Thus, we concluded that the gene isolated is the *Otx2* cognate in *Fugu*, and we called it *Fotx2*. Details of its genomic structure and DNA sequences will be reported elsewhere.

On the basis of the result in mouse that construct 1 gave β -gal expression in cephalic mesenchyme, we first tested the enhancer activity of the 2.4 kb 5' flanking region with the *lacZ* reporter (construct 11; see Fig. 6). The construct, however, did not give any β -gal expression in transgenic embryos (Fig. 6 and data not shown). Next, we added the 3' DNA fragment between the *Xba*I and *Bam*HI sites, located at +10.5 to +16 kb, to construct 11 (Fig. 6; construct 12). The transgenic embryos harboring this construct 12 exhibited β -gal expression in the cephalic mesenchyme as good as with the mouse *cis*-acting element (Figs 7A, 3E,F). Subdivision of this

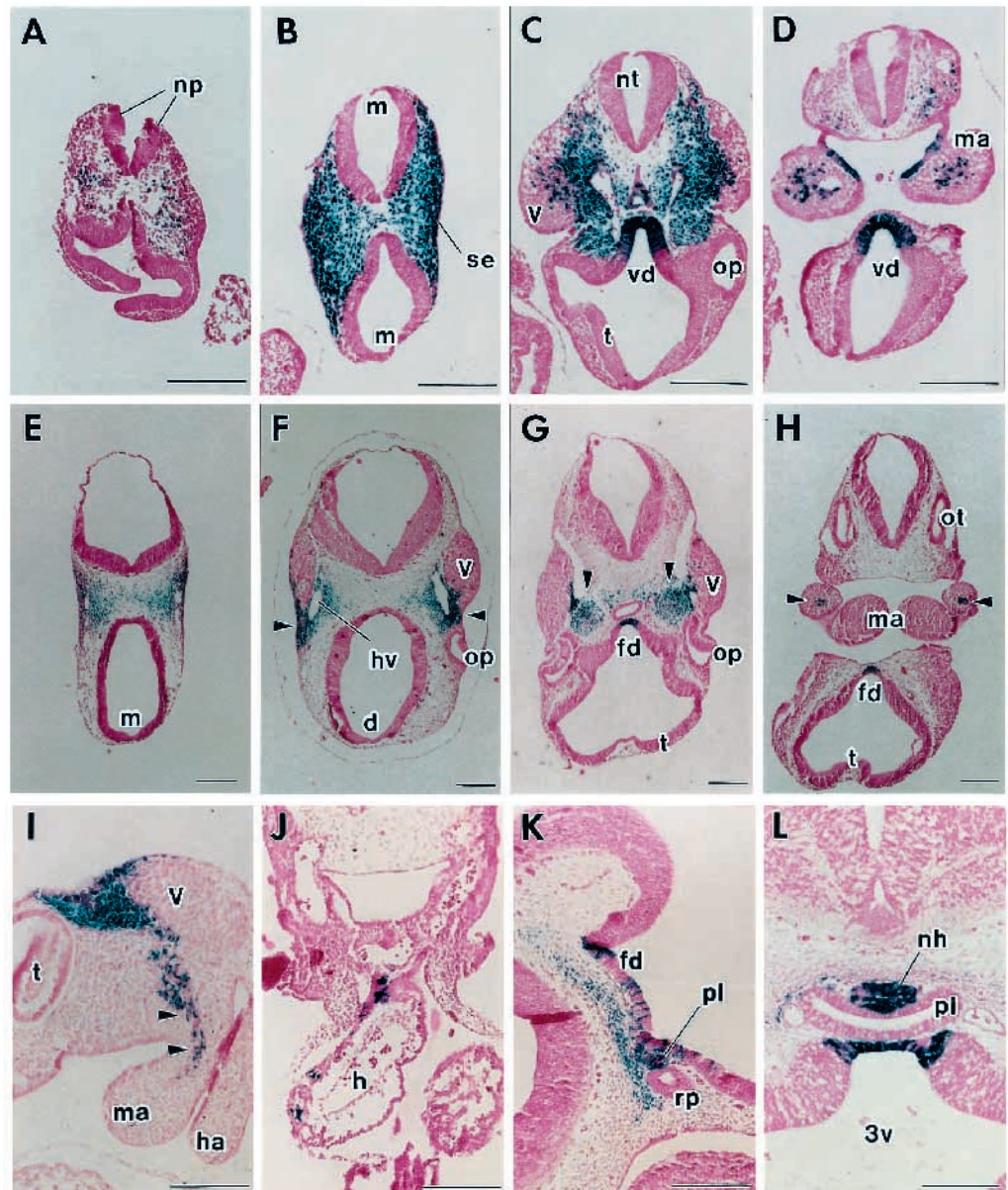
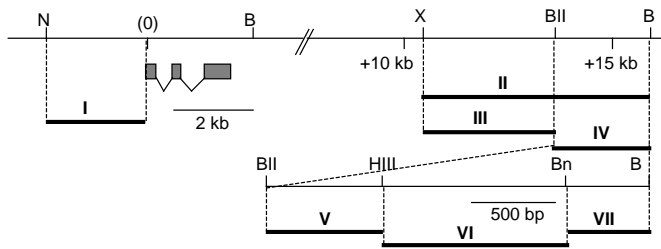
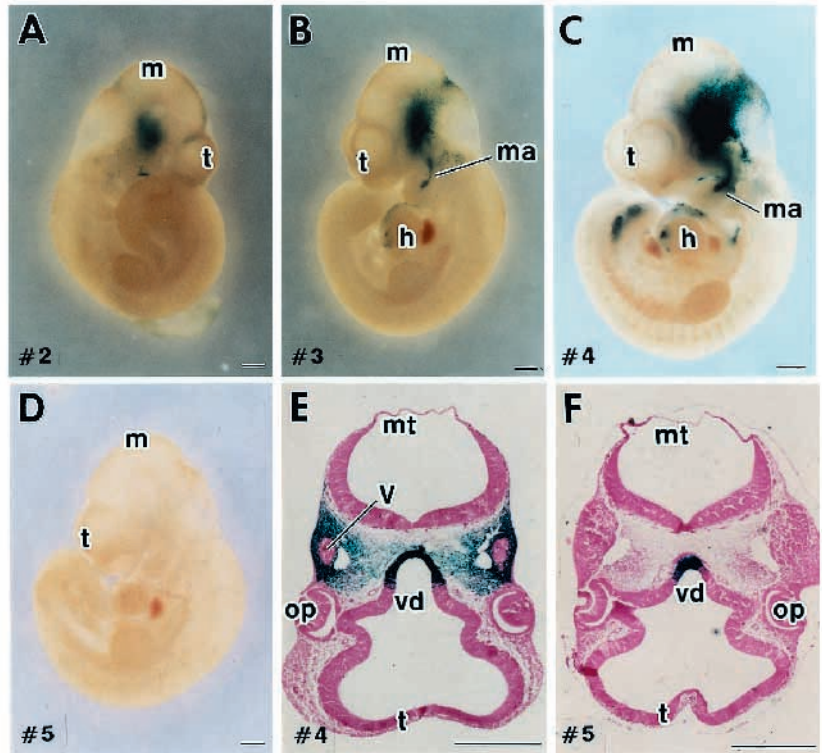


