

Genomes & Developmental Control

Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal *Sox9* expression pattern

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Abstract

SOX9 is an evolutionary conserved transcription factor that is expressed in a variety of tissues, with essential functions in cartilage, testis, heart, glial cell, inner ear and neural crest development. By comparing human and pufferfish genomic sequences, we previously identified eight highly conserved sequence elements between 290 kb 5' and 450 kb 3' to human *SOX9*. In this study, we assayed the regulatory potential of elements E1 to E7 in transgenic mice using a *lacZ* reporter gene driven by a 529 bp minimal mouse *Sox9* promoter. We found that three of these elements and the *Sox9* promoter control distinct subsets of the tissue-specific expression pattern of *Sox9*. E3, located 251 kb 5' to *SOX9*, directs *lacZ* expression to cranial neural crest cells and to the inner ear. E1 is located 28 kb 5' to *SOX9* and controls expression in the node, notochord, gut, bronchial epithelium and pancreas. Transgene expression in the neuroectoderm is mediated by E7, located 95 kb 3' to *SOX9*, which regulates expression in the telencephalon and midbrain, and by the *Sox9* minimal promoter which controls expression in the ventral spinal cord and hindbrain. We show that E3-directed reporter gene expression in neural crest cells of the first but not of the second and third pharyngeal arch is dependent on beta-catenin, revealing a complex regulation of *Sox9* in cranial neural crest cells. Moreover, we identify and discuss highly conserved transcription factor binding sites within enhancer E3 that are in good agreement with current models for neural crest and inner ear development. Finally, we identify enhancer E1 as a *cis*-regulatory element conserved between vertebrates and invertebrates, indicating that some *cis*-regulatory sequences that control developmental genes in vertebrates might be phylogenetically ancient.

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Introduction

Mutations in the human transcription factor gene *SOX9* cause the skeletal malformation syndrome campomelic dysplasia (CD) with associated XY sex reversal (Wagner et al., 1994; Foster et al., 1994). Accordingly, *Sox9* has been shown to be a master regulator of chondrogenesis and testogenesis in the mouse (Bi et

al., 1999; Akiyama et al., 2002; Chaboissier et al., 2004). CD patients also show defects in other organ systems where *Sox9* is expressed (Ng et al., 1997) such as the inner ear, brain, pancreas and heart (Houston et al., 1983; Mansour et al., 1995; Mansour et al., 2002), implying a wider role for *Sox9* in organogenesis. Among the symptoms seen in CD are deafness, Robin sequence (micrognathia, cleft palate and glossoptosis) and scoliosis, which, apart from defects in chondrocyte development, could also result from defective development of the otic vesicles, of cranial neural crest cells (CNCCs) and of the notochord, respectively. Indeed, studies in zebrafish, *Xenopus* and chicken revealed essential functions for *Sox9* in inner ear and cranial neural crest development (Spokony et al., 2002; Cheung and Briscoe, 2003; Liu et al., 2003; Saint-Germain et al., 2004). Furthermore, a frequent symptom seen in CD patients is

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macrocephaly with dilatation of the lateral ventricles, and, in about 25% of CD patients, the olfactory tracts and bulbs are missing, indicating an important role for *SOX9* in brain development (Mansour et al., 1995). In mice, *Sox9* is a major determinant of oligodendrocytes and astrocytes in the ventral neural tube, the two main types of glial cells in the central nervous system (Stolt et al., 2003). A role for human *SOX9* has also been proposed in pancreas development, as in all three CD patients analyzed, the pancreatic islets were less clearly formed and expression of the hormones insulin and glucagon was reduced to a variable degree (Piper et al., 2002). Finally, in accordance with the observed heart defects in CD patients, it has been shown that *Sox9* is needed for the formation of the endocardial cushions in mice and that homozygous *Sox9* knock-out mice die at 11.5 days post-coitum (dpc) from heart failure (Akiyama et al., 2004).

These data show that *Sox9* has essential early functions in the specification and/or differentiation of various tissues. The identification of the *cis*-regulatory elements controlling *SOX9* expression in these different tissues would greatly enhance the characterization of the *trans*-acting factors binding to these elements, allowing for a better definition of the regulatory network that specifies cells/organs like chondrocytes, testis, cranial neural crest cells, the otic placode and glial cells in humans or mice. The search for such *SOX9 cis*-regulatory elements has turned out to be challenging because *SOX9* has an extended regulatory region as indicated by CD translocation breakpoints that are scattered up to 1 Mb 5' to *SOX9* (Pfeifer et al., 1999). An initial study by Wunderle et al. (1998) approached the identification of these long-range elements by generating mice transgenic for human *SOX9-lacZ* yeast artificial chromosomes (YACs) which contained variable amounts of DNA sequences 5' to *SOX9*. They could show that the CD translocation breakpoints most likely remove multiple *cis*-regulatory elements from the human *SOX9* gene and that most of these elements should reside within a 600-kb interval extending 350 kb 5' and 250 kb 3' to *SOX9* (Wunderle et al., 1998). Moreover, they could define genomic subregions where tissue-specific enhancers should be located. However, with none of their transgenic YACs could they detect *lacZ* expression in the developing male gonads, implying that either the human sequences are not functional in the mouse or that the testis-specific enhancer(s) are located outside the 600-kb interval. By comparing 2.5 Mb of human with 195 kb of pufferfish (*Takifugu rubripes*) genomic sequences around the *Sox9* locus, we previously identified five highly conserved sequence elements, E1–E5, up to 290 kb 5' to human *SOX9*, and two such elements, E6 and E7, up to 95 kb 3' to human *SOX9*. These elements all reside within the interval identified by Wunderle and co-workers. Only element E8, located 450 kb 3' to *SOX9*, lies outside this interval. Most of these putative *long-range cis*-regulatory elements are around 100–150 bp in size, showing 68% to 80% sequence identity between human, mouse and *Takifugu* (Bagheri-Fam et al., 2001).

In this study, we have assayed the regulatory potential of elements E1 to E7 in transgenic mice, using *lacZ* as a

reporter gene that was driven by a minimal mouse *Sox9* promoter (Kanai and Koopman, 1999). We show that three of these elements, E3, E1 and E7, and the *Sox9* promoter itself can drive *lacZ* gene expression in a tissue-specific manner that recapitulates the endogenous *Sox9* expression pattern in various tissues including the cranial neural crest, inner ear, brain, ventral neural tube/hindbrain, node, notochord, gut, pancreas and bronchial epithelium. Moreover, we demonstrate that *Sox9* expression in a subset of CNCCs is dependent on beta-catenin and discuss highly conserved protein binding sites for enhancer E3 that are in good agreement with current models for cranial neural crest and inner ear development. Finally, we identify enhancer E1 as a *cis*-regulatory element conserved between vertebrates and invertebrates.

Material and methods

Generation of DNA constructs for microinjection

The conserved human elements E1–E7 with their 5'- and 3'-flanking sequences and the minimal mouse *Sox9* promoter (see below) were amplified by PCR with the proofreading polymerase *Pfu* using human and mouse genomic DNA, respectively. The oligonucleotides used for the PCR reaction contained at their 5'-end a restriction site for the subsequent cloning of the PCR product preceded by 2–6 nucleotides to facilitate the restriction digest. Digested PCR fragments E1 to E5 and the *Sox9* promoter were subcloned into the multiple cloning site of pBS SK (Stratagene) in order to generate constructs containing the mouse *Sox9* promoter with elements E1–E5 either as single fragments or in combination. The constructs generated within pBS SK were released with the restriction enzymes *SpeI* and *XhoI* and subcloned into the *lacZ* reporter vector pCMV β (CLONTECH). By this cloning step, the CMV promoter of the pCMV β vector was replaced by the element-*Sox9* promoter construct. Elements E6 and E7 were directly cloned as *SpeI* fragments into the pCMV β vector that already contained the *Sox9* promoter. The pCMV β vector used for our transgenic studies was a modification from the original vector with an insertion of a 40 bp *EcoRI-SalI-SmaI-SpeI-EcoRI* fragment into the *EcoRI* site. All finished constructs were sequenced using the ABI310 automated fluorescence sequencer in order to confirm the correct sequence, orientation and order of the different DNA fragments.

Constructs and primer sequences

Nucleotides in bold denote the restriction sites added to the 5' end, with the corresponding restriction enzyme given in brackets; nucleotides added to the 5' end to facilitate restriction are underlined. In all constructs, the *lacZ* gene is driven by the minimal 529 bp mouse *Sox9* promoter (*Sox9^P*).

Construct *Sox9^P* (529 bp) contains only the minimal mouse *Sox9* promoter (529 bp) that was amplified with primers 5'-GAATCGATACCTCTGGCTGAGCTCC-3' (*Clal*) and 5'-AGCTCGAGGAAGCGAGAAGCCGC-3' (*XhoI*).

Construct E1*Sox9^P* (1045 bp) contains the DNA fragment E1 (516 bp), consisting of element E1 (130 bp) extended by 206 bp in 5' and by 180 bp in 3' direction, that was amplified with primers 5'-GTATCGATAGACCAACTTCAAATATGCC-3' (*Clal*) and 5'-GTATCGATACAATTCATTAGTCCAC-3' (*Clal*).

Construct E2*Sox9^P* (1021 bp) contains the DNA fragment E2 (492 bp), consisting of element E2 (123 bp), extended by 189 bp in 5' and by 180 bp in 3' direction, that was amplified with primers 5'-AGGAATTCAAAGCCATTGATCCTCC-3' (*EcoRI*) and 5'-GTATCGATACACTTTGATTGTACTGTGG-3' (*Clal*).

Construct E3*Sox9^P* (1062 bp) contains the DNA fragment E3 (533 bp), consisting of element E3 (139 bp), extended by 144 bp in 5' and by 250 bp in 3' direction, that was amplified with primers 5'-AGGAATTCCTGGTCAAG-

CACTCATTGC-3' (*EcoRI*) and 5'-ACCATCGATATTGCTTTCAGAAA-CATGATTC-3' (*Clal*).

