Regulation of Six1 expression by evolutionarily conserved enhancers in tetrapods

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ABSTRACT

The Six1 homeobox gene plays critical roles in vertebrate organogenesis. Mice deficient for Six1 show severe defects in organs such as skeletal muscle, kidney, thymus, sensory organs and ganglia derived from cranial placodes, and mutations in human SIX1 cause branchio-oto-renal syndrome, an autosomal dominant developmental disorder characterized by hearing loss and branchial defects. The present study was designed to identify enhancers responsible for the dynamic expression pattern of Six1 during mouse embryogenesis. The results showed distinct enhancer activities of seven conserved non-coding sequences (CNSs) retained in tetrapod Six1 loci. The activities were detected in all cranial placodes (excluding the lens placode), dorsal root ganglia, somites, nephrogenic cord, notochord and cranial mesoderm. The major Six1-expression domains during development were covered by the sum of activities of these enhancers, together with the previously identified enhancer for the pre-placodal region and foregut endoderm. Thus, the eight CNSs identified in a series of our study represent major evolutionarily conserved enhancers responsible for the expression of Six1 in tetrapods. The results also confirmed that chick electroporation is a robust means to decipher regulatory information stored in vertebrate genomes. Mutational analysis of the most conserved placode-specific enhancer, Six1-21, indicated that the enhancer integrates a variety of inputs from Sox, Pax, Fox, Six, Wnt/Efy1 and basic helix-loop-helix proteins. Positive autoregulation of Six1 is achieved through the regulation of Six protein-binding sites. The identified Six1 enhancers provide valuable tools to understand the mechanism of Six1 regulation and to manipulate gene expression in the developing embryo, particularly in the sensory organs.

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Introduction

Six1 is a member of the homeobox gene family defined by its highly distinctive homeobox homologous to Drosophila sine oculis (so) (Kawakami et al., 2000; Oliver et al., 1995) and its homeodomain preferentially binds to the sequence G/A(C)(TATA) over the canonical TAAT core (Berger et al., 2008; Noyes et al., 2008). Progress in phylogenetic analysis of basal metazoans and unicellular choanoflagellates suggests that the ancestral Six gene originated from a TALE class homeobox gene before the emergence of metazoans (Derelle et al., 2007; Larroux et al., 2008). A single ancestral Six gene of Six1/2 class went through gene duplication and divergence, and gave rise to two other subfamily genes (Six3/6 and Six4/5) after the divergence of porifera (sponges) from other metazoans including ctenophores (Hill et al., 2010; Hoshiyama et al., 2007; Larroux et al., 2008; Pick et al., 2010).

In metazoans, Six1 or Six1/2 subfamily genes are known for their roles in sensory organogenesis. The founding member so is essential for the compound eye formation in Drosophila (Cheyette et al., 1994) and its misexpression is sufficient to induce ectopic eyes (Weasner et al., 2007). In other protostomes and cnidarians, Six1 homologs are also known for their roles in eye development (Arendt et al., 2002; Mannini et al., 2004; Stierwald et al., 2004). In vertebrates, disruption of Six1 function causes severe defects in multiple sensory organs derived from cranial sensory placodes such as the olfactory epithelium (Chen et al., 2009; Ikeda et al., 2010, 2007; Laclef et al., 2003b), inner ear (Bricula and Collazo, 2006; Kozlowski et al., 2005; Laclef et al., 2003b; Li et al., 2003; Ozaki et al., 2004; Xu et al., 1999; Zheng et al., 2003; Zou et al., 2004) and epibranchial ganglia (Ozaki et al., 2004; Zheng et al., 2003; Zou et al., 2004). Six1-deficient mice also show defective development of taste papillae (Suzuki et al., 2010, 2011). Defects in the trigeminal ganglion (Konishi et al., 2006) and the anterior pituitary (adenohypophysis), a derivative of adenohypophyseal placode (Li et al., 2003), were noted in Six1/Six4 and Six1/Eya1 double knockout mice, respectively. In addition, vertebrate Six1 is
involved in the development of structures other than sensory organs. For example, Six1-deficient mice show severe abnormalities in the skeletal muscle and skeleton derived from somites (Laclef et al., 2003a; Li et al., 2003; Ozaki et al., 2004). The kidney, salivary gland and branchial organs such as the thymus and parathyroid gland are also absent or severely reduced in size (Kobayashi et al., 2007; Laclef et al., 2003b; McCoy et al., 2009; Ozaki et al., 2004; Xu et al., 2003). In humans, mutations of SIX1 cause a severe auditory and renal disorder known as branchio-oto-renal syndrome (Kochhar et al., 2008; Ruf et al., 2004).

Consistent with the pleiotropic function, Six1 shows a dynamic expression pattern, particularly in tetrapods where Six1 is expressed in all three germ layers. In the mouse, the earliest expression of Six1 starts in the endoderm at embryonic day 7.5 (E7.5) (Gu et al., 2004). The expression subsequently appears in the non-neural ectoderm surrounding the anterior neural plate at E8.0 (Sato et al., 2010). The mouse ectoderm is thought to correspond to the pre-placodal region (PPR), previously described in chick and amphibians, which represents a unique territory of multipotent sensory precursor cells for all cranial placodes (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Schlosser and Ahrens, 2004; Streit, 2002). A robust mesodermal expression is already present at this stage. At the placodal stage (E8.5–E10.5), Six1 expression is detected in all placodes excluding the lens placode, pharyngeal arches and pouches, dorsal root ganglia (DRG), somites, nephrogenic cord and notochord (Chen et al., 2009; Laclef et al., 2003b; Oliver et al., 1995; Ozaki et al., 2004). A recent fate-mapping study demonstrated the expression of Six1 in the cardiogenic mesoderm as early as at E7.5, and also in cardiac progenitors in the secondary heart field and proepicardium during subsequent cardiovascular development (Guo et al., 2011).

With regard to the transcriptional control of Six1 in vertebrates, Eya1 is necessary for the expression of Six1 in several tissues such as the otic vesicles (Xu et al., 1999), cranial ganglia (Zou et al., 2004), and pharyngeal arches (Xu et al., 2002). Another important observation concerning the regulation of Six1 expression is that in Six1/Six4 double knockout mice, Six1 expression was severely reduced in the ventral otic vesicle and trigeminal placode/ganglion (Grifone et al., 2005), suggesting that the positive autoregulatory mechanism is important for Six1 expression in these structures. Recently, we identified a single conserved non-coding sequence at the tetrapod Six1 locus (Grifone et al., 2005), which is conserved in several rodent species (Dix5, Mx5 and Pax7) and Gata proteins in the regulation (Sato et al., 2010).

The present study was designed to identify enhancers responsible for the dynamic expression pattern of Six1, during mouse embryogenesis. For this purpose, we focused on conserved non-coding sequences (CNSs) shared among tetrapod Six1 loci. CNSs of mouse origin were initially assessed for enhancer activity using chick embryos. The results were confirmed by mouse transgenesis, and seven such CNSs exhibited specific enhancer activity in major Six1-expression domains including sensory placodes. Moreover, functional analysis of the most conserved placode enhancer (Six1-21) identified candidates for trans-acting factors and the potential molecular basis of the autoregulation of Six1 expression in the otic vesicle. The cis-regulatory elements and trans-acting factors that control Six1 expression and the evolution of Six1 enhancers are discussed.

Materials and methods

Animals

Mice were housed in an environmentally-controlled room in the RIKEN CDB and in the Center for Experimental Medicine of Jichi Medical University, under the guidelines for animal experiments. Fertilized eggs of chicken were purchased from Shiroyama Poultry Farm (Kanagawa, Japan), and incubated at 38 °C in a humidified rocking incubator. The developmental stage of chick embryos was determined according to Hamburger and Hamilton (1951). All animal experiments were carried out in a humane manner after receiving approval of the Institutional Animal Experiment Committee of the Jichi Medical University, and in accordance with the Institutional Regulation for Animal Experiment and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the MEXT of Japan.