Fig. 4. Analyses of the β -gal expression (blue) with construct 1 in sections. (A) Transverse section through the cephalic region of an 8.5 dpc transgenic embryo showing the onset of the β -gal expression in the cephalic mesenchyme. (B-D) Transverse sections through a 9.5 dpc transgenic embryo. (B) The entire cephalic mesenchyme is stained at the level of mesencephalon (m). (C) The mesenchyme caudal to the optic vesicle (op) and the ventral diencephalon (vd) are stained, but the trigeminal ganglion (V) or the cephalic mesenchyme at the level of telencephalon (t) is not. (D) β -gal is expressed in the mandibular arch (ma) and the ventral diencephalon. Transverse (E-H and J) and sagittal (I, K) sections through 10.5 dpc transgenic embryos. (E, F) Dorsally the transgene expressions are downregulated in mesencephalic mesenchyme, but the most rostral part of the trigeminal ganglion is stained (F, arrowheads). (G) Ventral β -gal staining remains in the mesenchyme bilaterally (arrowheads); it may contain the precartilaginous mesenchyme in the cranial base. The transgene is also expressed in the floor of diencephalon (fd). (H) β -gal expression in the mandibular arch converges in the center as spots (arrowheads). It probably contains the precartilaginous condensation of prospective mandibular bone. (I) The transgene expression in neural crest cells migrating into the mandibular arch (ma) located caudal to the telencephalic vesicle (v) (arrowheads). (J) β -gal is also expressed in a part of the myocardium (h). (K) β -gal is expressed in the floor of diencephalon and the posterior lobe (pl) but not in Rathke's pouch (rp). (L) A transverse section through an 11.5 dpc transgenic embryo. β -gal is expressed in the floor of diencephalon (fd) and neurohypophysis (nh) but not in the pars intermedia (pi). Abbreviations: ha, hyoid arch; hv, head vein; np, neural plate; nt, neural tube; ot, otic vesicle; se, surface ectoderm; 3v, third ventricle. Scale bars, 200 μ m.

Fig. 5. *Otx2/lacZ* transgene expressions with deletion constructs. (A-D) Lateral views of the transgene expressions in 10.5 dpc embryos harboring constructs 2 (A), 3 (B), 4 (C) and 5 (D) (cf. Fig. 2). β -gal expressions are virtually identical among constructs 2-4, being high in the cephalic mesenchyme (m) and mandibular arch (ma). In contrast, no β -gal expression is found in the cephalic mesenchyme with construct 5 (D). (E) Transverse section through a 10.5 dpc transgenic embryo containing construct 4. Expression of the transgene in the cephalic mesenchyme and the ventral diencephalon (vd) is almost identical to that seen in transgenic embryos with construct 1 (Fig. 4). (F) Transverse section of a 10.5 dpc transgenic embryo harboring construct 5. The transgene expression is lost in the cephalic mesenchyme but preserved in the ventral diencephalon. Abbreviations: h, heart; m, mesencephalon; mt, metencephalon; op, optic vesicle; t, telencephalon; V, trigeminal ganglion. Scale bars, 400 μ m.