Construct E4*Sox9^P* (1050 bp) contains the DNA fragment E4 (521 bp), consisting of element E4 (157 bp), extended by 170 bp in 5' and by 194 bp in 3' direction, that was amplified with primers 5'-AACCTGCAGTCCTTG-CAAAGGTAGTCC-3' (*PstI*) and 5'-AGGAATTCCTCTGTCAGCATG-CAG-3' (*EcoRI*).

Construct E5*Sox9^P* (956 bp) contains the DNA fragment E5 (427 bp), consisting of element E5 (129 bp), extended by 129 bp in 5' and by 169 bp in 3' direction, that was amplified with primers 5'-GGACTAGTTCCAAACAGC-CACAGTTATC-3' (*SpeI*) and 5'-AAACTGCAGGAGGTGCT-TATGTGGCTG-3' (*PstI*).

Construct E6*Sox9^P* (910 bp) contains the DNA fragment E6 (381 bp), consisting of element E6 (97 bp), extended by 129 bp in 5' and by 155 bp in 3' direction, that was amplified with primers 5'-ATCTAGACTAGTCTTCTGTT-TACACCTGAG-3' (*SpeI*) and 5'-ATCTAGACTAGTGGACTC-GAAGGCTCTG-3' (*SpeI*).

Construct E7*Sox9^P* (1667 bp) contains the DNA fragment E7 (1138 bp), consisting of element E7 (403 bp), extended by 453 bp in 5' and by 282 bp in 3' direction, that was amplified with primers 5'-ATCTAGACTAGT-GAAAGCTCCGAAGGACTC-3' (*SpeI*) and 5'-ATCTAGACTAGTGTTC-GAAGGACGCTTAG-3' (*SpeI*).

Construct E5–E3*Sox9^P* (2010 bp) contains the DNA fragments E3–E5.

Production and genotyping of transgenic mice

For microinjection, *lacZ* fusion gene constructs were separated from vector sequences by cutting with the restriction enzyme *SalI* followed by electrophoresis on 1% Seakem[®] GTG[®]-Agarose gels (Biozym), and excised insert fragments were purified with the QIAquick[®] PCR Purification Kit (Qiagen). The purified constructs were microinjected into the male pronuclei of fertilized FVB mouse oocytes using standard procedures (Hogan et al., 1994). Transgenic embryos and transgenic founder animals were identified by PCR with the *lacZ* primers 5'-CTGCGATGTCGGTTTCCG-3' and 5'-GGATGGTTCCGA-TAATGCG-3' using Proteinase K-digested yolk sacs and tail biopsies as DNA templates, respectively. To establish transgenic lines, founder animals were crossed with C57BL/6 wildtype mice.

Whole-mount β -galactosidase staining and histological analysis

Whole-mount β -galactosidase staining of 6.5 to 13.5 dpc embryos using X-gal as a substrate was performed as described (Hogan et al., 1994). 12.5 dpc and 13.5 dpc embryos were cut transversally prior to whole-mount β -galactosidase staining to facilitate penetrance of X-gal at these stages. After whole-mount staining, embryos were embedded in paraffin and 10- μ m sections were prepared. Staining was from 5 min to overnight depending on the strength of transgene expression.

Generation of *Wnt1-Cre;beta-cat^{flloxdel/fllox};E5–E3Sox9^P* embryos

Wnt1-Cre;beta-cat^{flloxdel/+} male mice in which one copy of the beta-catenin gene is inactivated and in which the Cre recombinase is under the control of the *Wnt1* promoter (Brault et al., 2001) were crossed with female mice carrying construct E5–E3*Sox9^P* (this study). Resultant *Wnt1-Cre;beta-cat^{flloxdel/+};E5–E3Sox9^P* male mice were crossed with female *beta-cat^{fllox/fllox}* mice in which both beta-catenin gene copies are flanked by *loxP* sites (Brault et al., 2001) to generate *Wnt1-Cre;beta-cat^{flloxdel/fllox};E5–E3Sox9^P* embryos. For genotyping, DNA from tail biopsies was analyzed by PCR using primers for *Cre* and beta-catenin as described (Brault et al., 2001).

SOX9 immunohistochemistry on paraffin and cryosections

C57BL/6 wildtype embryos were used for immunohistological analyses. For paraffin sections, embryos were collected in PBS, fixed in Serra (ethanol:37% formaldehyde:acetic acid, 6:3:1) at 4°C overnight, dehydrated through an ascending ethanol series, embedded in paraffin and sectioned at 7 μ m. For cryosections, embryos were collected in PBS and fixed in freshly

prepared 4% paraformaldehyde in PBS at 4°C overnight. The embryos were incubated in 15% sucrose for 2 h at 4°C followed by incubation in 30% sucrose at 4°C overnight. The embryos were then embedded in Tissue Freezing Medium (Jung) and frozen in a liquid nitrogen bath. 10–15- μ m sections were collected on SuperFrost slides (Roth) using a cryostat at –20°C and dried at room temperature. For SOX9 immunohistochemistry on paraffin and frozen sections, the Peroxidase Rabbit IgG Vectastain[®] ABC Kit (Vector) was used following the instructions of the manufacturer. The primary rabbit anti-SOX9 antibody (kind gift of M. Wegner) was applied at 1:200 dilution on the sections.

Results

In this study, we have assayed the regulatory potential of the putative human *long-range Sox9 cis*-regulatory elements E1 to E7 in transgenic mice using *lacZ* as a reporter gene that was driven by a minimal mouse *Sox9* promoter (Kanai and Koopman, 1999). We first examined the regulatory potential of the minimal mouse *Sox9* promoter which has been described to be, at least in part, responsible for testis-specific expression of the *Sox9* gene in vitro (Kanai and Koopman, 1999). In order to investigate the effects of the potential human enhancers E1 to E7 on mouse *Sox9* promoter activity, we performed a series of transgenic assays with DNA constructs containing these elements either as single DNA fragments or in combination. As the sequence conservation of elements E1 to E7 between human and mouse extends beyond the 80–120 bp of sequence conserved between human or mouse and pufferfish, we included additional 5' and 3' sequences flanking the highly conserved elements in our transgenic analyses (Fig. 1). *LacZ* expression of the different constructs was compared to the previously described *Sox9* gene expression pattern (Ng et al., 1997; Zhao et al., 1997). As *Sox9* expression in some tissues such as gut and lung had so far not been studied in detail, we performed immunohistochemistry in these tissues using a SOX9 antibody.

The 529 bp minimal mouse *Sox9* promoter controls early gene expression in the ventral spinal cord and ventral hindbrain

The mouse *Sox9* promoter used in our study spans 529 bp of genomic DNA and extends 202 bp 5' and 327 bp 3' relative to the transcription start site (construct *Sox9^P*). In contrast to human elements E1 to E8 that are highly conserved in *Takifugu* (Bagheri-Fam et al., 2001 and Figs. 6A and B), the mouse *Sox9* promoter is well conserved only in human and shows some sequence similarity to chicken (Kanai and Koopman, 1999). Of the two established transgenic mouse lines, none expressed the transgene between 6.5 and 13.5 dpc. However, 4 of 11 transient transgenic embryos showed a very restricted reporter gene expression in the ventral hindbrain and ventral neural tube at 9.5 dpc and 10.5 dpc (Fig. 2). As all *lacZ* expressing transgenic mouse lines established in this study exhibited an almost identical expression pattern in the ventral hindbrain and ventral neural tube (Fig. 1), we could study the spatiotemporal expression controlled by the *Sox9* promoter in detail.

During the development of the spinal cord, the mouse SOX9 protein is initially detected at 9.5 dpc in the ventral neural tube (Fig. 2C and Stolt et al., 2003). From 10.5 dpc on, SOX9 protein expression is found in the proliferating ventricular zone