**In situ hybridization**

Whole mount in situ hybridization was performed using single-stranded digoxigenin-UTP (Roche, Mannheim, Germany)-labeled riboprobes as described previously (Ishihara et al., 2008a). Hybridization was performed at 65 °C. Signals were detected with an anti-digoxigenin antibody conjugated AP (Roche) and NBT/BCIP (Roche) for chromogen. The placod harboring the full-length chick Six1 cDNA (AB199734 in pBluescript II SK+) was kindly provided by Dr. Atsushi Kuroiwa, and used as a template. Antisense RNA probe was synthesized with T3 RNA polymerase using the HindIII-cut linearized template. After whole mount in situ hybridization, embryos were refixed with 4% paraformaldehyde, immersed in 18% or 30% sucrose/PBS, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek), then frozen on dry ice, and cut into 16- to 20-µm thick sections.

**Genomic sequence analysis**

The genomic sequences containing Six1 and neighboring Six4 and Six6 genes were downloaded from Ensembl: Human (NCBI 36 assembly, November 2005), Mouse (NCBI m37 assembly, April 2007), Opossum (MonDom5, Oct 2006), Chicken (release 2.1, May 2006), Xenopus (assembly version 4.1, August 2005), medaka (HdrR, Oct 2005), fugu (FLJU 4.0, Jun 2005), tetraodon (TETRA-ODON 8.0, Mar 2007), stickleback (BROAD S1, Feb 2006) and zebrafish (Zv8, Dec 2008). Global pairwise alignment of the above sequences were carried out using shufle-LAGAN (Brudno et al., 2003), and the results were visualized using the VISTA Browser (Frazier et al., 2004). Direct BLAST search was also used to examine the presence/absence of some of CNSs. Conserved transcription factor binding sites (TFBSs) were identified using rVISTA (Loots and Ovcharenko, 2004), Mulan (Ovcharenko et al., 2005) or TESS (Schug, 2008). Six protein binding sequences were searched manually using the consensus sequence TCAGGTNNC common to all the six known Six1 target genes (Fig. 5C). The sequence partially overlaps with the recently identified Six1 homeodomain-binding site G(A/G)TATCA.

**Reporter plasmid and transgene construction**

Reporter plasmids were constructed as described previously for the Six1 rostral PPR enhancer, Six1-14 (Sato et al., 2010). DNA fragments containing 14 CNSs were amplified by PCR from 200 ng of C57BL/6 mouse genomic DNA using LA Taq DNA polymerase (Takara, Ohtsu, Japan) or PfuTurbo DNA polymerase (Stratagene/Agilent Technologies, Santa Clara, CA). Table S1 provides a list of the PCR primers. The amplified fragment containing Six1-8 was cut with HincII. The genomic DNA fragment containing Six1-17 was isolated as an Nhel-Spi1 fragment from a Six1 genomic subclone. The amplified and isolated DNA fragments containing all 15 CNSs (mSix1-8 to mSix1-29) were then ligated into the multiple cloning site of ptkEGFP vector (Uchikawa et al., 2003).
and named as ptkEGFP mSix1-8 to ptkEGFP mSix1-29. For construction of mRFP1 reporter plasmids and multimerized (2x and 4x) reporters, ptkmRFP1ver2 (Inoue et al., 2007) was used. For construction of mutated ptkEGFP mSix1-21 reporters, LA PCR in vitro mutagenesis system (Takara) or cassette mutagenesis with PfTurbo DNA polymerase, was performed using the oligonucleotide primers listed in Table S2.

The DNA fragments containing CNs were ligated into the ASHsp68lacZpA (Sasaki and Hogan, 1996) vector for mouse transgenesis. One of the fragments, mSix1-8, showed a highly variable enhancer activity in transgenic mouse embryos, so flanked by insulators from the chicken β-globin locus. Two sets of two tandem copies of the core sequence of Hs4 insulator (Recillas-Targa et al., 2002) separated by the synthetic BsmBI (compatible with SalI ends) and BbsI (compatible with NotI ends) restriction sites were inserted into the Cla1-BamHI sites of ASHsp68lacZpA, and the resultant new plasmid, ASSinsBBins, was used to add insulators. For ASSinsBBins mSix1-8-8tkintronlacZpA, the mouse hsp68 gene promoter was replaced by the thimidine kinase gene (tk) promoter from human herpes simplex virus (HSV) plus the downstream synthetic intron (tktron) from ptkEGFP-mSix1-8. All transgenes for microinjection were excised from ASHsp68lacZpA or ASSinsBBins vectors as SalI- or NotI-fragments, run on an agarose gel and purified using QIAEX II gel extraction kit (Qiagen, Hilden, Germany).

The DNA sequences of all plasmids were verified by dyeoxy sequencing and purified by QIAfilter Plasmid Midi Kit or EndoFree Plasmid Maxi Kit (Qiagen). Table S3 provides a list of the plasmids used in this study.

Electroporation into chick embryos and detection of enhancer activity

Chick embryos at stages HH4 and slightly older HH4+ were electroporated using the CUY21 electroporator with a pair of 2 × 2 mm square platinum plates (7 V/v mm, 50 ms pulse, 950 ms interval, 5 pulses) and cultured on albumen-agar plates, as described previously (Ishihara et al., 2008b; Sato et al., 2010). For the initial screening of enhancer activity, 1.5 µl (per embryo) of DNA solution containing 2.0–2.5 mg/ml of EGFP reporters, 0.5–1.0 mg/ml of PCAG-HcRed (Matsuda and Cepko, 2004), and 0.01% Fast Green in 10 mM Tris–HCl (pH 7.5) was injected in the space between the blastoderm and vitelline membrane. PCAG-HcRed was included to verify electroporation as it drives ubiquitous expression of HcRed under the control of the strong CAG promoter/enhancer. The embryos were examined at 24 and 48 h post-electroporation (h.p.e.) using a stereo microscope (M205A, Leica Microsystems, Wetzlar, Germany, or SXZ16, Olympus, Tokyo, Japan).

To determine the enhancer activity of CNs in the DRG, DNA solutions containing 1x reporter plasmids were injected into the central canal of the neural tube at HH9-10 in ovo (Uchikawa et al., 2003), and electroporated using a pair of platinum electrodes with 0.5 mm diameter (25 V/v mm, 50 ms pulse, 950 ms interval, 4 pulses). Embryos were examined at 6, 12 and 24 h post-electroporation. We performed electroporation until more than 5 embryos showed homogeneous DNA distribution and normal morphology during 48 h-culture (HH4-4+ electroporation) or 48 h incubation (HH9-10 electroporation) periods. The patterns of enhancer activities were highly reproducible, and essentially the same results were obtained from those embryos. For histological analysis, embryos were embedded in OCT compound, then frozen on dry ice, and cut into 14-µm thick sections.

For mSix1-21 mutation analysis, mutated EGFP reporters at 2 mg/ml (Ebox-12m, Fox-12m, Lef1-m, Pax-m1, Pax-m2, Sox-1m, Sox-12m and Six-12m) and wild-type mRFP1 reporter at 1.6 mg/ml were co-electroporated at HH4-4+. The ratio of EGFP reporters to mRFP1 control was kept constant (EGFP:mRFP1 = 1:0.8) to adjust fluorescence intensity, embryos were fixed at 48 h.p.e. and EGFP and mRFP1 images were taken at the same exposure time from both the left and right sides. To evaluate the effects of mutation(s), the mean values (gray values) of the EGFP and mRFP1 channels of a fixed area (about 0.2 × 0.2 mm rectangle) that covered the otic vesicle, from each side of the embryo were measured separately. The mean EGFP levels normalized to the mRFP1 levels were calculated, and presented relative to the value obtained from the wild-type reporter.