Construct #	Construction scheme	Number of β -gal positive embryos / transgenics
#11	I \rightarrow lacZ	0 / 6
#12	II \rightarrow I \rightarrow lacZ	5 / 7
#13	III \rightarrow I \rightarrow lacZ	0 / 9
#14	IV \rightarrow I \rightarrow lacZ	10 / 13
#15	V \rightarrow I \rightarrow lacZ	0 / 5
#16	VI \rightarrow I \rightarrow lacZ	11 / 15
#17	VII \rightarrow I \rightarrow lacZ	0 / 8

Fig. 6. Schematic diagram of the *Ftx2/lacZ* transgene constructs used to identify *Fugu cis*-acting elements. The restriction map of the *Ftx2* genomic locus surveyed in this study is shown. Coding regions are indicated by filled boxes underneath, and the translational start site by (0). Each fragment examined is identified by bars marked I-VII. The table gives a summary of the transgenic analyses. In all β -gal-positive embryos, the patterns of expressions were virtually the same though their levels were variable. Abbreviations: B, *Bam*HI; BII, *Bg*III; Bn, *Ban*II; HIII, *Hind*III; N, *Nsp*V; X, *Xba*I.

5.5 kb fragment at the *Bg*III site further localized the activity to the 2.3 kb *Bg*III-*Bam*HI fragment (construct 14, Figs 6, 7B). The *Xba*I-*Bg*III fragment did not give any β -gal expression (construct 13, Fig. 6; data not shown).

In 9.5 dpc whole-mount embryos harboring construct 14, transgene expression was found in the cephalic mesenchyme at the levels of diencephalon, mesencephalon, metencephalon and in the mandibular arch, but not in the most anterior region of cephalic mesenchyme or the neural tube (Fig. 7C). Some of the β -gal-positive cells were distributed further posteriorly and distally to the mesenchyme of hyoid arch and the myocardium (Fig. 7C; and data not shown). In sections, β -gal-positive cells were distributed throughout the entire dorsal mesenchyme at the mesencephalic level, but not in the trigeminal ganglia (Fig. 7G,H). The β -gal-positive mesenchyme extended caudally to the optic vesicle but not rostrally (Fig. 7H,I), and it was also found in the mandibular arch (Fig. 7I). This distribution pattern of β -gal expression in cephalic mesenchyme was almost identical to that seen with the mouse *cis*-element. In contrast to the latter, however, no β -gal expression was observed in the neurohypophysis or ventral diencephalon (Fig. 7H).

The 2.3 kb region was then further subdivided into *Bg*III-*Hind*III 0.7 kb, *Hind*III-*Ban*II 1.1 kb and *Ban*II-*Bam*HI 0.5 kb fragments; these were combined with construct 11 to generate constructs 15, 16 and 17, respectively (Fig. 6). Neither construct 15 nor construct 17 gave any β -gal staining (Fig. 7D,F), but construct 16 containing the 1.1 kb subfragment yielded the same pattern of β -gal expression in the cephalic mesenchyme (Fig. 7E; data not shown). These results suggest that the 1.1 kb subfragment is responsible for the *Ftx2* expression in the cephalic mesenchyme.

Critical DNA motifs conserved between mouse and *Fugu* cis-elements

A question remains as to whether the mouse 49 bp sequences corresponding to the 5' endpoint of constructs 4 and 5 is sufficient for the transgene expression in cephalic mesenchyme (Fig. 8A). The three tandem repeats of the 49 bp cis-element were then linked to the *Fotx2* promoter construct 11 (construct 21), and the β -gal expression in transgenic embryos harboring this construct was analyzed. Four out of seven transgenic embryos exhibited β -gal expression in the cephalic mesenchyme as good as with the mouse 1.8 kb cis-acting element (Fig. 9A). Thus it was concluded that the mouse 49 bp cis-element is not only essential but also sufficient for driving the transgene expression in cephalic mesenchyme. The result also indicates that the mouse 49 bp cis-element is functionally equivalent to the *Fugu* 1.1 kb element.

To determine the DNA motif(s) essential to the expression in the cephalic mesenchyme, our strategy is to find DNA sequences conserved between mouse and *Fugu* cis-elements. The comparison of nucleotide sequences between mouse 49 bp and pufferfish 1.1 kb DNA fragments revealed four candidate sequences (Fig. 8A): the consensus sequences for binding of *snail* transcription factor and three DNA motifs, A, B and C. To determine whether these DNA motifs were indeed crucial for the enhancer activity, we introduced mutations in each of them (Fig. 8B). The mutation in two motifs, *snail* and the first A (construct 23; Fig. 8B), gave β -gal expression that was indistinguishable from the expression by the control construct 22 (Fig. 9B,C). With the mutation in both motif A sites (construct 24; Fig. 8B) transgene expression in the premandibular region was completely lost but that in the mandibular region was retained (Fig. 9D). With construct 25, introducing the mutation into both of two B motifs (Fig. 8B), no transgene expression was found at all (Fig. 9E). The mutation in motif C (construct 26; Fig. 8B) did not affect the expression (Fig. 9F). These results indicate that motifs A and B are crucial for the transgene expression in the cephalic mesenchyme. Motif B, CTAATTA, is essential for expression in both premandibular and mandibular mesenchyme while motif A, TAAATCTG, is essential for expression only in premandibular mesenchyme.