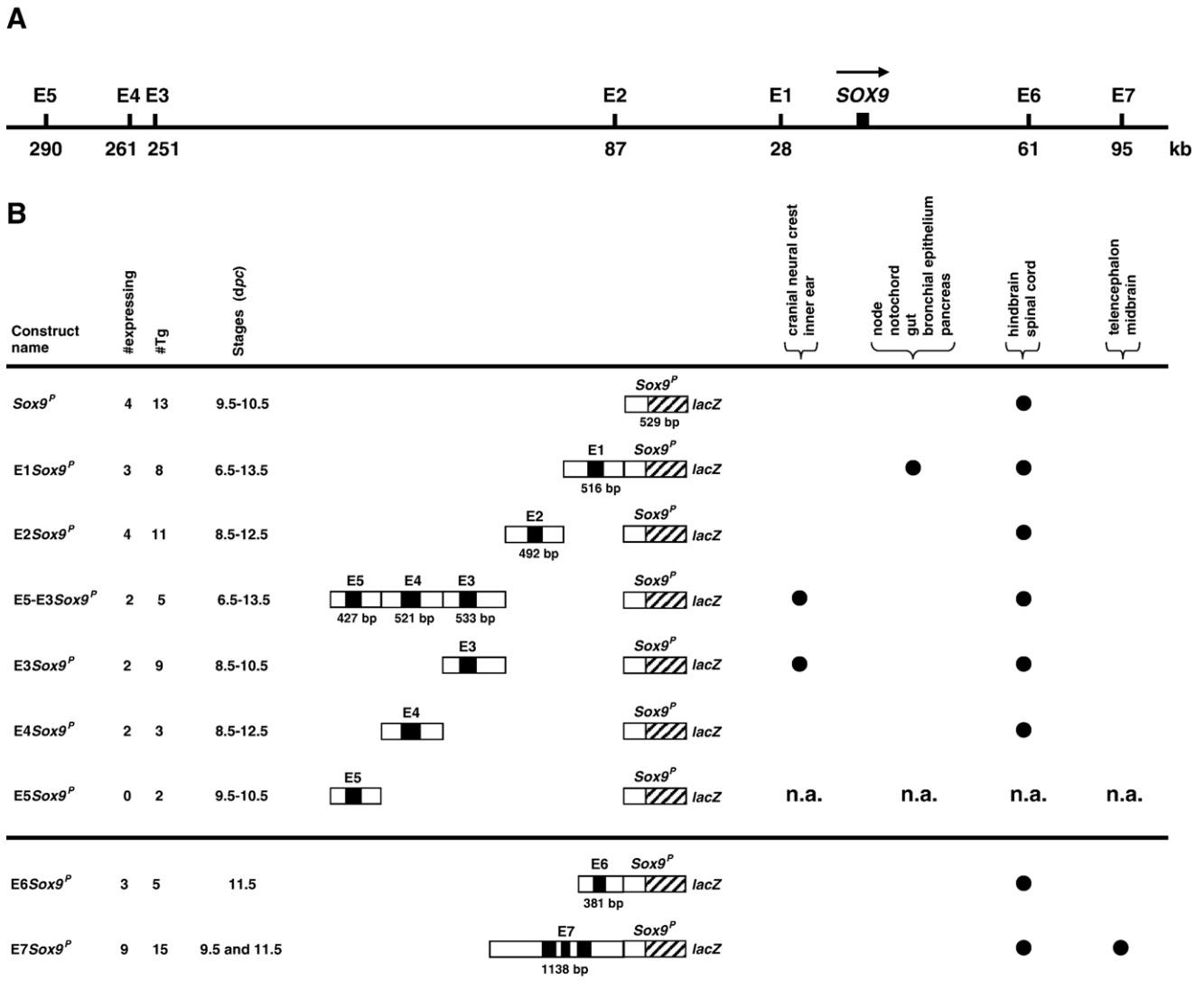


Fig. 1. Conserved sequence elements from the human *SOX9* locus function as tissue-specific enhancers in transgenic mouse embryos. (A) Position of conserved sequence elements located 5' (E1–E5) and 3' (E6 and E7) to the human *SOX9* gene, with distances given in kb. The direction of transcription of the *SOX9* gene is indicated by an arrow. (B) Summary of constructs, transgenic lines or embryos and observed expression pattern. Elements E1 to E7 were assayed either as single DNA fragments or in combinations for their regulatory potential in transgenic mice using a *lacZ* reporter gene driven by a 529 bp minimal mouse *Sox9* promoter. The shaded box within the *Sox9* promoter represents the region 3' to the transcription start site. As the sequence conservation of elements E1 to E7 between human and mouse extends beyond the 80–120 bp of sequence conserved between human or mouse and pufferfish (black boxes), additional 5'- and 3'-flanking sequences were included (white boxes). The first column at left gives the name of the construct, the second column indicates the number of lines or embryos with β -gal activity (#expressing), the third column indicates the total number of transgenic lines or embryos (#Tg) and the fourth column denotes the stages in dpc (days post-coitum) at which the transgenic embryos were analyzed. The tissues expressing the *lacZ* transgene are listed at right. Note that for construct E5*Sox9^P* no expressing transgenic line/transient transgenic embryo could be obtained (n.a., not analyzed).

throughout the whole neural tube (Fig. 2F). Consistent with the early and restricted expression of SOX9 at 9.5 dpc, β -galactosidase staining was first weakly visible at 9.5 dpc in the ventral neural tube, extending from the hindbrain to the spinal cord beyond the forelimb bud level (Figs. 2A and B). At 10.5 dpc, transgene expression was up-regulated and extended up to the hindlimb bud level (Figs. 2D and E). However, except for a small region in the ventral part, no transgene expression was detected at 10.5 dpc and at later stages in the SOX9-expressing ventricular zone (compare Fig. 2E with Fig. 2F, and data not shown). Thus, the *Sox9* promoter fragment can mediate only the early aspect of SOX9 expression in the spinal cord and

hindbrain. The delay of strong β -galactosidase staining in the ventral part of the neural tube by 1 day in comparison to the SOX9 protein may be explained by the fact that some time is needed for the β -galactosidase protein to accumulate in these cells. It should be mentioned that no expression, at any stage analyzed, could be detected in the testis.

Enhancer E1 mediates expression in the node, notochord, gut, bronchial epithelium and pancreas

The conserved 116 bp element E1 (Fig. 6A), located 28 kb 5' to human *SOX9*, was cloned as a 516 bp fragment in front of the

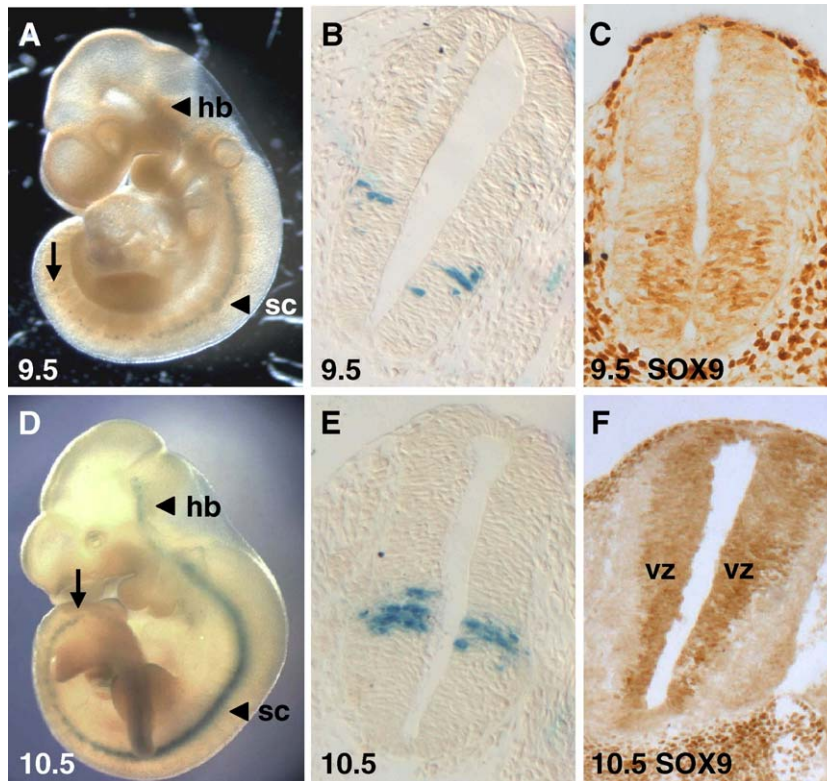


Fig. 2. The 529 bp mouse *Sox9* promoter mediates *lacZ* expression in the early ventral hindbrain and early ventral spinal cord. (A and D) Whole-mount β -galactosidase staining and (B and E) paraffin transverse sections of transient transgenic embryos carrying construct *Sox9^P*. (C and F) SOX9 immunohistochemistry on cryosections. The arrows in A and D indicate the posterior expression boundary in the spinal cord. The *Sox9* promoter initiates *lacZ* expression in the ventral hindbrain/spinal cord at 9.5 dpc (A and B). Up-regulated *lacZ* expression was observed in both the hindbrain and spinal cord at 10.5 dpc (D and E). Abbreviations used: hb, hindbrain; sc, spinal cord; vz, ventricular zone.

Sox9 promoter, resulting in construct E1*Sox9^P* (Fig. 1). We could establish three expressing transgenic mouse lines which all exhibited a similar expression pattern. Enhancer E1 mediated *lacZ* expression in a variety of tissues with an expression pattern closely resembling that of endogenous *Sox9*/SOX9 (Fig. 3).

Similar to endogenous *Sox9* expression (Ng et al., 1997), *lacZ* expression was first detected in the node at 7.5 dpc (Fig. 3A) and at 8.25 dpc also in the notochord (Fig. 3B). Expression of β -galactosidase and of SOX9 protein in the notochordal plate and notochord continued through subsequent development (Figs. 3D–M) at least until 13.5 dpc (data not shown). At 8.5 dpc, we could also detect strong *lacZ* expression in mesodermal cells immediately lateral to the notochordal plate (Fig. 3D, inset), similar to endogenous *Sox9* (Ng et al., 1997).

Like the SOX9 protein, β -galactosidase staining in the gut was already observed at 8.5 dpc (Figs. 3D–G). However, while the reporter gene seemed to be uniformly expressed from anterior to posterior (Figs. 3D and E), the SOX9 protein, while clearly detectable in the posterior region, was only barely detectable in the anterior region of the gut (Figs. 3F and G). This difference in expression pattern could also be seen at later stages. Thus, from 9.5 dpc on, *lacZ* was almost equally highly expressed in the esophagus, stomach, duodenum and hindgut (Figs. 3H, I, L and O), while SOX9 protein expression was significantly weaker in the esophagus than in the more distal parts of the gut (Figs. 3J, K, M and P).

The expression pattern of β -galactosidase and of SOX9 was almost identical, however, during lung development. Both proteins were already detectable at 9.5 dpc in the laryngo-tracheal groove of the foregut (Figs. 3I and K). By 11.0 dpc, β -galactosidase/SOX9 expression could be seen in the bronchial epithelium of the two lung buds (Figs. 3L and M). Later on, the expression of both proteins was lost in the two main bronchi and was confined to the newly forming bronchial branches of the lung (Fig. 3N and data not shown).

LacZ expression was also observed in the developing pancreas. Similar to endogenous *Sox9* (Lioubinski et al., 2003), the β -galactosidase protein was already present when the pancreatic buds first become visible at 9.5–10.0 dpc (Fig. 3C). Histological sections of 11.0 dpc transgenic embryos showed that β -galactosidase was expressed, like SOX9, in the epithelium of both pancreatic buds (Figs. 3O and P). At 13.5 dpc, the reporter gene was still strongly expressed in the entire pancreatic epithelium (Fig. 3Q), similar to *Sox9* (Lioubinski et al., 2003).

Apart from these specific sites of expression recapitulating endogenous *Sox9*/SOX9 expression, in all three expressing lines analyzed, we observed ectopic reporter gene expression in the floor plate (Fig. 3L), in cells surrounding the neural tube (data not shown), within the presomitic mesoderm (data not shown) and in the newly formed somites (Fig. 3C).