Production and genotyping of transgenic mice

Transgenic mice were generated by microinjection of DNAs into the fertilized eggs of the CD-1 (ICR) strain using a standard protocol (Nagy et al., 2003). Transgenic embryos at E10.5 were identified by PCR analysis of yolk sac DNA. Briefly, embryos and yolk sacs were dissected carefully in ice-cold phosphate buffered saline (PBS), and the yolk sacs were incubated overnight at 55 °C in 100 µl of Direct PCR Lysis Reagent (Viagen Biotech, Los Angeles, CA) as recommended by the manufacturer. After heat-inactivation (85 °C for 45 min), 0.2–1.0 µl of the heat-inactivated DNA solution was subjected to PCR using a pair of primers specific to each CNS (mSix1-8–3 to mSix1-21-1) and the adjacent mouse hsp68 promoter (mhsp68) or each CNS, and the adjacent HSV tk promoter (ptkEGFP-RP), Table S4 lists the primers used for genotyping.

X-gal staining and histological analysis of embryos

Mouse embryos at E10.5 were fixed and processed for X-gal staining as described previously (Kimura et al., 1997). The dissected embryos were fixed in 1% formaldehyde, 0.8% glutaraldehyde and 0.02% NP-40 in PBS for 10 min, followed by three washes with PBS for 10 min at room temperature. Staining was carried out 0.5–24 h at 37 °C in a solution containing 1 mg/ml X-gal, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6 and 2 mM MgCl2 in PBS. The stained embryos were washed twice with PBS and stored in 10% formaldehyde or 4% paraformaldehyde in PBS. For histological analysis, the embryos were embedded in OCT compound, and cut into 16-µm thick sections.

Immunofluorescence examination

Immunofluorescence was carried out as described previously (Ikeda et al., 2007; Ishihara et al., 2008a). Anti-human CD57 antibody (clone HNK-1) (mouse IgM, 1:200 dilution, Becton-Dickinson) was used to stain migrating neural crest cells. Anti-Pax3 mouse monoclonal antibody from Developmental Hybridoma Bank, University of Iowa, was used to stain the trigeminal placodes. The secondary antibodies were Alexa Fluor 633 anti-mouse IgG (Molecular Probes/Invitrogen, Carlsbad, CA). DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO) was used for nuclear staining. Images of immunofluorescence samples were obtained with a microscope (BX51, Olympus, or DM5000B, Leica Microsystems).

Results

Six1 expression pattern is largely conserved in vertebrates

In the chick, previous studies have shown the expression of Six1 in the PPR and underlying mesoderm and endoderm at stages HH5–8, otic vesicle at about HH16, and myotomes, migrating limb muscle myoblasts and posterior limb mesenchyme at HH20 (Heanue et al., 1997; Litsiou et al., 2005), and cranial mesenchyme and trigeminal ganglion at HH19/20 (Mootooosamy and Dietrich.
However, Six1 expression in the sensory placodes derived from the PPR and sensory organs, such as the olfactory epithelium, remains unclear. Thus, in order to characterize the expression pattern of chick Six1 and to examine whether the pattern is conserved between chick and other vertebrates, particularly mouse, we carried out whole mount in situ hybridization using chick embryos at stages HH5-21. At HH5 and HH7, Six1 expression was detected in the anterior part of the embryo (Fig. 1A and B). In the ectoderm, Six1 mRNA was specific to the non-neural domain, i.e., PPR (Streit, 2002, 2004), though it was also detected in the underlying mesoderm and endoderm (Fig. 1A) as was described previously (Litsiou et al., 2005; Streit, 2004). At HH8, Six1 expression appeared in the cranial ectoderm, particularly in the otic placode area, foregut endoderm and cranial mesenchyme (Fig. 1C and S1B) (Seifert et al., 1993). At HH10 and HH11, hybridization signals became intense in the otic placode area (Fig. 1D, E and S1C). Signals were also present in other parts of the embryo such as the foregut endoderm, cranial mesenchyme, ventral cranial ectoderm that give rise to future oral ectoderm and adenohypophyseal placode, olfactory placode, somites and notochord (Fig. 1D, E and S1C). At HH15, the hybridization signals were detected in the ventral portion of the otic vesicle, in the pharyngeal arches and in the maxillary region (Fig. 1F, G). Sectioning of the stained embryo showed the presence of Six1 mRNA in the trigeminal (V) and epibranchial (VII, IX and Xth) placodes and ganglia, pharyngeal pouch endoderm, pharyngeal arch mesenchyme, cranial mesenchymes, Rathke's pouch, myotomes, notochord and nephrogenic cord (Fig. 1F, G, and S1D, E, F). At HH21, Six1 expression was detected in the posterior mesenchyme of the limb buds and dorsal root ganglia (DRGs), in addition