DISCUSSION

Gans and Northcutt (1983) proposed the vertebrate head rostral to otic vesicles as a 'New Head' that has no homologues in protochordates. According to their proposal, the cranial neural crest, a novel feature in vertebrates, plays an essential role in development of this region. The role of cephalic neural crest in the patterning of rostral head, however, has remained uncertain.

Our previous study indicated the essential role of the *Otx2* gene in the cephalic crest cells, and we have attempted to determine the regulatory mechanism of *Otx2* expression in these cells. The current study specified the 49 bp 5' flanking sequences of mouse *Otx2* gene that are essential and sufficient for the β -gal expression in the mesencephalic neural crest cells of transgenic embryos. Furthermore, the 1.1 kb DNA fragment of *Fotx2* gene, a *Fugu* homologue of *Otx2* gene, had almost the same activity. Two DNA motifs, A and B, conserved between these two species, were crucial for the expression. This is the first cis-element identified for gene expression in mesencephalic

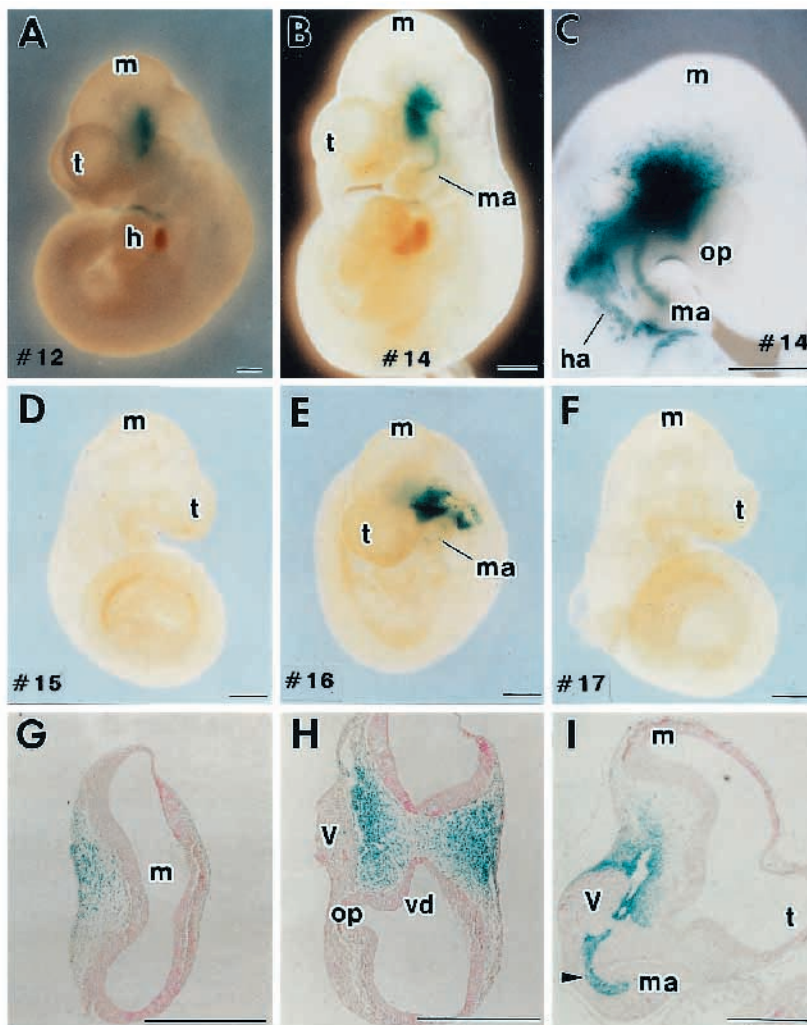


Fig. 7. Expression patterns of *Fotx2/lacZ* reporter constructs. (A-F) Lateral views of β -gal expression in transgenic embryos with constructs 12 (A), 14 (B, C), 15 (D), 16 (E) and 17 (F) (cf. Fig. 6). (A,B) Transgenic embryos at 10.5 dpc. (C-F) Transgenic embryos at 9.5 dpc. β -gal expression patterns are virtually identical among constructs 12, 14 and 16, being high in the cephalic mesenchyme and in the mandibular arch (ma), but neither construct 13 (data not shown), 15 (D) nor 17 (F) gives any expression. (G,H) Transverse and (I) sagittal sections through 9.5 dpc transgenic embryos containing construct 14. (G) The entire cephalic mesenchyme is stained at the level of mesencephalon. (H) β -gal is expressed in the cephalic mesenchyme caudal to optic vesicles (op) but not in the trigeminal ganglions (V) nor in the ventral diencephalon (vd). (I) The transgene is expressed in crest cells migrating into the mandibular arch (ma) (arrowhead). Abbreviations: h, heart; ha, hyoid arch; m, mesencephalon; t, telencephalon. Scale bars, 400 μ m.

crest cells, and it will be useful to analyze the complex genetic controls that regulate *Otx2* gene in these cells and thereby to understand the role of *Otx2* gene in patterning of the rostral head.