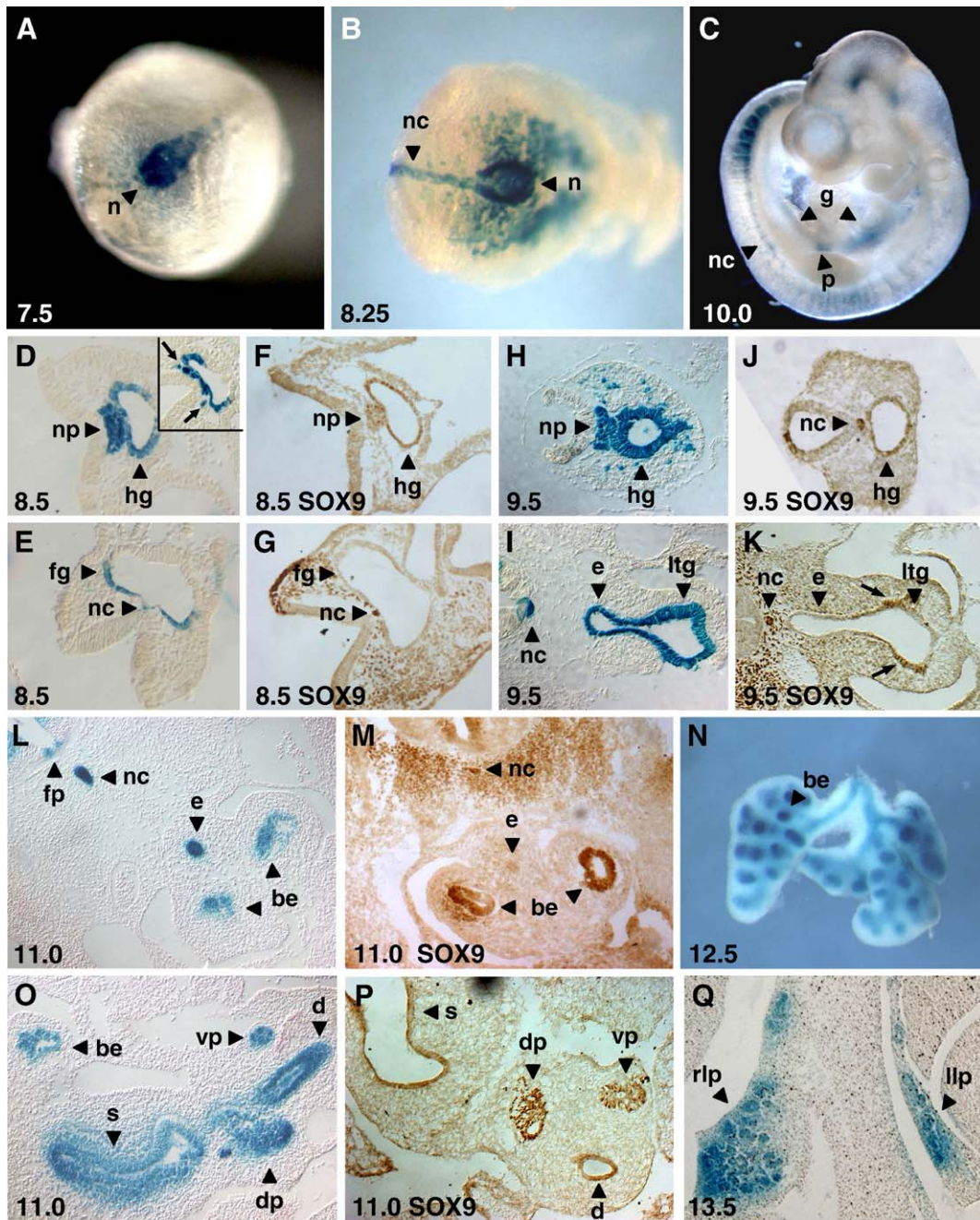


Fig. 3. Enhancer E1 is responsible for transgene expression in the node, notochord, gut, bronchial epithelium and in the pancreas. (A–C) Whole-mount β -galactosidase staining, (N) dissected lung after whole-mount β -galactosidase staining and (D, E, H, I, L, O, Q) paraffin transverse sections of transgenic embryos carrying construct E1*Sox9^P*. (F, G, J, K, M, P) SOX9 immunohistochemistry on cryosections. The arrows in the inset of D indicate β -galactosidase-positive cells in paraxial mesodermal cells immediately lateral to the notochordal plate. Note that, from 8.5 dpc on, *lacZ* expression is present throughout the entire gut (D, E, H, I, L, O), while SOX9 protein expression is hardly detectable in the foregut (G) but is specifically initiated in the laryngo-tracheal groove of the foregut at 9.5 dpc (arrows in K). Abbreviations used: be, bronchial epithelium; d, duodenum; dp, dorsal pancreatic primordium; e, esophagus; fg, foregut; fp, floor plate; g, gut; hg, hindgut; llp, left lobe of pancreas; ltg, laryngo-tracheal groove; n, node; nc, notochord; np, notochordal plate; p, pancreas; rlp, right lobe of pancreas; s, stomach; vp, ventral pancreatic primordium.

Enhancer E3 mediates expression in cranial neural crest cells and in the inner ear

The regulatory potential of DNA fragments E5 (427 bp), E4 (521 bp) and E3 (533 bp) containing the conserved elements E5 (129 bp), E4 (157 bp) and E3 (139 bp), respectively, was first tested in combination (construct E5–E3*Sox9^P*, Fig. 1).

Two expressing transgenic mouse lines could be established which exhibited a similar *lacZ* expression pattern in CNCCs and in the developing inner ear (Fig. 4), where endogenous *Sox9* is strongly expressed (Ng et al., 1997).

Sox9 expression in CNCCs has been reported to initiate as early as 8.0 dpc (Takahashi et al., 2001). In agreement with this early expression, E5–E3-directed *lacZ* expression was first

weakly detectable at the dorsal tips of the hindbrain neural folds at 7.75 dpc (Fig. 4A), becoming up-regulated after 8.0 dpc (Figs. 4B and E). At 8.5 dpc, both SOX9 and β -galactosidase were expressed in pre-migratory CNCCs at the dorsal tips of the hindbrain neural folds, in migrating CNCCs immediately contiguous to these dorsal edges outside the neural tube and in CNCCs within the first and second

pharyngeal arches (Figs. 4B, E and F and data not shown). However, the SOX9 protein exhibited a broader expression pattern than β -galactosidase that included the more anteriorly located cranial NCCs of the forebrain and midbrain and the more posteriorly located trunk NCCs (data not shown). At 9.5 dpc, both SOX9 and β -galactosidase were strongly expressed in migrating CNCCs originating from rhombomeres 2 and 4