Fig. 1. Expression patterns of Six1 mRNA in chick embryo examined by whole mount in situ hybridization. (A, B) Six1 is expressed in the anterior region of the embryo at HH5 (A) and HH7 (B). Six1 expression is detected in the pre-placodal region (PPR, indicated by white arrowheads) in the ectoderm, and in the underlying mesoderm and endoderm. Dorsal view. (C) Six1 expression pattern at HH8+. Six1 is expressed in the cranial ectoderm, including the otic placode area (indicated by dotted line) and somites. Dorsal view. (D, E) Six1 expression pattern at HH10 (D) and HH11 (E). Six1 expression is evident in the otic placode area (dotted line), and in other parts of the embryo such as the olfactory placode and somites. Dorsal view. (F, G) Six1 expression pattern at HH15. Six1 is expressed in the mesenchyme in the first pharyngeal arch, maxillary region, trigeminal ganglion, antero-ventral portion of the otic vesicle, epibranchial (VIIth, IXth and Xth) placodes/ganglia, pharyngeal pouch endoderm, and somites. Lateral view. (H) Six1 expression pattern at HH21. Six1 is expressed in the head in the structures that were positive for Six1 in the earlier stages and posterior mesenchyme of the limb buds (the pattern is similar between the forelimb and hindlimb buds). Lateral view. (G–J) Six1 expression in the olfactory placode area. Lateral view. At HH14 (I), Six1 is expressed only in a small number of placodal cells (I, white arrow). The hybridization signals become stronger with advanced developmental stages (HH15, G) both in a cluster of cells (G, white arrow) and in the placode. At HH18 (J), Six1 is expressed in the olfactory placode and a process (J, white arrow) that extends medially from the placode (J, indicated by a dotted line). At HH21 (H), the olfactory nerve is positive for Six1 hybridization (indicated by white arrowheads). In A–E, anterior is to the top. In F, G, I, J, anterior is to the right. In H, anterior is to the left. The planes of sections shown in Fig. S1 are indicated by the yellow dotted lines in B–D, F, H. ce: cranial ectoderm; fl: forelimb bud; hl: hindlimb bud; oe: olfactory epithelium; ol: olfactory placode; ov: otic vesicle; so: somites; v: trigeminal ganglion; vii, ix and x: epibranchial ganglia; vii: VIIth ganglion. Scale bars: 0.5 mm, except 1 mm in H. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 2. Position of 16 evolutionarily conserved sequences around Six1 exons. (A, B) The VISTA plot of the 190 kb (A) and the central 26 kb (B) interval containing mouse Six1. The plot shows conserved sequences between mouse and human, opossum, chicken, Xenopus, fugu, Tetraodon, medaka, stickleback, zebrafish chromosome 20 (containing six1a) and zebrafish chromosome 13 (containing six1b) (Bessarab et al., 2008). Abscissa: mouse sequence, ordinate: percentage identity in a 100 bp window. The conserved regions above the level of 50%/100 bp are highlighted under the curve, with pink indicating conserved non-coding sequence (CNS), blue, conserved exon, and cyan, untranslated region. Among such CNSs located in the region between the flanking Six6 and Six4, 16 CNSs were conserved in mammals (mouse, human and opossum), and chicken and/or Xenopus. They are termed Six1-8 to Six1-29 and the positions are indicated in Arabic numerals on each plot. Four CNSs (Six1-8, 10, 12 and 21) were retained both in tetrapods and teleosts. Six1-8 of zebrafish (on Chromosome 20), Six1-12 of fugu, Six1-13 of Xenopus and Six1-24 of fugu and zebrafish (on Chromosome 20) are not shown in this plot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 3. Enhancer activity of 8 CNSs in electroporated chick embryos. (A) A schematic representation of the positions of CNSs harboring enhancer function. (B–G) Enhancer activity of ptkEGFP mSix1-8. Whole mount embryo at 24 h post-electroporation (h.p.e.) (dorsal view, B) and transverse section through the trunk region (E). In the head, EGFP expression is detected in individual cells located in the ectoderm between the lens and otic placodes (B). The otic placode (ot) is weakly positive for EGFP expression (B). In the head, EGFP-positive cells are located both in and under (indicated by white arrowheads) the ectoderm, and most of the cells are positive for Pax3 expression (transverse section, C), a specific marker for the trigeminal placode. In contrast, EGFP positive cells are negative for HNK-1 antigen, a specific marker for migrating neural crest cells (D). In the trunk (E), EGFP expression was detected in somites (so). Whole mount embryo at 48 h.p.e. (HH9-10 electroporation) (lateral view, F) showing EGFP expression in the cranial ganglia (v, vii–x) and otic vesicle (ov). Weak expression is also observed in the posterior optic cup and brain. Whole mount embryo at 48 h.p.e. (HH9-10 electroporation) after electroporation into the central canal of the neural tube (lateral view, G) showing EGFP expression in the dorsal root ganglia (drg) and spinal cord (sc). (H, I) Enhancer activity of ptkEGFP mSix1-9 × 4. Whole mount embryo at 48 h.p.e. (lateral view, H) showing EGFP expression in the cranial ganglia (v and viii) and epibranchial ectoderm (ep, marked with dotted line). Transverse section at the level of trigeminal ganglia (H). (J–M) ptkEGFP mSix1-12 × 4. Whole mount embryo at 24 h.p.e. (J) and 48 h.p.e. (K) showing expression in the otic vesicle (ov) and epibranchial ectoderm (ep). Coronal section of the head (L) showing expression in the Rathke’s pouch (rp) and transverse section of the trunk (M) showing expression in the nephrogenic cord (nc) at 48 h.p.e. EGFP expression was also detected in the anterior neural tube. (N–R) Enhancer activity of ptkmRFP1 mSix1-21 × 4. Whole mount embryo at 24 h.p.e. (dorsal view, N) and 48 h.p.e. (lateral view, O), showing the expression in the olfactory (ol) placode, otic placode (ot)/vesicle (ov), the VIIIth ganglion (viii) and epibranchial ectoderm (ep). Transverse section at the otic vesicle level (P) showing EGFP expression in the otic vesicle (ov) and epibranchial ectoderm (ep) at 48 h.p.e. Also, coronal section (Q) showing EGFP expression in the olfactory placode (ol) and another coronal section (R) showing expression in the Rathke’s pouch (rp). (S, T) Enhancer activity of ptkmRFP1 mSix1-10. Whole mount embryo at 24 h.p.e. (trunk region, ventral view, S) and transverse section (T) showing EGFP expression in the somites. (U–W) Enhancer activity of ptkmRFP1 mSix1-11 × 4. Whole mount embryo at 24 h.p.e. (dorsal view, U) and transverse section (V) showing EGFP expression in the cranial mesenchyme (cm). Immunohistochemical detection of migrating neural crest cells using HNK-1 antibody indicates EGFP-positive cells are negative for HNK-1 antigen and thus belong to cranial mesoderm (W). (X–Z) Enhancer activity of ptkmRFP1 mSix1-17. Whole mount embryo at 24 h.p.e. (trunk region, ventral view, X) and transverse section (Y) showing EGFP expression in the notochord (nc). Weak EGFP expression is also detected in the mesodermal structures surrounding the notochord (Y), the cranial ectoderm (ce) (dorsal view, Z) and the floor plate of the cervical neural tube (data not shown). In all panels of whole mount embryos, anterior is to the right, and all but panels S and X are ventral views. Panels S and X are ventral views. In all panels of whole mount embryos, the green channel (EGFP) is superimposed on each bright field image (grayscale). DAPI is used for nuclear staining (blue in C, D, W). br: brain, ce: cranial ectoderm, cm: cranial mesenchyme, drg: dorsal root ganglia, ep: epibranchial ectoderm, nc: nephrogenic cord, no: notochord, ol: olfactory placode, ot: otic placode, ov: otic vesicle, rp: Rathke’s pouch, so: somites, sc: spinal cord, tg: trigeminal placode, v-: trigeminal ganglion, vii, ix and x: epibranchial ganglia, viii: VIIIth ganglion. Scale bars: 0.5 mm (B, F–H, J, K, N, O, S, U, X, Z), 0.2 mm (C–E, P, T, V, W, Y), 0.1 mm (I, L, M, Q, R). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
to the structures that were positive for Six1 in the previous stages (Fig. 1H, and S1L–N).

Overall, the above expression patterns were very similar to those described in mouse (Laclef et al., 2003b; Li et al., 2003; Oliver et al., 1995; Ozaki et al., 2004; Sato et al., 2010). However, there was one intriguing exception. In the olfactory placodal region of the chick embryo, clear hybridization signals were observed in a small number of cells in the slightly thickened olfactory placode at HH14 (Fig. 1I and S1C) whereas the rest of the placodal cells were negative for such signals. With advancement of embryogenesis, a cluster of cells with clear hybridization signals were found in the underlying mesenchyme (HH15, Fig. 1G and S1H). By HH18, the Six1-strongly positive cell clusters changed their morphology by becoming longer, extending from the olfactory placode (also positive for Six1 expression) toward the forebrain on each side (Fig. 1J and S1I). This contrasts with a strong placodal expression of Six1 and the shutdown of its expression in cells that delaminate from the placode/epithelium in mouse embryos (Chen et al., 2009; Ikeda et al., 2007). Finally, at HH21, the olfactory nerves were positive for the hybridization signals and became visible both in whole mount preparations and sections (Fig. S1J, K). This is also different from Six1 expression pattern in the mouse embryo.

Identification of CNSs flanking Six1 exons

To identify the cis-regulatory elements that control mouse Six1 expression during embryogenesis, we first searched for evolutionarily conserved CNSs surrounding Six1 exons. It has been shown that CNSs conserved between mammals and non-mammalian tetrapods, such as chicken and Xenopus, often possess tissue-specific enhancer activity (Gottgens et al., 2000; Ishihara et al., 2008b; Ogino et al., 2008; Uchikawa et al., 2004). Fig. 2A shows the VISTA plot of the 190 kb interval containing Six1 and the neighboring Six6 and Six4 on mouse Chr 12 compared with syntenic regions of human, opossum, chicken, Xenopus and five teleost genomes. Sequence blocks that showed higher than 50% identity over 100 bp both in mammals and in chicken or Xenopus were defined as CNSs. We focused on CNSs located in the 150-kb region between the neighboring Six6 and Six4 gene exons. In our previous study (Sato et al., 2010), one CNS (Six1-14) was identified as an enhancer specific to the rostral PPR and early endoderm. Among 15 other CNSs ranged from 0.1 to 0.7 kb (Six1-8 to Six1-29), the majority clustered in the 20-kb region flanking Six1 exons (Fig. 2B), which is reminiscent of the distribution of multiple independent enhancers around the Sox2 exon (Uchikawa et al., 2003). It is noteworthy that one CNS, Six1-17, overlapped with three transcribed sequences (ESTs) located 3’ to Six1 exons, AK035085, AK034831 and AK142897, in which only AK142897 was in the same orientation with Six1 transcripts, indicating that the region is transcribed from both strands.