The *cis*-acting element for mesencephalic crest cells

The mouse and *Fugu* *Otx2* regulatory elements exhibited almost identical expression in cephalic mesenchyme. However, this is not true in all the mesenchymal cells, which raises the question of which cell populations they correspond to. The cephalic mesenchyme comprises the neural crest cells and mesoderm (Noden, 1988). No marker is available at present that distinguishes neural crest cells and cephalic mesoderm, so that no definite conclusion can be drawn. The distribution pattern of β -gal-positive cells, however, was quite similar to that of neural crest cells, as revealed by transplantation of avian neural crest and by dye-labeling experiments in mammals (Noden, 1984; Lumsden et al., 1991; Imai et al., 1996). In mouse, the emigration of crest cells first commences in the mesen-/metencephalic regions at the 4-somite stage, and is completed by the 7- to 14-somite stage (Nichols, 1981; Serbedzija et al., 1992). The β -gal-positive mesenchymal cells were first detected at the mesencephalic level at 8.5 dpc. The strong β -gal expression at 9.5 dpc at the level of dorsal mesencephalon, its disappearance in dorsal and its shift to ventral at 10.5 dpc, coincide well with migration of the crest cells. Migration is far less extensive in the mesodermal mesenchyme cells (Noden, 1984), and the possibility is less likely that these cells express β -gal at 9.5 dpc but not at 10.5 dpc, though such expression in the mesodermal mesenchyme cannot be excluded.

The precise cell lineages of cephalic mesenchyme are still not known in mouse, but cephalic crest cells are believed to yield most of the craniofacial skeleton including mandible, cephalic connective tissues and cranial nerves in avian species (Le Douarin, 1982; Noden, 1984; Couly et al., 1993). These are structures primarily affected in *Otx2* heterozygous mutants (Matsuo et al., 1995), although in our previous study it remained uncertain

A)

mouse <i>Otx2</i>		
A	ATAAATCTGTTACTTACTTCCGAAATCTTAATTATCCAGACTACTAATTAG	49
#4	A	snail
	A	B
	C	B
#5		
<i>Fotx2</i>		
	AAGCTTGTGGCTGCCACACACTGACAAGCACACACATTAAGAGTGACAGCAAACACAAC	60
	CTCAGACAAGTCAATCACATCATCATGTCAACACAGACAGGCGTTAATGGCCTAATTCC	120
	GCCCAATACACATCGGTAAATCTA TAGGAAATAATTTATCTTCATTAGCAAAACTCTCAA	180
	CACATATGGGTTGGGTTTTGCTGTGAGAGTTGCGGTCTCAGGTAGGAGCTGCAACTTA	240
	ATCCAGAGAATAACAACAACCCTGAACCTGAGAAATGTCTGTGGAGTTTAGCGGCAGGCA	300
	TTGGCAGTCTGCACAAAAGATGGGTGGCACCTCGTAAACATCC TTGAAACAGGTTTCTCA	360
	TGCTTAAAGGGGATCTGCAGGCAAAGTTTTTCCAGAGTGTGTGAGATTCTGCAAAATCTG	420
	AGGCCAGCTCAGTATATCATGCCTCTCACATCCTCTATTCTCTCCCAAACACTCACCAG	480
	CGTCCCCCACCATCCCCACACCTGCCCTCCACCCTCTCCACTCCAAGCTCCC ATCCA	540
	GC CAGGGAGAGCAGACGAGAGGAAGAGAAAGAGGAGATAGGCCAGTTGCTAAAGCTGCC	600
	TTTTTCAGCCACTGCTCCCGCTCAGTCTATCTGAGATACACAGCAAGCGGAGGTGTCGCTT	660
	TTCCGCTGGCATTTCGGACAGTATCTACTTATAGACAGGAGAGAAAGAGAAAAGAAAAAC	720
	AGAAGGGGTATTATCAGGGAGGGATGAGAGCAGCAAAGGCATTTTGTCTTTTTATTCCCC	780
	TCCTAAGCCCTGGGCTTTGGTCCCAATCCGATTGGTTCGATACGGCCAGGATGTAAATGA	840
	AATGCAAAGAGGAATAAGGCTGGCTTCCCATCTCCAGTGTGCACCCCCCTAAGTCTCC	900
	ACCCCTCTTTCTCTGCTCACTCTGCTTGTCTCTCGCAGTTGTTTATGTTGTTGTTGTTG	960
	GTTGTGTGCTCTCCATTACAGACTTGCTGCTCTCCCGCTTCCGTCGCCCTTCTCTCACT	1020
	CTGCCACCCCCCCCCACACACACACACACACACTCTCGCTCCATCCCTCCCCT	1080
	CTCCCTGGCTGGGCTC	

B)