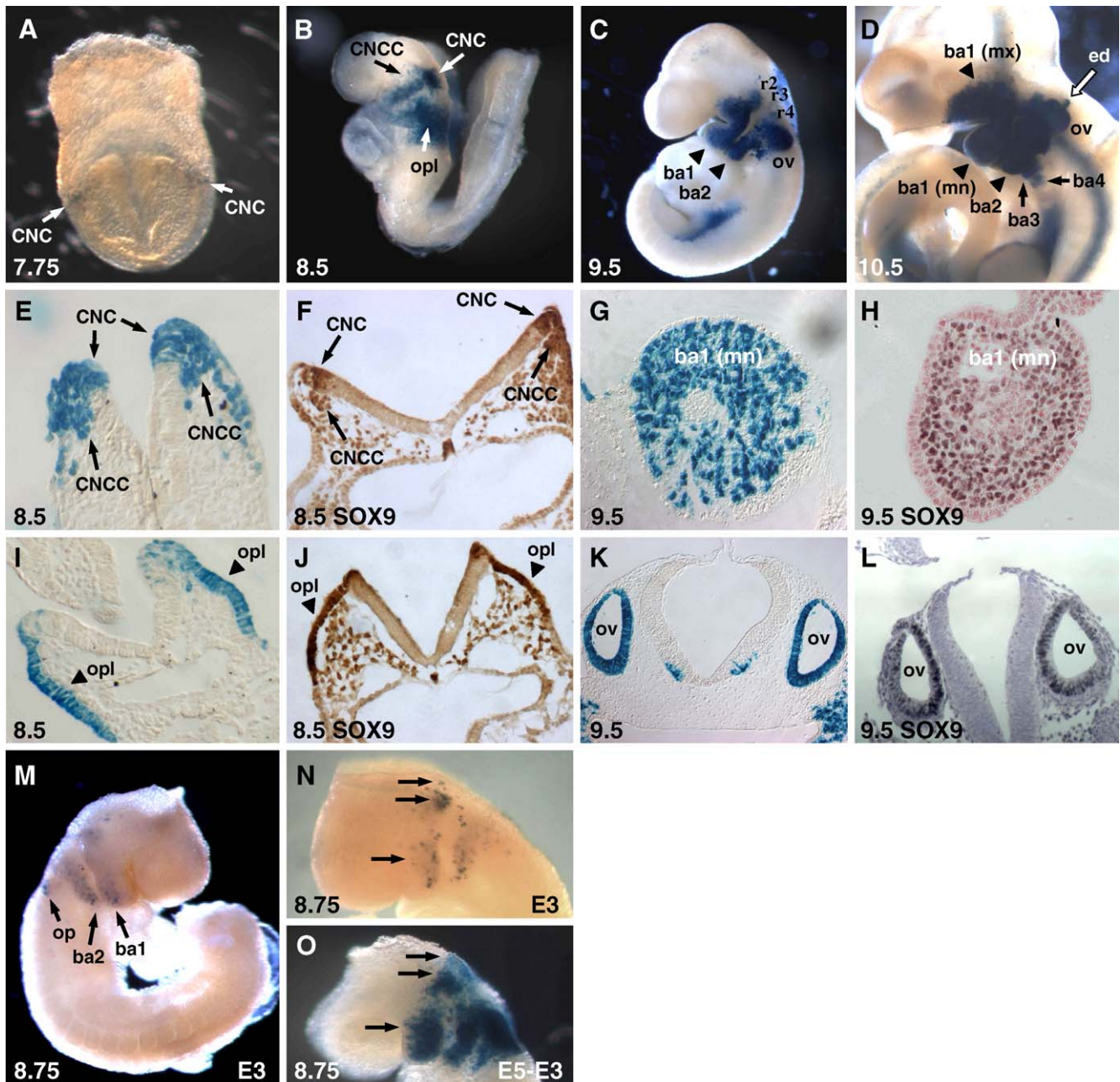


Fig. 4. Constructs E5–E3*Sox9^P* and E3*Sox9^P* direct *lacZ* expression to CNCCs and to the inner ear. (A–D and O) Whole-mount β -galactosidase staining and (E, G, I, K) paraffin transverse sections of transgenic embryos carrying construct E5–E3*Sox9^P*. (F, H, J, L) SOX9 immunohistochemistry on cryosections. (M and N) Whole-mount β -galactosidase staining of transgenic embryos carrying construct E3*Sox9^P*. Note that *lacZ* expression at the dorsal tips of the hindbrain can be seen as early as 7.75 dpc (A). In contrast to construct E5–E3*Sox9^P* (O), construct E3*Sox9^P* (M and N) shows only very weak expression in the CNCCs and in the inner ear. However, construct E3*Sox9^P* expresses the *lacZ* gene in a manner similar to construct E5–E3*Sox9^P* (the three arrows in N and O indicate similar *lacZ* expression at the dorsal tips of the hindbrain, in migrating CNCCs and in CNCCs within the pharyngeal arches, respectively). Abbreviations used: ba1, first pharyngeal arch; ba2, second pharyngeal arch; ba3, third pharyngeal arch; ba4, fourth pharyngeal arch; ba1 (mx), maxillary process of the first pharyngeal arch; ba1 (mn), mandibular process of the first pharyngeal arch; CNC, cranial neural crest; CNCC, cranial neural crest cells; ed, endolymphatic duct; op, otic pit; opl, otic placode; ov, otic vesicle; r2, rhombomere 2; r3, rhombomere 3; r4, rhombomere 4.

and within the first, second and third pharyngeal arches (Figs. 4C, G and H and data not shown). At 10.5 dpc, β -galactosidase continued to be expressed in the CNCCs of the pharyngeal arches (Fig. 4D), as did the SOX9 protein (not shown).

Regarding inner ear development, the transgene was already strongly expressed at 8.0–8.5 dpc in the otic placode (Figs. 4B and I), consistent with the early endogenous expression of SOX9 (Fig. 4J). At 9.0 dpc (data not shown) and 9.5 dpc, high reporter gene expression could be found in the invaginating otic placode (otic pit) and in the entire otic vesicle, respectively (Figs. 4C and K). In contrast, the SOX9 protein showed stronger expression in the medial part of the otic vesicle at 9.5 dpc (Fig. 4L). At 10.5 dpc, the transgene was still expressed in the otic vesicle and was also detectable in the developing endolymphatic duct (Fig. 4D). From 11.0 dpc on, expression of *lacZ* decreased significantly and was not detectable any more at 12.5 dpc (data not shown), similar to endogenous *Sox9* (Ng et al., 1997).

In order to determine the element(s) responsible for the expression in the cranial neural crest and in the inner ear, we examined reporter constructs containing DNA fragments E5, E4 and E3 as single DNA fragments (constructs *E5Sox9^P*, *E4Sox9^P* and *E3Sox9^P*, Fig. 1). Unfortunately, no expressing line could be established for construct *E5Sox9^P*. For construct *E4Sox9^P*, we generated three transgenic lines, two of which expressed the transgene. As both lines only revealed the *Sox9* promoter expression pattern (data not shown), E4 seems to have no independent enhancer activity. Finally, for construct *E3Sox9^P*, we could establish six transgenic mouse lines of which only one expressed the *lacZ* gene. Since transgene expression was very weak in the E3 transgenic mouse line, we could not analyze *lacZ* expression in the same detail as we did for fragments E5–E3. However, we found that enhancer E3 directed reporter gene expression exclusively to CNCCs and to the inner ear from approximately 8.0 to 8.5 dpc on (Figs. 4M and N), similar to DNA fragments E5–E3 (Fig. 4O). This expression pattern was confirmed by an 8.5 dpc embryo transient transgenic for construct *E3Sox9^P* (data not shown). Thus, enhancer E3 seems to recapitulate at least some if not all aspects of the expression pattern mediated by fragments E5–E3.

Enhancer E7 regulates early SOX9 expression in the telencephalon and midbrain

To obtain construct *E7Sox9^P* (Fig. 1), we cloned a 1138 bp DNA fragment containing the conserved 403 bp element E7 in front of the *Sox9* promoter. The regulatory potential of this construct was analyzed in transient transgenic embryos at 9.25 dpc and 11.5 dpc. At 9.25 dpc, six out of nine transgenic embryos showed strong β -galactosidase staining in the telencephalon and in the midbrain (Figs. 5A–C). This expression is consistent with endogenous *Sox9* which starts to be expressed in the forebrain neuroepithelium as early as 8.5 dpc and is expressed in the midbrain from 9.5 dpc on; at 11.5 dpc, *Sox9* expression is confined to the ventricular zone of the forebrain, midbrain, hindbrain and spinal cord (Zhao et al., 1997). However, none of the three out of six transgenic *lacZ*-expressing embryos at 11.5 dpc showed β -galactosidase staining in the ventricular zone, indicating that enhancer E7 can only mediate the early aspect of *Sox9* expression in the telencephalon and in the midbrain.

Like E4, elements E2 and E6 have no independent enhancer activity

We assayed a construct with a 492 bp DNA fragment containing human sequence element E2 (123 bp) (construct *E2Sox9^P*, Fig. 1). Four of the eleven established transgenic mouse lines expressed the *lacZ* gene. However, all four lines only showed the *Sox9* promoter expression pattern (data not shown). We also assayed a construct with a 381 bp DNA fragment containing the conserved 97 bp element E6 (construct *E6Sox9^P*, Fig. 1). The regulatory potential of this construct was only analyzed in transient transgenic embryos at 11.5 dpc. Three out of five transgenic embryos expressed the *lacZ* gene. Again, all three embryos exclusively showed the *Sox9* promoter expression pattern (data not shown). Therefore, like E4 (see above), E2 and E6 do not seem to have an independent enhancer activity; however, it is still possible that E6 has a regulatory potential at earlier or later embryonic stages.

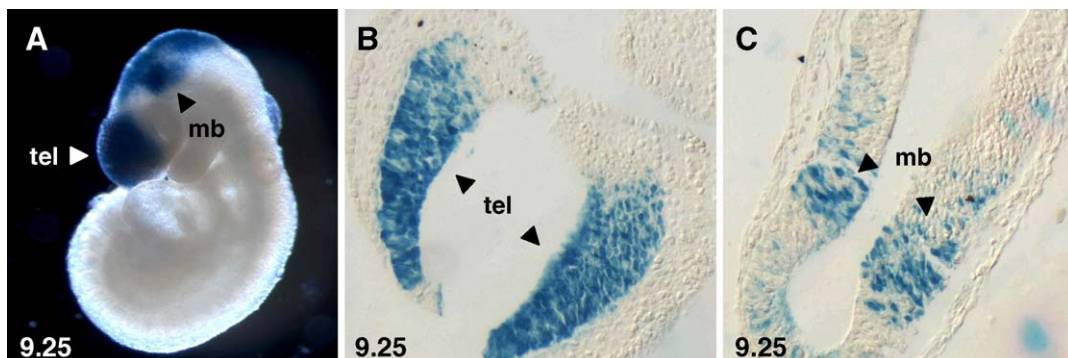


Fig. 5. Enhancer E7 drives transgene expression in the early telencephalon and early midbrain. (A) Whole-mount β -galactosidase staining and (B and C) paraffin transverse sections of a 9.25 dpc transient transgenic embryo carrying construct *E7Sox9^P*. Abbreviations used: mb, midbrain; tel, telencephalon.

Identification of evolutionarily highly conserved transcription factor binding sites within enhancer E1 and E3

By using the online program MatInspector (Quandt et al., 1995; www.genomatix.de) and by visual inspection, the enhancers E3, E1 and E7 and the *Sox9* promoter were searched for potential DNA binding sites for transcription factors that are expressed in the β -galactosidase-positive tissues of the transgenic embryos before or concomitant with *Sox9* expression.

In agreement with the *lacZ* expression pattern of enhancer E1 in the node, notochord, gut, bronchial epithelium and pancreas, we previously identified a highly conserved binding site for the transcription factor Foxa2 (Hnf3 β) (Bagheri-Fam et al., 2001) that is co-expressed with *Sox9* in all these tissues (Monaghan et al., 1993; Ng et al., 1997). While the mouse and *Takifugu* FOXA2 binding sites differ only by one base, the human FOXA2 binding site is identical to the 12 bp Foxa2 consensus sequence (C/G)AAT(G/A)TTTG(C/T)(C/T)(C/T) (Overdier et al., 1994; Fig. 6A).

In agreement with the *lacZ* expression pattern of enhancer E3, we could identify several binding sites conserved between human, mouse and *Takifugu* for transcription factors that play a central role in the development of the cranial neural crest and of the inner ear. In close proximity to each other, we found binding sites for Tcf/Lef1 (mediators of the canonical Wnt signaling pathway), Ets (mediators of the FGF signaling pathway), Gli (mediators of the Shh signaling pathway)/Krox20, Dlx, Otx and two binding regions for the trimeric protein complex Meis/Pbx/Hox1–5. Furthermore, we identified an AP2 binding site that is only conserved between human and mouse. Remarkably, all these human and mouse transcription factor binding sites differ by only one base from or are even identical to the described consensus binding sites (Fig. 6B).

Within enhancer E7 (forebrain/midbrain) and the *Sox9* promoter (ventral hindbrain/spinal cord), we also found some conserved transcription factor binding sites (data not shown), but none of these transcription factors could be specifically correlated with the observed *lacZ* expression pattern.