CNS enhancer activities in chick embryos

To examine the potential enhancer activities of the 15 CNSs identified by comparative genomics, we isolated 15 CNSs from the C57BL/6J mouse genomic DNA as 0.5 to 0.8 kb fragments, termed mSix1-8 to mSix1-29, and constructed EGFP reporter plasmids. Initially, the reporters were introduced into the entire epiblast of chick embryos (HH4-4+, gastrulae) by electroporation, cultured on albumen-agar plate and enhancer activities were assessed as EGFP expression. mSix1-24, a CNS closest to Six6 exons, showed EGFP expression in the forebrain and the neural tube (data not shown). Since Six1 is not expressed in the central nervous system while the expression of Six6 is specific to the forebrain (Jean et al., 1999), we excluded this CNS from further analysis. Among the remaining 14 CNSs, seven CNSs showed EGFP expression in specific subdomains in the Six1 expression domains in a 48-hr culture period (mSix1-8, 9, 10, 11, 12, 17 and 21, Fig. 3A) while

Table 1
Summary of enhancer activities directed by the conserved sequences*.

<table>
<thead>
<tr>
<th>Conserved sequence</th>
<th>Location</th>
<th>Mouse embryo E10.5</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSix1-8</td>
<td>Trigeminal placode/ganglion</td>
<td>Trigeminal placode/ganglion</td>
<td>Sato et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Otic placode</td>
<td>Vllth ganglion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Otic vesicle/Vllth ganglion</td>
<td>Epibranchial placodes/ganglia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epibranchial placodes/ganglia</td>
<td>Somites</td>
<td></td>
</tr>
<tr>
<td>mSix1-9</td>
<td>Trigeminal ganglion</td>
<td>Trigeminal ganglion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epibranchial placodes/ganglia</td>
<td>Vllth ganglion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Somites</td>
<td>Epibranchial placodes/ganglia</td>
<td></td>
</tr>
<tr>
<td>mSix1-10</td>
<td>Epibranchial placodes/ganglia</td>
<td>Somites</td>
<td></td>
</tr>
<tr>
<td>mSix1-11</td>
<td>Cranial mesenchyme</td>
<td>Cranial mesenchyme</td>
<td></td>
</tr>
<tr>
<td>mSix1-12</td>
<td>Otic placode</td>
<td>Otic vesicle/Vllth ganglion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epibranchial placodes/ganglia</td>
<td>Epibranchial placodes/ganglia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rathke’s pouch</td>
<td>Rathke’s pouch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nephrogenic cord</td>
<td>Nephrogenic cord</td>
<td></td>
</tr>
<tr>
<td>mSix1-13</td>
<td>Rostral pre-placodal region</td>
<td>Rostral pre-placodal region</td>
<td></td>
</tr>
<tr>
<td>mSix1-14</td>
<td>Notochord</td>
<td>Notochord</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mesoderm</td>
<td>Endoderm</td>
<td></td>
</tr>
<tr>
<td>mSix1-17</td>
<td>Cranial ectoderm</td>
<td>Olfactory placode/epithelium</td>
<td></td>
</tr>
<tr>
<td>mSix1-21</td>
<td>Olfactory placode/epithelium</td>
<td>Otic placode</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Otic vesicle/Vllth ganglion</td>
<td>Epibranchial placodes/ganglia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epibranchial placodes/ganglia</td>
<td>Pharyngeal pouch endoderm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rathke’s pouch</td>
<td>Rathke’s pouch</td>
<td></td>
</tr>
</tbody>
</table>

* Major enhancer activities of 16 CNSs at Six1 were assayed by chick electroporation (examined at 6, 12, 24 and 48 h post-electroporation [h.p.e.]). Then, 8 CNSs that showed enhancer activity in chick and one additional CNS, Six1-13 (5 transgenic embryos only showed variable lacZ expression patterns), were assayed by mouse transgenesis (E10.5). For Six1-14, the enhancer activity was also analyzed by Xenopus transgenesis (examined at St. 15 and St. 19) (Sato et al., 2010).
the remaining seven CNSs showed no EGFP expression. Two Six1 exons (the most conserved sequences in the 150-kb region surveyed in our study) also did not show any enhancer activity on its own (data not shown). We also confirmed the enhancer activities of all positive CNSs using multimerized (2x and/or 4x) reporters by electroporation into chick embryos, since EGFP expression levels of some CNSs were relatively low. Multimerization significantly augmented enhancer activities and allowed earlier detection of EGFP in some cases, but the main expression domains of EGFP were unaltered compared with the original reporters. Also, all positive CNSs were assessed for enhancer activity in the DRGs by electroporation into the HH9-10 neural tube in ovo. The results are summarized in Fig. 3 and Table 1.

Enhancers for cranial placodes

Four CNSs (mSix1-8, 9, 12 and 21, Fig. 3A) activated EGFP expression in the cranial placodes, each driving the expression in a unique subset of placodes, except the lens placode, which is negative for endogenous Six1 expression (Oliver et al., 1995).

mSix1-8 activated EGFP expression in the otic placode and the ectoderm at the level of developing mid- to hindbrain at 24 h.p.e. (Fig. 3B). To determine the identity of EGFP-positive cells at the mid- to hindbrain level, immunohistochemical analyses for Pax3 (a marker of ophthalmic trigeminal placode in chick) and HNK-1 antigen (a marker of migrating neural crest) (Xu et al., 2008) were carried out. As shown in Fig. 3C and D, EGFP was expressed in the ectodermal cells and few ingressing cells, which were mostly Pax3-positive (Fig. 3C) but distinct from migrating neural crest cells labeled by HNK-1 antibody (Fig. 3D). These findings indicate that mSix1-8 activated EGFP expression in the trigeminal placode. At 48 h.p.e., EGFP expression was found in the Vth, VIIth and epibranchial (VII, IX and Xth) sensory ganglia and otic vesicle (Fig. 3F).