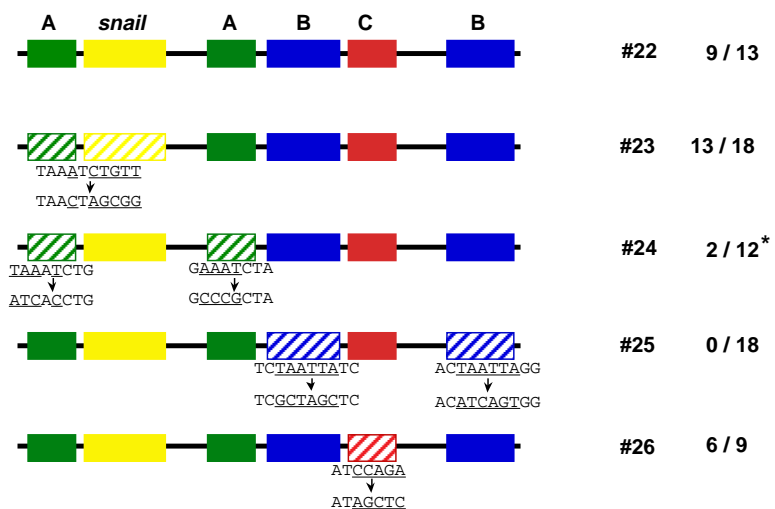
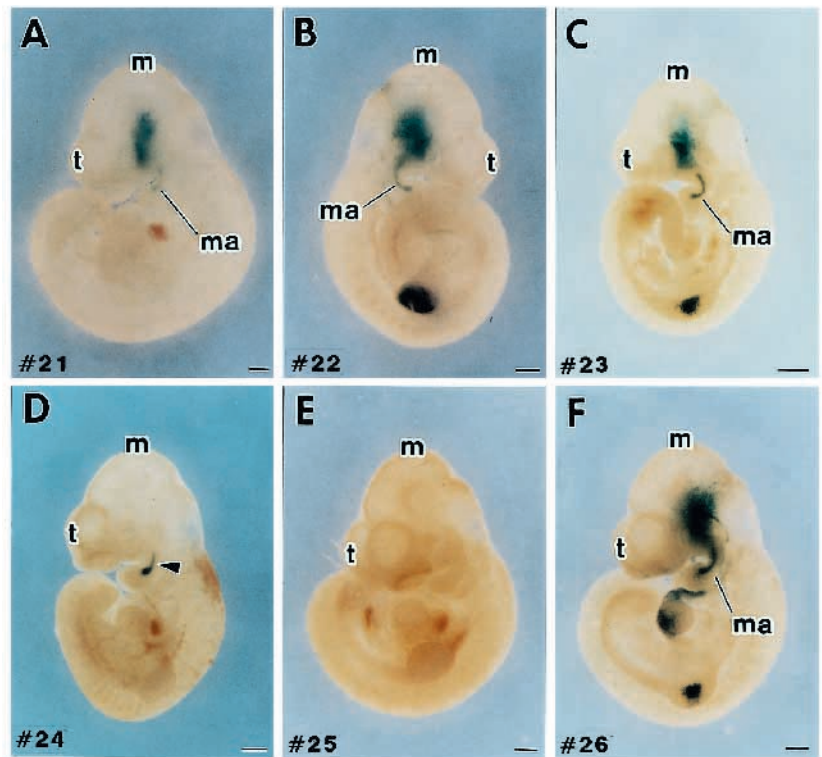


Fig. 8. DNA motifs conserved between mouse and pufferfish *Otx2* *cis*-elements.

(A) Nucleotide sequences of 49 bp mouse *Otx2* (top) and 1.1 kb *Fotx2* (below) *cis*-acting region. Homologous DNA sequences conserved between them are indicated: motifs A (green color), B (blue) and C (red) as well as the consensus binding site for *snail* (yellow; Kasai et al., 1992). Gene bank accession no. U96489. (B) Schematic diagram of the mutation constructs. The number of β -gal positive embryos in the cephalic mesenchyme among transgenic embryos with each construct is indicated on the right. In all β -gal-positive embryos, the patterns of the expressions were virtually the same except for construct 24. *The transgene expression by construct 24 was restricted to the mandibular mesenchyme (see Fig. 9D). Analyses with constructs 22-26 were performed in transgenic embryos carrying the mouse 84 bp *cis*-element, which corresponds to the sequences from the 5' end of construct 4 to that of the presumptive TATA box (Fig. 2B), in three tandem repeats. Construct 22 is wild type, construct 23 has mutations in motifs, *snail* and the first A, construct 24 in both A sites, construct 25 in both B sites and construct 26 in motif C. The wild-type and substituted sequences of motifs for the mutational analyses are underlined.

Fig. 9. Roles of DNA motifs conserved between mouse and pufferfish *Otx2* cis-elements for expression in cephalic mesenchyme. (A) Lateral view of the transgene expression at 10.5 dpc with construct 21, in which the three tandem repeats of the 49 bp cis-element are linked to the upstream region of *Fox2* promoter construct 11. High expression in the cephalic mesenchyme and in the mandibular arch (ma) demonstrates the sufficiency of the 49 bp cis-element for expression in cephalic mesenchyme. (B-F) Mutation analyses of the DNA motifs; lateral views of the transgene expressions at 10.5 dpc are given. (B) Wild-type construct 22; (C) construct 23 bearing mutations in motifs *snail* and the first A; (D) construct 24 in both A sites; (E) construct 25 in both B sites; (F) construct 26 in motif C (see Fig. 8b). No change in β -gal expression is found with constructs 23 (C) and 26 (F) from wild-type construct 22; both are high in the cephalic mesenchyme and in the mandibular arch. In contrast, with construct 24 no β -gal expression is found in the cephalic mesenchyme at the level of mesencephalon, while the expression at the level of mandibular arch is preserved (arrowhead, D). Construct 25 shows no β -gal expression at all (E). Constructs 22, 23 and 26 sometimes give ectopic expression in the forelimb. Abbreviations: m, mesencephalon; t, telencephalon. Scale bars, 400 μ m.