Enhancer E1 has evolved prior to the separation of the Deuterostomia and Protostomia lineages 700 million years ago

The *Drosophila* gene *Sox100B*, the likely precursor of the vertebrate group E *Sox* genes *Sox8*, *Sox9* and *Sox10*, has been shown to share some aspects of the tissue-specific expression pattern with the group E *Sox* genes, in particular in gut (Loh and Russell, 2000). As the evolutionarily highly conserved element E1 mediates strong reporter gene expression in the murine gut, we speculated that some sequences of this enhancer could be conserved in *Drosophila*. By visual inspection, we analyzed genomic sequences around the *proto SoxE* gene of *Drosophila* and of the urochordate *Ciona intestinalis*, the lineages of which separated approximately 700 and 550 million years ago

from the vertebrate lineage, respectively. We indeed found some sequence similarity to element E1 in both species, with remarkable conservation of the Foxa2 binding site (Fig. 6A). While in *Ciona* this sequence resides approximately 55 kb 5' to the *SoxE* gene, in *Drosophila*, this sequence is located only 3 kb 3' to *Sox100B*. The relatively large distance between element E1 and the *Ciona SoxE* gene may be incorrect since the genomic *Ciona* sequences are still in draft form. To better visualize the conservation of element E1 between these evolutionarily distant species, we subdivided this element into the conserved boxes A–K (Fig. 6A). While the Foxa2 binding site and boxes A, D, F and K are conserved from human to *Drosophila*, the boxes C and I are only conserved up to *Ciona*, and boxes E, G, H and J are vertebrate-specific. Furthermore, the invertebrates *Ciona* and *Drosophila* share a common sequence element that differs from the vertebrate-specific box E, and, finally, box B is specific for *Takifugu*, *Ciona* and *Drosophila*.

Beta-catenin is essential for E5–E3-directed β -galactosidase protein expression in the first pharyngeal arch

It has been shown in *Xenopus* that *Sox9* expression in CNCCs is dependent on the canonical Wnt signaling pathway (Luo et al., 2003). This pathway leads to the stabilization of cytosolic beta-catenin, which then associates with TCF transcription factors to regulate expression of Wnt-target genes. As the cranial neural crest/inner ear-specific enhancer E3 contains a highly conserved Tcf/Lef1-binding site (Fig. 6B), we speculated that this enhancer could be regulated by the canonical Wnt pathway. To assess if the E3 enhancer-mediated *lacZ* expression in the cranial neural crest is dependent on beta-catenin in vivo, we crossed mice carrying construct E5–E3*Sox9*^P with mice where the beta-catenin gene has been conditionally inactivated in CNCCs by the Cre recombinase under the control of the *Wnt1* promoter. Inactivation of beta-catenin in these mice (*Wnt1-Cre;beta-cat*^{flloxdel/fllox}) was reported to start at 8.5 dpc, with complete absence of beta-catenin protein in the pharyngeal arches at 9.25 dpc (Brault et al., 2001). We analyzed *lacZ* expression of 8.5–9.5 dpc *Wnt1-Cre;beta-cat*^{flloxdel/fllox};E5–E3*Sox9*^P embryos and compared this expression with mice carrying only construct E5–E3*Sox9*^P. In embryos of the former genotype, we observed a dramatic decrease or even a complete loss of β -galactosidase-positive cells migrating to and within the first pharyngeal arch from 8.75 to 9.0 dpc on, while the total cell number within the first pharyngeal arches seemed to be unaltered compared to the E5–E3*Sox9*^P embryos (compare Figs. 7A and B with C and D). In contrast, the second and third pharyngeal arches showed only a mild reduction in β -galactosidase staining intensity, and the total number of β -galactosidase-positive cells seemed to be unaltered. As expected, β -galactosidase staining was normal in control tissues such as the otic vesicle, where beta-catenin has not been inactivated (Figs. 7A and C). It seems that Wnt/beta-catenin signaling is essential for E5–E3-directed *lacZ*

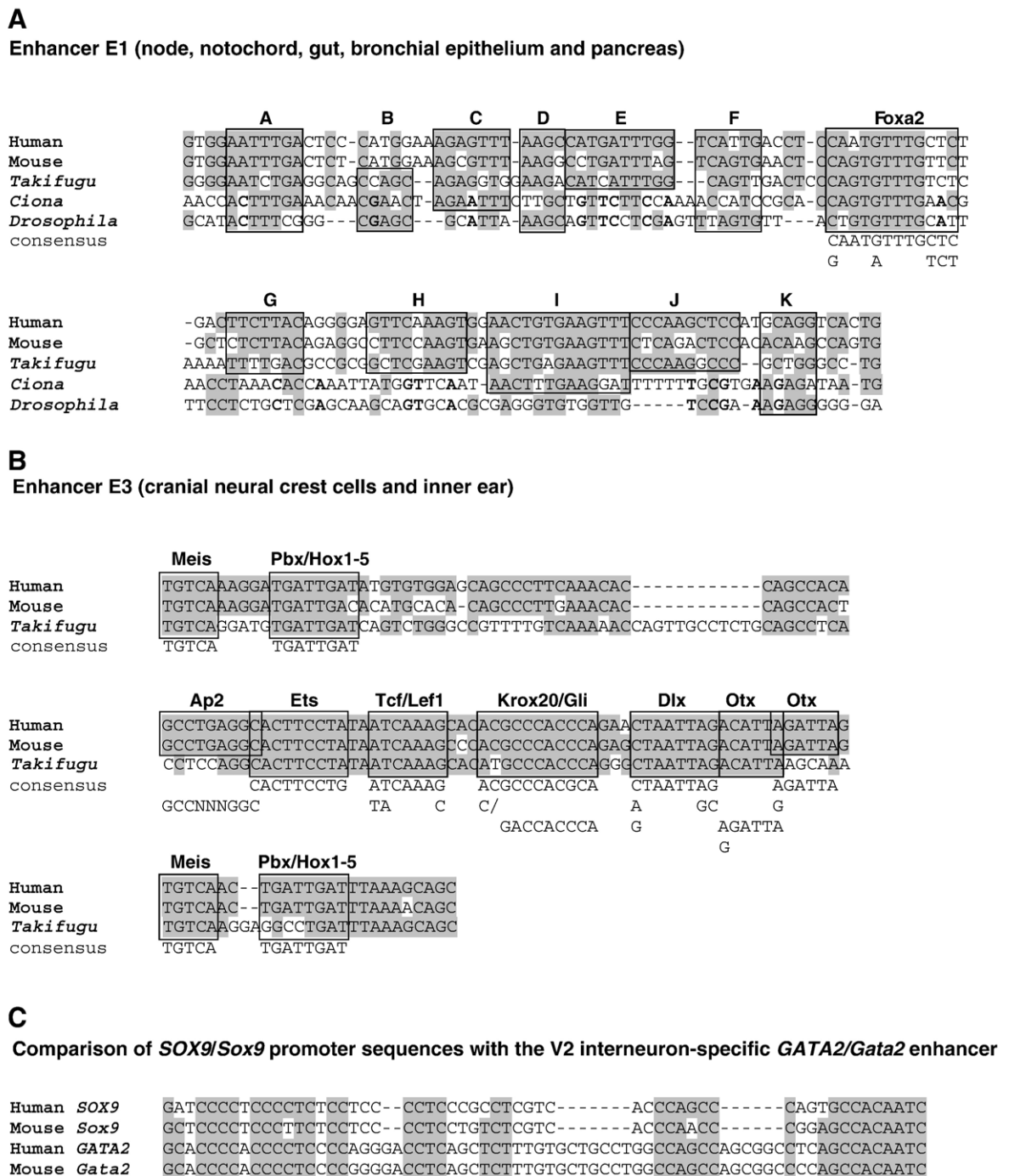


Fig. 6. Conservation of *Sox9* E1 and E3 enhancer and promoter sequences indicates multiple transcription factor binding sites. (A) Sequence comparison of *Sox9* enhancer E1 between human, mouse, *Takifugu*, *Ciona* and *Drosophila* that controls expression in the node, notochord, gut, bronchial epithelium and pancreas. A potential FOXA2 binding site could be identified that is highly conserved even in *Drosophila*. To better visualize the conservation of element E1 between these evolutionarily distant species, we have subdivided this element into conserved boxes A–K that are discussed in the text. Nucleotides in bold are exclusively conserved between the invertebrates *Ciona* and *Drosophila*. (B) Sequence comparison of *Sox9* enhancer E3 between human, mouse and *Takifugu* that mediates expression in cranial NCCs and in the inner ear. This enhancer contains two potential binding sites for the trimer Meis/Pbx/Hox1–5 and for Otx and single binding sites for Ap2 (only conserved between human and mouse), Ets, Tcf/Lef-1, Krox20/Gli and Dlx. Note that all human transcription factor binding sites differ only by one base or are even identical to the consensus sequence. (C) Comparison of human and mouse *SOX9/Sox9* promoter sequences with the V2 interneuron-specific *GATA2/Gata2* enhancer (Zhou et al., 2000) reveals several highly conserved sequence blocks. In panels A, B and C, conserved nucleotides are highlighted in gray, gaps are shown by dashes and consensus sequences for the predicted transcription factor binding sites are indicated below the sequences. Except for Ap2 (Mitchell et al., 1991) and Meis/Pbx/Hox1–5 (Shen et al., 1997), binding sites were taken from the online program MatInspector (www.genomatix.de).