mSix1-9 showed a similar but weak EGFP expression only at 48 h.p.e. in the Vth and VIIth ganglia and epibranchial ectoderm, but not in the otic vesicle (Fig. 3H, I). mSix1-12 activated EGFP expression in the notochord (no), floor plate (fp), epibranchial ectoderm (ep) and pharyngeal pouch endoderm (pe). Expression in the floor plate is limited to the anterior region. In panels C–G, I, J, R and S, particularly in E, lacZ expression is detected in cells located in the ventral portion of the hind brain and spinal cord. Since this characteristic expression pattern of reporter gene was observed in most of the CNSs but never in chick electroporation that used the tk promoter and EGFP reporter, we reasoned that the hsp68 promoter or another part of the transgene backbone is responsible for the reporter expression. In all panels of whole mount embryos, anterior is to the left and all panels are lateral views. The planes of sections are indicated by yellow dotted lines in A, D, F, J, N, P. br: brain, cm: cranial mesenchyme, dp: dorsal root ganglia, ep: epibranchial ectoderm, nc: nephrogenic cord, no: notochord, ol: olfactory placode, ov: otic vesicle, rp: Rathke's pouch, sc: spinal cord, v: trigeminal ganglion, vii, ix and x: epibranchial ganglia, viii: VIIIth ganglion. Scale bars: 1 mm (A, D, F, J, N, P, R), 0.2 mm (B, C, E, G–I, K–M, O, Q, S). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
expression in the otic placode/vesicle and epibranchial ectoderm at both 24 h.p.e. (Fig. 3J) and 48 h.p.e. (Fig. 3K). Sectioning of the head of a 48 h-embryo showed EGFP expression in the Rathke’s pouch (Fig. 3L). mSix1-21 activated EGFP expression in the olfactory and otic placodes and epibranchial ectoderm at 24 h.p.e. (Fig. 3N) and the strong otic expression was still maintained at 48 h.p.e. (Fig. 3O, P). At that stage, EGFP expression also appeared in the VIIIth ganglion (Fig. 3O), epibranchial ectoderm (Fig. 3O, P) and Rathke’s pouch (Fig. 3R). In the olfactory region (Fig. 3O, Q), EGFP expression was detected in the olfactory placode/epithelium (Fig. 3Q). Interestingly, several cells in the olfactory placode/epithelium and a cluster of cells in the mesenchyme beneath the placode were strongly positive for EGFP signals as were scattered cells adjacent to the forebrain (Fig. 3Q). The EGFP expression pattern was fairly similar to the endogenous expression pattern of chick Six1 (Fig. S1H, I) but was different from that of mouse Six1 described previously (Chen et al., 2009; Ikeda et al., 2007).

Enhancers for other structures

One of the CNSs with placode enhancer activity, mSix1-8, activated EGFP expression in somites (Fig. 3E). Also, following electroporation of the mSix1-8 reporter into the neural tube at HH9-10, EGFP expression was detected in the DRG at 48 h.p.e. (at ~HH21, Fig. 3G). Other CNSs were negative for such activity. Another CNS with placode enhancer activity, mSix1-12, directed EGFP expression in the nephrogenic cord (Fig. 3M). In addition, the following three enhancers activated transcription primarily in mesodermal structures: Six1-10 activated EGFP expression in the somite/myotome (Fig. 3S, T), mSix1-11 activated EGFP expression in the cranial mesenchyme (Fig. 3U, V). Interestingly, the EGFP-positive cells did not overlap with the HNK-1-positive neural crest cells (Fig. 3W), indicating that mSix1-11 induced the expression in the cranial mesoderm. mSix1-17 activated EGFP expression strongly in the notochord, weakly in the surrounding mesoderm (Fig. 3X, Y), and weakly in the cranial ectoderm and the floor plate of the cervical neural tube (Fig. 3Z).

In summary, 7 out of 15 CNSs isolated from the mouse genome exhibited specific enhancer activity in all placodes positive for Six1 expression, DRG, and other non-ectodermal structures, such as somites, cranial mesoderm, nephrogenic cord and notochord, in chick embryos. Together with the previously identified enhancer (mSix1-14), the combined enhancer activities of 8 CNSs cover the major conserved expression domains of Six1.

Enhancer activities of CNSs in transgenic mouse embryos

To provide evidence that the enhancer activities detected in the present study in the chick embryo reflect the endogenous activity of each CNS (mSix1-8, 9, 10, 11, 12, 17 and 21, Fig. 3A) in mouse embryos, we constructed transgenes with the reporter lacZ gene and generated transgenic mouse embryos. The results were summarized in Fig. 4 and Table 1.

Enhancers for cranial placodes

Four CNSs (mSix1-8, 9, 12 and 21) that activated EGFP expression in the cranial placodes of chick embryos also activated lacZ expression in the same unique subsets of placodes and their derivatives in E10.5 transgenic mouse embryos. The mSix1-8 reporter with flanking insulators derived from the chick b-globin locus (Recillas-Targa et al., 2002) activated lacZ expression specifically in the Vth, VIIIth and epibranchial sensory ganglia in all (5/5) transgenic embryos (Fig. 4A and B). mSix1-9 also showed similar lacZ expression in the cranial ganglia (2/4 transgenic embryos, Fig. 4D, E). mSix1-12 activated lacZ expression in the otic vesicle, VIIIth and epibranchial sensory ganglia (3/5 transgenic embryos, Fig. 4F, G) and the Rathke's pouch (3/5 transgenic embryos, Fig. 4H). mSix1-21 activated lacZ expression in the olfactory placode/epithelium (Fig. 4J, K and S2), otic vesicle (Fig. 4J, L), the VIIIth and epibranchial sensory ganglia (3/5 transgenic embryos, Fig. 4J, L), and the Rathke's pouch (2/5 transgenic embryos, Fig. 4M). In the epibranchial area, LacZ staining was not limited to the thickened placodes but extended to the flanking region composed of flat ectodermal cells (Fig. 4J, L).

Enhancers for other structures

The mSix1-8 reporter activated lacZ expression in the DRG (5/5 transgenic embryos, Fig. 4A, C). In contrast to the result of chick electroporation (Fig. 3E), somites were negative for lacZ expression in the DRG (Fig. 4A). mSix1-12 activated lacZ expression in the nephrogenic cord (2/5 transgenic embryos, Fig. 4I). Also, Six1-10 activated lacZ expression in myotomes (3/3 transgenic embryos, Fig. 4N, O), mSix1-11 in the cranial mesenchyme (4/8 transgenic embryos, Fig. 4P, Q) and mSix1-17 in the notochord (3/8 transgenic embryos, Fig. 4R, S). In some embryos, mSix1-10 (3/3 transgenic embryos) and mSix1-17 (3/8 transgenic embryos) activated LacZ expression in the branchial region (Fig. 4N, R, S), mSix1-21 activated LacZ expression in the pharyngeal pouch endoderm (Fig. 4J and data not shown).

Therefore, all the 8 CNSs (including mSix1-14) initially identified using the chicken electroporation system exhibited essentially the same enhancer activities in the mouse, which collectively covered the major Six1 expression domains. The enhancer activity of mSix1-14 in the early endoderm, in which it was difficult to introduce DNA in chick embryos at HH4 in our hands, was identified by mouse transgenesis (Sato et al., 2010). Also, while enhancer activity in the epibranchial placode/ganglia of the cultured chick embryos was difficult to demonstrate due to the relatively underdeveloped pharyngeal arches, mSix1-8, 9, 12 and 21 exhibited enhancer activity in the epibranchial sensory ganglia of transgenic mouse embryos.

Identification of essential cis-elements for mSix1-21 enhancer activity

mSix1-21 is the most conserved CNS in the Six1 locus, based on BLAST comparison of mouse sequences against chick and Xenopus genomes. It was also identified in all teleost genomes surveyed in the present study. Another characteristic of mSix1-21 is that the enhancer activated reporter gene expression in the largest number of placodes and placode-derived structures among the identified Six1 enhancers (Figs. 3 and 4 and Table 1). In addition, among the three enhancers that activated the expression in the otic placode/vesicle of chick embryos, mSix1-21 always yielded the strongest signal, suggesting that it is the major conserved otic enhancer of Six1. Furthermore, mSix1-21 was the only identified enhancer that activated gene expression in the olfactory placode/epithelium area. These findings prompted us to examine the regulatory mechanisms of mSix1-21 enhancer activity. For this purpose, we characterized the cis-elements required for the activity of mSix1-21 in vivo.