whether the defects reside in the *Otx2* function in these neural crest cells themselves or are secondary to abnormal neuroectoderm. Endogenous *Otx2* is expressed in cephalic mesenchyme, and the sites of β -gal expression identified under the cis-element correspond well with the mesencephalic neural crest cells. Thus the *Otx2* heterozygous defects may relate primarily to the *Otx2* function in cephalic crest cells rather than secondarily to the *Otx2* function in the midbrain neuroectoderm.

The next question is the spectrum of neural crest cell lineages in which the cis-element is active. Most dorsal mesenchymal cells were β -gal-positive at the level of mesencephalon at 9.5 dpc. The β -gal-positive cells extended from diencephalon to the mesen/metencephalon boundary. No transgene expression was found rostral to the optic vesicles. No crest cells arise at the telencephalic level, and the majority of rostral crest cells are known to migrate from the most anterior edge of the diencephalon and to end up in the frontonasal region (Nichols, 1981; Noden, 1988; Osumi-Yamashita et al., 1994). The cis-element may thus not direct expression in the crest cells from the anterior diencephalon. β -gal was not expressed in most trigeminal ganglion cells by the cis-element, but the most rostral part of the cells expressed β -gal at 10.5 dpc. The trigeminal ganglia consist of crest cells originating from at least two different axial levels (Lumsden et al., 1991). Rhombencephalic crest cells fill the maxillo-mandibular lobe. The ophthalmic lobe, however, is likely to be composed of mesencephalic crest cells which may migrate into the rostral part of the trigeminal ganglia. In *Otx2* heterozygous trigeminal ganglion, the maxillary and mandibular branches developed normally while the ophthalmic branch was specifically affected (Matsuo et al., 1995). This suggests that most, if not all, of the neural

crest cells at the level of mesencephalon express β -gal under the cis-element.

The last question concerns the developmental stages of mesencephalic neural crest cells when the cis-element directs the expression. The mouse cis-acting element that we identified was unable to direct the expression in neural fold or neural crest, so that its activity apparently commences when the crest cells leave the neural crest, indicating that the regulatory mechanism of the *Otx2* gene expression in mesencephalic neural crest cells was independent of that in the premigratory neural crest and neural plate. A similar situation is known in *Hoxa-2* expression at the level of rhombomere 4 (Prince and Lumsden, 1994; Nonchev et al., 1996). At the same time, most of the transgene expression disappeared until 11.5 dpc when the crest cells began to differentiate into tissues. Mesencephalic crest cells contribute to the viscerocranium, peripheral nerves and connective tissues, as stated above (Noden, 1975, 1984; Couly et al., 1993, 1996; Köntges and Lumsden, 1996). β -gal expression apparently converged into the procartilagenous mesenchyme or peripheral nerves of premandibular and mandibular regions at 10.5 dpc, where it ceased; nor were expressions found in other differentiated neural crest-derived structures. β -gal expression generally lasts longer than RNA expression, and it is most likely that the activity of the cis-element is characteristic of the migratory phase of mesencephalic crest cells.

Besides the cephalic mesenchyme, the 1.8 kb mouse *Otx2* region mediated the expression in the ventral diencephalon at 9.5 dpc. Later, the β -gal-positive cells were distributed to the posterior lobe but not in the placodal ectoderm of stomodeal roof, Rathke's pouch. The former subsequently forms the neurohypophysis and the latter the adenohypophysis of the pituitary gland. Consistent with this, *Otx2* heterozygous mutant mice showed defects in development of neurohypophysis

(Matsuo et al., 1995). The 2.3 kb *Fugu* regulatory region, however, did not have the activity for expression in the ventral diencephalon. In addition, even in mouse, this activity appears to reside in sequences different from those for cephalic mesenchyme; the activity persisted even after deletion of 49 bp sequences that were essential for activity in the mesenchyme.

At this point, however, we are not able to conclude the exact cell populations in which the *cis*-acting element is active and therefore how structures originating from these cells correspond to structures affected in *Otx2* heterozygous mutants. To deduce more conclusively the cephalic mesenchyme cell lineages in which the *cis*-element is active, the production of mice that lack this proximal *cis*-element is eagerly awaited. The mice should coincidentally reveal the role of the *Otx2* gene in mesencephalic crest cells and, more clearly the role of these crest cells in development of the rostral head.