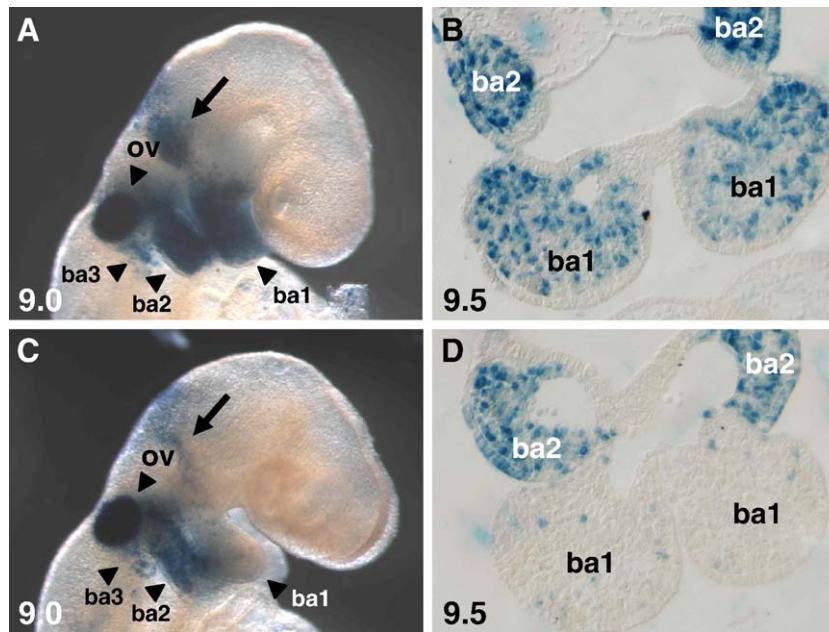


Fig. 7. E5–E3-directed β -galactosidase protein expression in the first but not in the second and third pharyngeal arch is dependent on beta-catenin. (A) Whole-mount β -galactosidase staining and (B) paraffin transverse section of a 9.0 dpc and a 9.5 dpc transgenic embryo carrying construct E5–E3*Sox9^P*. (C) Whole-mount β -galactosidase staining and (D) paraffin transverse section of a 9.0 dpc and a 9.5 dpc E5–E3*Sox9^P* transgenic beta-catenin-deficient embryo (genotype: *Wnt1-Cre; beta-cat^{floxdel/flox}; E5–E3Sox9^P*). The arrow in A and C points to CNCCs that migrate into the first pharyngeal arch. In beta-catenin-deficient embryos (C and D), the number of β -galactosidase-positive cells within the first pharyngeal arch is drastically reduced compared to wildtype (A and B) embryos, while, in the second and third pharyngeal arches, β -galactosidase staining intensity is only mildly reduced. Abbreviations used: ba1, first pharyngeal arch; ba2, second pharyngeal arch; ba3, third pharyngeal arch; ov, otic vesicle.

expression within the first pharyngeal arches, but not within the second and third pharyngeal arches.

Discussion

Genomic organization of the *SOX9* cis-regulatory domain

This study shows that *SOX9* is regulated by a complex set of widely spaced tissue-specific enhancers, located in the immediate vicinity of the transcription start site, up to 251 kb 5' and up to 95 kb 3' to *SOX9* (Fig. 8). The combined *lacZ* expression patterns mediated by the *Sox9* promoter (neural tube and hindbrain), enhancer E1 (node, notochord, gut, bronchial epithelium and pancreas), fragments E5–E3/enhancer E3 (CNCCs and inner ear) and enhancer E7 (forebrain and midbrain) recapitulate many aspects of the complex endogenous *Sox9* expression pattern. All four *cis*-regulatory sequences initiated *lacZ* expression at stages very similar to endogenous *Sox9*. However, while fragments E5–E3 and enhancer E1 were able to recapitulate *Sox9* expression also at later stages, both neural enhancers could reproduce only the early aspect of *SOX9* expression (see also below). Elements E2, E4 and E6 did not show independent enhancer activity. These elements could be general enhancers that up-regulate an already specified expression or they could function as silencers.

The presence of multiple *cis*-regulatory elements between 350 kb 5' and 250 kb 3' to *SOX9* has previously been inferred from expression analyses in mice transgenic for human *SOX9-lacZ* yeast artificial chromosomes (YACs) containing variable amounts of DNA sequences 5' to *SOX9* (Wunderle et al.,

1998). In accordance with the findings of Wunderle et al. (1998) that *cis*-regulatory elements for *SOX9* expression in the neuroectoderm are located between 75 kb 5' and 250 kb 3' to *SOX9*, both neural-specific enhancers (E7 located 95 kb 3' to *SOX9* and the *Sox9* promoter) reside within this region. Furthermore, apart from the E3 inner ear enhancer identified in this study that lies 251 kb upstream of *SOX9*, Wunderle et al. (1998) found a weak inner ear enhancer located between 75 kb 5' and 250 kb 3' to *SOX9* and an enhancer potentially up-regulating this expression between 150 kb and 75 kb 5' to *SOX9*. Thus, *SOX9* expression in the inner ear seems to be controlled by at least three different widely spaced enhancers. In contrast to the YAC transgenes that showed robust *lacZ* expression in the chondrocytes of the vertebral column, pharyngeal arches and limbs, we could so far not identify enhancers for *SOX9* expression in chondrocytes. However, elements E2, E4 and possibly E5 which showed no independent enhancer activity could be involved in the up-regulation of chondrocyte expression. In agreement with this, E2 resides within an interval (150 kb to 75 kb 5' to *SOX9*) where up-regulating enhancers for pharyngeal arch chondrocyte expression were assumed to exist, and elements E5 and E4 are located within an interval (350 kb to 200 kb 5' to *SOX9*) where up-regulating enhancers for *SOX9* expression in chondrocytes of the limbs and vertebral column were suggested to exist by Wunderle et al. (1998). The expression pattern of enhancer E1 and the E3-mediated expression in CNCCs could not be compared with the expression pattern of the YAC transgenes as the corresponding tissues have not been analyzed by Wunderle et al. (1998).

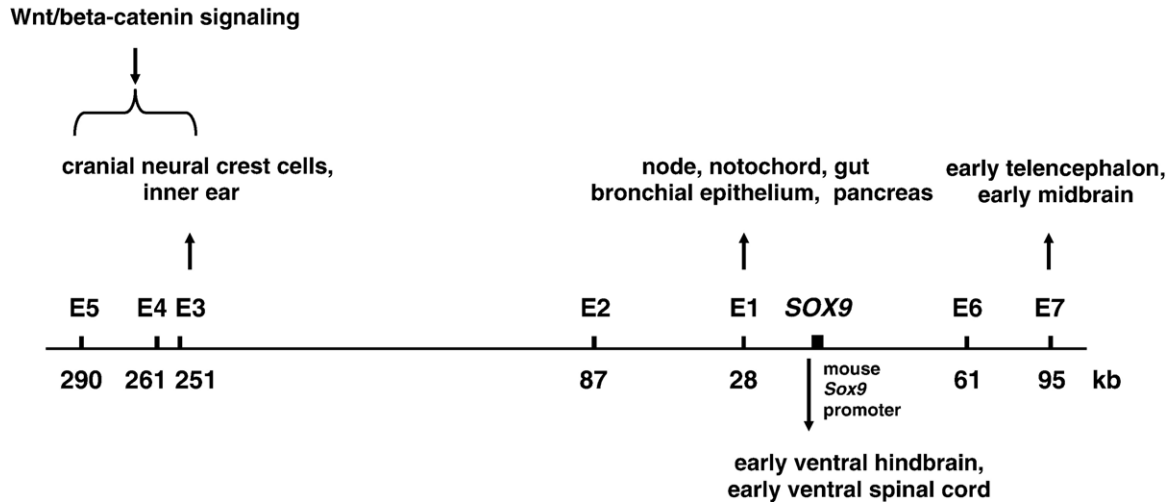


Fig. 8. Location of functional enhancers within the *SOX9* cis-regulatory domain identified in this study. Three (E3, E1 and E7) of the seven conserved sequence elements from the human *SOX9* locus (E1–E7) analyzed in this study and the 529 bp mouse *Sox9* promoter mediated *lacZ* expression in a tissue-specific manner. Arrows point to the tissue specificity of enhancers E3, E1 and E7 and of the *Sox9* promoter. The expression patterns of these cis-regulatory elements are non-overlapping. Expression of *Sox9* in the node, notochord, in the gut and gut-derived tissues (bronchial epithelium and pancreas) is controlled by enhancer E1. For the control of *Sox9* expression in neural tissues, two cis-regulatory elements have been identified: while E7 directs the early aspect of expression in the telencephalon and midbrain, the 529 bp *Sox9* promoter mediates the early aspect of expression in the hindbrain and spinal cord. Finally, enhancer E3 is responsible for *Sox9* expression in cranial neural crest cells and inner ear.

The minimal mouse *Sox9* promoter used in this study has been shown to be at least partly responsible for the testis-specific expression of *Sox9* in vitro (Kanai and Koopman, 1999). However, we found that the *Sox9* promoter can mediate *lacZ* expression only in the ventral neural tube/hindbrain in transgenic mice, suggesting that the *Sox9* promoter itself has no significant testis-specific activity in vivo.

Control of *SOX9* expression in neural tissues

We identified two distinct regulators for *Sox9* expression in neural tissues along the anteroposterior (AP) axis. While enhancer E7 directs reporter gene expression in the telencephalon and midbrain, the 529 bp *Sox9* promoter mediates expression in the hindbrain and spinal cord. Since both neural enhancers could reproduce only the early aspect of *SOX9* expression, these enhancers are most likely involved in the onset of *SOX9* expression in neural tissues, while the maintenance of *SOX9* expression is regulated by so far unidentified enhancers.

Sox9 has been shown to be a major molecular component in the ventral neural tube for the switch from neurogenesis to gliogenesis in mice (Stolt et al., 2003). Mice in which *Sox9* has been inactivated in neural stem cells show an early dramatic reduction in progenitors of oligodendrocytes and astrocytes and a concomitant transient increase in motoneurons and V2 interneurons (Stolt et al., 2003). The 529 bp minimal mouse *Sox9* promoter region contains enhancer sequences that, similar to endogenous *SOX9* (Stolt et al., 2003 and this study), initiate *lacZ* expression specifically in the ventral neural tube at 9.5 dpc. Since little is known as to what causes the neural stem cells to switch from neurogenesis to gliogenesis, this enhancer provides an opportunity to identify the essential transcription factors that initiate this process. By comparing the *SOX9/Sox9*

promoter sequence with the 190 bp V2 interneuron-specific *GATA2/Gata2* enhancer (Zhou et al., 2000) which mediates *lacZ* expression exclusively in the spinal cord and in the hindbrain, similar to the *Sox9* promoter, we could identify several sequence blocks conserved between human and mouse (Fig. 6C). The high conservation of these sequences suggests that they might be important for *SOX9* expression in the ventral neural tube.