Fig. 5A shows the alignment of the core conserved regions of Six1-21 from four tetrapod species. Although the sequences are highly conserved, initially we could only identify the binding sites of the following transcription factors: Sox, Fox, Pax, Lef1/Tcf and E-box-binding basic-helix-loop-helix (bHLH) protein. To address the role of these sites, we introduced wild-type (mRFP1) and mutated (EGFP) reporters into chick embryos and quantified the change in reporter gene expression in the otic vesicle at 48 h.p.e. (Fig. 5Ba). As shown in Fig. 5Bb and Bc, the most dramatic
reduction in mSix1-21 enhancer activity (EGFP expression levels) was noted in association with mutation of one (Sox-1m) or two (Sox-12m) putative Sox-binding sites. Mutations of Pax (Pax-m2 and Pax-m1 mutations) and two Fox binding sites (Fox-12m) also resulted in significant reduction of the enhancer activity (Fig. 5Bd). In contrast to the mutations of the aforementioned binding sites, mutations of two E-boxes (Ebox-12m) and one unique Lef1/Tcf binding site (Lef1-m) reduced the enhancer activity only by about half (Fig. 5Bd).

Previous studies indicated that Six1 expression in the otic vesicle is dependent on the presence of functional Six1 and Six4 (Grifone et al., 2005), indicating the importance of positive autoregulatory mechanism. To explore the role of mSix1-21 in this process, we searched for potential Six protein binding sites...
(TCAGGTNNC) predicted from known Six1/4/5 target genes listed in Fig. 5C (Chai et al., 2006; Kawakami et al., 1996; Ohto et al., 1999; Yu et al., 2006). As shown in Fig. 5Bc and Bd, Six-12m mutation (disruption of two of the three predicted binding sites conserved in tetrapods) reduced the enhancer activity in the olfactory vesicle. Single mutations (Six-1m and Six-2m) were also analyzed, but they had only a marginal effect (data not shown).

To confirm the importance of Sox- and Six-binding sites for the mSix1-21 enhancer activity, we generated mouse embryos carrying mutated transgenes. Fig. 5Da shows lacZ expression patterns driven by the wild-type mSix1-21 enhancer at E10.5. Unexpectedly, mSix1-21-Sox-1m modified the expression pattern of lacZ (4/5 transgenic embryos, Fig. 5Db). The lacZ expression in the olfactory placode/epithelium was severely reduced although the expression in the otic and epibranchial placodes/ganglia seemed relatively preserved. In the case of mSix1-21-Six-12m, lacZ expression was reduced both in the olfactory and otic/epibranchial areas (4/5 transgenic embryos, Fig. 5Dc, Dd) and ectopic expression of lacZ was observed in all transgenic embryos. Although it is difficult to control the number of transgenes integrated into the individual embryos during mouse transgenesis, and one cannot compare directly lacZ expression levels in different embryos/transgenes (Fig. 5D), the result demonstrating the importance of Sox- and Six-binding sites in normal mSix1-21 activity was consistent with the data obtained in chick and confirmed the importance of both sites in the regulation of mSix1-21.

Discussion

Multiple independent enhancers control Six1 expression

Six1 is expressed in a wide variety of organs derived from all three germ layers and plays essential roles in organogenesis. The present study demonstrated that eight CNSs, including one CNS already described in a previous study (Sato et al., 2010) and seven CNSs in this study, surrounding Six1 exons acted as tissue-specific enhancers and each activated the transcription in a subset of Six1 expression domains (Table 1). Importantly, the major Six1 expression domains during development were covered by the sum of the enhancer activities. In addition, all the eight CNSs clustered within the 20-kb region surrounding Six1 exons (Figs. 2 and 3) while another CNS (Six1-24) with enhancer function located next to Six5 exons at about 100 kb downstream from the Six5 exons activated transcription in the central nervous system, in which Six6 (Jean et al., 1999) but not Six1 is expressed. Previous studies described enhancers for developmentally important genes, such as Sox2 (Uchikawa et al., 2003), Scl (Gottgens et al., 2000), Irx3/5/6 cluster (de la Calle-Mustienes et al., 2005), N-cadherin (Matsumata et al., 2005) and Eya1 (Ishihara et al., 2008b), in the DNA regions sandwiched between upstream and downstream flanking genes (exons). Our results indicate that the eight CNSs identified in this study represent major evolutionarily conserved enhancers responsible for the endogenous expression of Six1. It is plausible, however, that sequences outside the 150-kb region or coding sequences are also involved in the regulation of Six1.

Chick electroporation has been successfully used in the past to identify enhancers of small number of genes (Ishihara et al., 2008b; Izumi et al., 2007; Matsumata et al., 2005; Uchikawa et al., 2003). In the present study, we provided another example showing the effectiveness of using chick electroporation for systematic identification of conserved enhancers, as well as provided confirmative data with mouse transgenesis (Figs. 3 and 4, and Table 1). Admittedly, the enhancer activities detected by chick electroporation were not always reproduced in mouse transgenesis. mSix1-8 activated transcription in somites in chick embryos only and mSix1-21 activated transcription in the olfactory placode area differently in chick and mouse embryos, which could primarily reflect species differences (discussed below). Thus, we advocate the use of chick embryo for the assessment of enhancer activity of any DNA fragments that act from gastrulation through early organogenesis stages in vertebrate embryos. The new improved gene transfer technique that enables the introduction of DNA into early endoderm (Voiculescu et al., 2008) should also facilitate such studies.

Six1 enhancers as a new tool to understand development

The identification of major Six1 enhancers led to the understanding of the regulatory mechanism that controls Six1 expression in tetrapod embryos. (1) Six1 is regulated by at least eight discrete enhancers and each of which activates transcription in a unique subset of Six1 expression domains. (2) During sensory organogenesis, Six1 expression is controlled differently in the PPR and its derivatives, i.e., Six1-14 is a single characterized enhancer involved in the expression in the PPR (none of the seven enhancers described here are expressed early on in the PPR) while there are four placodal enhancers with distinct activities. (3) The expression of Six1 in each structure [e.g., PPR (Six1-14), early endoderm (Six1-14), olfactory placode (Six1-21), trigeminal placode/ganglia (Six1-8), DRG (Six1-8), cranial mesoderm (Six1-11), somites (Six1-10), notochord (Six1-17) and nephrogenic cord (Six1-12)] is driven by a single major enhancer. The above information should help us identify upstream signals and transcription factors that directly control Six1 expression during various organogenesis by focusing on the response of enhancers of less than 1 kb. Indeed, we were able to identify promising candidates that regulate Six1 expression in the PPR (Sato et al., 2010) and the potential regulatory mechanism in the olfactory/otic/epibranchial placodes (Fig. 5 and see below). Further analysis should enhance our understanding of the etiology of BOR and related syndromes: mutations that affect some of the identified Six1 enhancers might be causative for those disorders as described previously for mutations of SHH (Lettice et al., 2003), RET (Emison et al., 2005) and MYC (Wasserman et al., 2010). Recently, the Six1-12 enhancer was identified as a Six1 cardiac enhancer and its analysis stressed the role of Six1 in postnatal cardiac homeostasis (Delgado-Olguín et al., 2012) although Six1-12 variants that affect its integrity are yet to be identified. As to the reasons why we failed to identify Six1-12 as the cardiac enhancer is as follows: (1) we could not introduce DNA into the cardiac mesoderm by electroporation in chick embryos, and (2) only one embryo out of five transgenic mouse embryos showed any cardiac lacZ expression (data not shown). A weak cardiac enhancer activity was detected in retrospect but the result was not included in Fig. 4 or Table 1.

Another important application of the identified Six1 enhancers would be to use them as switches to control the expression of desired genes in specific tissues in transgenic mouse lines, particularly in the sensory placodes. Also, the identified enhancers would be ideal to create tissue-specific Six1 knockout mouse lines in order to determine the precise role of Six1 in a given germ layer or a cell type.