Activity in branchial arches

β -gal expression under the *cis*-element appeared to largely coincide with endogenous *Otx2* expression, but there were a few discrepancies. This was observed in the cells that had emigrated into the 2nd branchial arch and heart by both mouse and *Fugu* *Otx2* *cis*-elements, though they apparently arise in the mesencephalic region. No endogenous *Otx2* expression was found in the hyoid arch or heart. Since the β -gal expression is generally sustained longer than endogenous RNA and protein expression, it is possible that a minor population of mesencephalic crest cells emigrates into the 2nd arch and myocardial wall of the heart and suppresses the *Otx2* expression when it enters these regions. The migration of the mesencephalic crest cells into the hyoid arch has also been demonstrated with chick/quail chimera analyses (Couly et al., 1996).

The strong β -gal staining in the mandibular arch probably reflects the crest-derived population, which corresponds to the prospective Meckel's cartilage and mandible. Mapping experiments in chick have suggested that the distal elements of the mandibular arch derive from the mesencephalic crest and proximal elements from the rhombencephalic crest (Köntges and Lumsden, 1996; Couly et al., 1996). Indeed, in *Otx2* heterozygous mutants, distal elements of mandibular arch skeletons are lacking or severely affected but proximal elements develop normally (Matsuo et al., 1995). In contrast, the *Hoxa-2* mutant mice duplicated only the proximal portions of the mandibular arch skeletons, and no duplication occurred in the distal element, the mandible (Rijli et al., 1993). Thus, the mandibular arch ectomesenchyme is composed of heterogeneous crest cell components at different neuraxial levels with different genetic codes (Matsuo et al., 1995); the distal part of this arch is under the control of the *Otx2* gene, which is directed by the *cis*-element identified in this study.

Motifs A and B are conserved between mouse and fish *cis*-elements

In addition to *snail* consensus sequences, three DNA sequences were conserved between mouse and *Fugu* *Otx2* *cis*-elements (A, B and C sequences in Fig. 8A). Mutational analyses of these sequences indicated that motifs A and B were essential for the transgene expression in cephalic mesenchyme, but

A)	B)
mouse <u>TAAATCTG</u> <u>GAAATCTA</u>	mouse TCTA <u>ATTATC</u> ACTA <u>ATTAGG</u>
<i>Fugu</i> TAAATCTA TAAATATT GAAATGTG AAAATCTG TAAAGCTG	<i>Fugu</i> TCTACTTATA
A motif TAAATCTG	B motif CTAATTA
	<i>En</i> GGTA <u>ATTACA</u>
	<i>otd</i> <u>G</u> GGATTAG <u>C</u>
	<i>MHox</i> T _A <u>ATTAT</u> T _A A
	<i>Msx1</i> CAATTA _C <u>G</u>

Fig. 10. Comparison of the A and B sequences in 49 bp mouse and 1.1 kb pufferfish *Otx2* *cis*-elements. The sequences substituted in the mutational analyses (Figs 8B,9) are underlined. Binding sites of *en*, *otd*, *MHox* and *Msx1* homeodomain products are also listed for comparison (Gehring et al., 1994; Cserjesi et al., 1992; Catron et al., 1993). The ATTA core motif of homeodomain proteins is shaded.

motifs *snail* and C were not (Figs. 8B, 9). Motif B is required for the cephalic mesenchyme at the level of premandibular and mandibular regions, while motif A is not required for the expression at the level of mandible. As stated above, our previous study has suggested that the *Otx2* gene, or *Otx2*-positive mesencephalic neural crest cells, might have played an essential role in the establishment of the masticating system upon transition from agnatha to gnathostome. It is our interest to examine the enhancer structure, especially motif B, in extant agnatha lamprey *Otx2* for its expression in mesencephalic neural crest cells. Motif B, CTAATTA, contains the core motif, ATTA, for binding of homeodomain proteins (Fig. 10A,B; Gehring et al., 1994). The 5' TA dinucleotide preceding the ATTA core is known to bind with a higher affinity to the Gln residue at position 50 in the recognition helix of such homeodomains as those of *en*, *MHox* and *Msx1* products. In contrast, 5' GG dinucleotide is known to bind with a higher affinity to the Lys residue at position 50, as seen in *otd* and *Otx* homeodomains (Gehring et al., 1994; Cserjesi et al., 1992; and references therein; Fig. 10B). Among the homeodomain proteins that bear the Gln side chain at position 50 in the recognition helix, *MHox*, *Cart-1* and the *Msx* and *Dlx* families are expressed in the cephalic mesenchyme (Cserjesi et al., 1992; Zhao et al., 1994; Bulfone et al., 1993; Dollé et al., 1992; MacKenzie et al., 1991). In addition, mutations in these mouse genes are reported to cause defects in cephalic mesenchyme-derived structures such as cranial skeletons (Satokata and Maas, 1994; Qiu et al., 1995; Martin et al., 1995; Zhao et al., 1996).

The potential role of motif A, TAAATCTG, cannot be discussed here since it does not match any known consensus binding sequences for transcriptional factors. In addition, the present mutational analyses do not exclude the possibility that motifs *snail* and/or C also regulate the expression in cooperation with other motifs. To test this possibility, transgenic analyses are required in combination with a series of detailed mutations. Further analysis, including in vitro binding experiments on these motifs, will be important steps towards the elu-

cidation of the role of the *Otx2* gene in development of the gnathostome rostral head.

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