Evolution of Six1 enhancers in vertebrates

Identification of conserved Six1 enhancers allow us to speculate on the origin and evolution of the expression pattern of Six1. We have shown that four CNSs (Six1-8, 10, 12 and 21) are conserved between tetrapods and teleosts. What about more basal vertebrates or invertebrate chordates? The presence of eight Six1 enhancers in the lamprey Petromyzon marinus is unclear due
to the sequence gaps around Six1 exons and no sequences similar to the eight enhancers were found in the ascidian Ciona intestinalis (data not shown). In addition, related sequences were neither found around Six2 (Six1 paralog), Eya1/2 (genes often co-expressed with Six1) (Ishihara et al., 2008a) nor any other part of the mouse genome (data not shown). Thus, the most parsimonious scenario regarding the evolution of eight conserved Six1 enhancers identified in our study would be as follows: (1) The common ancestors of agnathans (lampreys and hagfishes) and gnathostomes may not have possessed any of the eight conserved CNSs as deduced from the recently described model for the expansion of vertebrate specific conserved non-coding sequences (McEwen et al., 2009). (2) At least four Six1 enhancers, Six1-8, 10, 12 and 21, have been (acquired and) present before the divergence of teleosts and tetrapods. It is noteworthy that the combined activities of the above four enhancers is sufficient to drive transcription in all Six1-positive placodes, somites and nephrogenic cord. Six1-14, the tetrapod PPR/endoderm enhancer, may also have been present although it was difficult to detect sequences overtly similar to Six1-14 in extant teleost genomes (see Fig. 2 and discussion in Sato et al., 2010). (3) Acquisition of the remaining three enhancers, Six1-9, 11, and 17 occurred in the tetrapod lineage after the teleost-tetrapod divergence. The fact that Six1 expression has not been described in tissues such as cranial mesoderm and notochord in zebrafish (Bessarab et al., 2004, 2008) is consistent with this. To test the validity of the above scenario and to investigate the origins of vertebrate Six1 enhancers and structure such as PPR and placodes, we need to understand the regulatory mechanism of Six1 expression in agnathans and basal chordates (Bassham and Postlethwait, 2005; Mazet et al., 2005; Schlosser, 2008).

Importance of positive autoregulation for Six1 expression in placodes

Functional analysis of the major olfactory/otic/epibranchial placode enhancer, mSix1-21, indicates that the enhancer integrates a variety of inputs from Sox, Pax, Fox, Six, Wnt/lef1 and bHLH proteins. The fact may reflect the importance of Six1 as one of the core transcription factors governing otic development (Ozaki et al., 2004; Zou et al., 2004). Among the aforementioned factors, particularly important are Sox and Six proteins (Figs. 3–5). Sox2 and Sox3 are expressed in all placodes except trigeminal placode (Abu-Elmagd et al., 2001; Schlosser, 2006; Uchikawa et al., 2003). Also, Sox9 and Sox10 are expressed in the otic placode (Schlosser, 2006). In chick electroporation, mutation of Sox-binding site resulted in severe reduction in the reporter gene expression in the otic vesicle (Fig. 5B) and mouse transgenesis revealed that the mutation has a more profound effect on the expression in the olfactory placode (Fig. 5D). The implication of Sox protein on Six1 expression in the olfactory placode is consistent with the finding of normal Sox2 expression in the placode of Six1 knockout mouse embryos (Ikeda et al., 2007).

With regard to the involvement of Six proteins in the regulation of Six1 expression in the sensory placodes, Six1, Six4, Six3 and Six6 are expressed in the olfactory placode, and Six1 and Six4 are also expressed in the trigeminal placode, otic placode and epibranchial placodes (Jean et al., 1999; Laclef et al., 2003b; Oliver et al., 1995; Ozaki et al., 2001). Thus, it is likely that Six1-21 is positively regulated by Six proteins in the olfactory and otic/epibranchial placodes, and this is the molecular basis for the severe reduction of Six1 expression in the otic placode of Six1/4 double knockout embryos (Grifone et al., 2005). Given that Six1 and Six4 are present in the PPR before the start of placode specification, it is not clear whether Six proteins are required for the initiation of placode-specific upregulation of Six1 or whether they are required for the maintenance of Six1 in the otic vesicle. While it is also possible that Six proteins are involved both in the initiation and maintenance, this is the first report on the potential molecular mechanism of positive autoregulation of Six1 expression during development.

Conserved and diverged expression patterns of Six1 in vertebrates

The present study showed different Six1 expression patterns in chick and mouse in the olfactory placode area (Fig. 1). In chick, Six1 is strongly expressed in a cluster of cells located underneath the olfactory placode that perfectly match the description of the first-born cells that migrate out from the placode and express N-CAM, HuC/D, Lhx2, Ngn1, Gap43 (Croucher and Tickle, 1989; Drapkin and Silverman, 1999; Fornaro et al., 2001, 2003; Maier and Gunhaga, 2009; Mendoza et al., 1982) and Eya2 (Ishihara et al., 2008a). While the function of these early-delaminating neurons remains unclear, it has been suggested to perforate the basal lamina to create openings and the scaffold later utilized by the emerging olfactory axons and to migrate towards the forebrain (Croucher and Tickle, 1989; Drapkin and Silverman, 1999; Fornaro et al., 2001, 2003; Maier and Gunhaga, 2009; Mendoza et al., 1982). In mammals, a group of cells known as the pioneer neurons is produced during the development of olfactory epithelium and are considered to play similar roles (Bystron et al., 2006; Ikeda et al., 2007). In mouse, Six1 expression is initiated in the olfactory placode but rapidly downregulated in pioneer neurons that emigrate outside the placode while the expression of markers, such as HuC/D, N-CAM, Gap43, Scg10, Ebf1 and Phd1, persists in migrating pioneer neurons (Bystron et al., 2006; Ikeda et al., 2007). Given that a single conserved enhancer (Six1-21) directs Six1 expression in the olfactory area both in mouse and chick (Figs. 2–5, S2), how can we explain this difference? There are two potential explanations: (1) differences in the enhancer activity itself defined by the number, order and the affinity of various TFBSs, and (2) differences in the cellular environment, i.e., repertoire of trans-acting factors present in the olfactory placode/epithelium lineage cells. We have shown in chick embryos that EGFP driven by Six1-21 from mouse appeared more intense in a cluster of cells subjacent to the olfactory placode than in the epithelial cells comprising the placode (Fig. 3). On the other hand, in mouse embryos, the same mouse enhancer activated transcription in the thickened placode but not in the cells that emigrated from the placode (Fig. 4 and S2). Thus, mouse Six1-21 activates transcription in a pattern that closely matches the endogeneous expression pattern of chick Six1 in chick and in a pattern that closely matches that of mouse Six1 in mouse, pointing to the difference in the cellular environment as the primary cause. The result also suggests that the function of Six1 may not be conserved during olfactory development between chick and mouse, suggesting evolutionary changes in the olfactory developmental program.

Conclusions

The present study identified seven discrete enhancers that collectively cover the major Six1 expression domains including cranial placodes. Together with the previously identified PPR/endoderm-specific enhancer, these represent major evolutionarily conserved enhancers responsible for the complex expression pattern of Six1 in tetrapods. Mutational analysis of the most conserved placode-specific enhancer (Six1-21) demonstrated the involvement of several transcription factors in the regulation of this enhancer and the molecular basis of positive autoregulation of Six1 expression in the otic/epibranchial placode area. The results confirmed that chick electroporation is a robust means...
to decipher regulatory information stored in the vertebrate genomes, and the identified enhancers provide useful tools to understand the mechanism of Six1 regulation and to manipulate gene expression during development.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.023.

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