SOX9 expression in the node, notochord, gut and gut-derived tissues is regulated by a common enhancer

Enhancer E1, located 28 kb 5' to *SOX9*, directs *lacZ* expression to the node, notochord, gut, bronchial epithelium and pancreas, with an expression pattern closely resembling that of endogenous *Sox9* (Ng et al., 1997). Thus, enhancer E1 seems to contain most or all of the cis-regulatory sequences needed to control *Sox9* expression in these tissues. Previously, we had identified a potential *Foxa2* binding site within enhancer E1 that is well conserved between human, mouse and *Takifugu* (Bagheri-Fam et al., 2001) and that differs by only one base or is identical to the 12 bp *Foxa2* consensus sequence (C/G)AAT(G/A)TTTG(C/T)(C/T)(C/T) (Overdier et al., 1994, Fig. 6A). In the present study, we found that this site is even conserved up to the invertebrates *Ciona* and *Drosophila*. *Foxa2* is essential for node and notochord formation in mice and is a good candidate to regulate *Sox9* expression via enhancer E1 since it is co-expressed with *Sox9* in all tissues in which *lacZ* expression is mediated by E1 (Monaghan et al., 1993; Ang and Rossant, 1994; Ng et al., 1997). In the fetal intestinal epithelium, *SOX9* expression has been shown to be dependent on Wnt/beta-catenin signals that are mediated by the transcription factor TCF4 (Blache et al., 2004). We could not identify a conserved Tcf/Lef1 binding

site within enhancer E1. However, Sinner et al. (2004) could show that beta-catenin physically interacts with Sox17 (essential for endoderm formation) and potentiates its transcriptional activation on target genes, particularly on *Foxa2*. Therefore, the Wnt/beta-catenin signaling pathway could act indirectly through *Foxa2* in the activation of *Sox9* in the gut.

The notochordal cells are enveloped within a sheath of extracellular matrix that contains different collagens (e.g. *Col2a1*), proteoglycans and glycoproteins, typically found in cartilage. *Sox9* is essential for chondrogenesis, is expressed in chondrocytes and in the notochord and directly regulates *Col2a1* through its 48 bp intronic enhancer (Ng et al., 1997; Lefebvre et al., 1998; Akiyama et al., 2002). In contrast to the *Col2a1* enhancer that is active both in chondrocytes and in the notochord (Sakai et al., 2001), we found that enhancer E1 is only active in the notochord. This indicates that *Sox9* is controlled by a different set of transcription factors in its function as a regulator of extracellular matrix genes in chondrocytes on the one hand and in the notochord on the other hand. *Sox9* expression in the notochord, in the gut and in gut-derived organs, however, seems to be under the control of similar regulatory mechanisms. As both the *Drosophila Sox100B* gene, the likely precursor of *Sox8*, *Sox9* and *Sox10*, and the *Drosophila forkhead* gene (*fkh*), the likely precursor of *Foxa1*, *Foxa2* and *Foxa3*, are expressed in the gut (Loh and Russell, 2000; Weigel et al., 1989) and as element E1 with a highly conserved Foxa binding site is already present in *Drosophila* (Fig. 6A), we speculate that element E1 originally evolved as a gut-specific enhancer under *fkh* control already some 700 million years ago. Later during evolution, with the appearance of the notochord in Urochordates such as *Ciona*, which separated from the vertebrate lineage approximately 550 million years ago and which also contains an E1 element (Fig. 6A), this element acquired the function to regulate *SoxE* expression also in the notochord. In accordance with an evolutionary early E1-driven expression of *Sox9* in the notochord, the zebrafish *sox9b* gene has also been shown to be expressed in the notochord (Li et al., 2002), and the human enhancer E1 is able to drive reporter gene expression in the zebrafish notochord (I. Hess, U. Straehle and G. Scherer, unpublished), demonstrating functional conservation over at least 400 million years of evolutionary separation.

To our knowledge, the evolutionary conservation of a *cis*-regulatory element between vertebrates and invertebrates has not been reported so far. Recently, by using a whole-genome comparison between humans and the pufferfish, *T. rubripes*, Woolfe et al. (2005) could identify nearly 1400 highly conserved non-coding sequences that associate with developmental regulators. However, no significant matches to any of these conserved sequences were found in the invertebrates *Ciona*, *Drosophila* or *C. elegans*. Our data indicate that, by a more in-depth analysis, e.g. by visual inspection, some non-coding, functionally relevant sequences may indeed be found to be conserved between vertebrates and invertebrates.

Regulation of SOX9 expression in cranial neural crest cells and in the otic placode/vesicle

In *Xenopus*, *Sox9* is essential for the specification of CNCCs (Spokony et al., 2002; Lee et al., 2004a) and of the otic placode (Saint-Germain et al., 2004). Similarly, in homozygous *Sox9* knock-out mice, the population of CNCCs is drastically reduced, and the development of the otic vesicle is disrupted from the earliest stages on (Akiyama et al., 2004; Barrionuevo et al., unpublished). These data suggest that *Sox9* has an evolutionarily conserved role in the early development of both tissues. We found that the highly conserved enhancer E3 (80% identity over 98 bp between human and *Takifugu*, Fig. 6B) mediates *lacZ* expression specifically in CNCCs and in the otic placode/vesicle, suggesting that also the regulatory mechanisms activating *Sox9* expression in these tissues are conserved across species. Indeed, within enhancer E3, we could identify several conserved binding sites for transcription factors that have important and early functions in cranial neural crest and inner ear development (Meis/Pbx/Hox1–5, Ap2, Tcf/Lef1, Ets, Gli/Krox20, Dlx and Otx, Fig. 6B).

In *Xenopus*, *Sox9* expression in CNCCs is regulated by the Wnt/beta-catenin- and the Fgf signaling pathways (Lee et al., 2004a) which are both essential and sufficient for neural crest formation in *Xenopus* (Villanueva et al., 2002). Similarly, in chicken, Wnt signals can function as inducers of neural crest fate (Garcia-Castro et al., 2002), and, in the mouse, Wnt signals are involved in the regulation of neural crest fate decisions (Lee et al., 2004b). *Sox9* expression in CNCCs in *Xenopus* is also dependent on Ap2 α which itself is essential for neural crest formation in *Xenopus* (Luo et al., 2003; Saint-Germain et al., 2004). In the mouse, Ap2 α is expressed before *Sox9* during neural crest development (Mitchell et al., 1991; Ng et al., 1997), and, similar to *Sox9* knock-out mice (Akiyama et al., 2004; Barrionuevo et al., unpublished), Ap2 $^{-/-}$ mosaic knock-out mice show hypoplasia of the pharyngeal arches (Nottoli et al., 1998). In accordance with the activation of *Sox9* by Ap2 α and by both the Wnt/beta-catenin and the Fgf signaling pathway, enhancer E3 contains, within a region of only 26 bp, conserved binding sites for Ap2 α (only conserved between human and mouse), Tcf/Lef1 (transcription factor that activates Wnt/beta-catenin dependent target genes by interaction with beta-catenin) and Ets (transcription factor involved in the activation of Fgf-dependent target genes) (Fig. 6B). It is thus possible that these factors regulate *Sox9* expression across species and that this regulation is direct. Indeed, by analyzing E5–E3-directed *lacZ* expression in mouse embryos with targeted inactivation of beta-catenin in the dorsal neural tube, we could show that beta-catenin is essential for *lacZ* expression in CNCCs migrating to and within the first pharyngeal arch. Future studies will have to show if this regulation by beta-catenin is direct or indirect. *LacZ* expression in the second and third pharyngeal arches, however, was only mildly affected in beta-catenin-deficient mice.

Our data indicate that distinct regulators control *Sox9* expression in NCCs along the AP axis. First, enhancer E3 mediates *lacZ* expression exclusively in cranial NCCs migrat-

ing into the pharyngeal arches, while endogenous *Sox9* is also expressed in the more anterior located forebrain and midbrain cranial NCCs as well as in the more posterior located trunk NCCs (Ng et al., 1997; Zhao et al., 1997). Second, as described above, regulation of enhancer fragments E5–E3 in the first pharyngeal arch differs from its regulation in the second and third pharyngeal arches. A possible explanation for this complex regulation is indicated by the high accumulation of conserved potential binding sites for homeodomain transcription factors within enhancer E3. We found two binding regions for the ternary protein complex Meis/Pbx/Hox, in which both Hox binding sites are specific for the most anterior expressed Hox groups 1–5 (Shen et al., 1997), two potential binding sites for the Otx homeobox proteins and one potential binding site for Dlx. These homeodomain transcription factors display distinct expression domains in CNCCs along the AP and dorsoventral (DV) axis of the developing vertebrate head, thereby providing positional identity to CNCCs (see Santagati and Rijli, 2003, for review). While Hox genes are expressed in CNCCs of the second pharyngeal arch and at more caudal levels, *Otx2* is expressed in the Hox-negative CNCCs that are rostral to the second pharyngeal arch (Kimura et al., 1997). Along the dorsoventral axis of the pharyngeal arches, molecular identity is provided by the nested expression of *Dlx* genes (Panganiban and Rubenstein, 2002). We hypothesize that a specific combination of these homeodomain transcription factors binding to enhancer E3 restricts reporter gene expression to CNCCs migrating into the pharyngeal arches. Moreover, since Hox proteins are not expressed in CNCCs of the first pharyngeal arch where *lacZ* expression is mainly dependent on beta-catenin, but in CNCCs of the second and third pharyngeal arch where *lacZ* expression is independent of beta-catenin, we speculate that *lacZ* expression in the second and third pharyngeal arches could be largely controlled by Hox proteins. The importance of Meis/Pbx/Hox1–5 sites for the expression in rhombomere R4/second pharyngeal arch neural crest cells has been well documented for the *Hoxb2*-R4 enhancer that mediates the cross-regulation of *Hoxb2* by *Hoxb1* in vivo (Jacobs et al., 1999).

We also found a potential binding site for Gli or Krox20. The Gli protein family members are transcriptional mediators of Sonic hedgehog (Shh) signaling. Shh is required for the proliferation and survival of CNCCs (Ahlgren and Bronner-Fraser, 1999; Jeong et al., 2004). *Shh* null mice show loss of the pharyngeal arches and frontonasal mass (Chiang et al., 1996), and inhibition of Shh signaling in the chick results in reduced pharyngeal arch structures and frontonasal tissue (Ahlgren and Bronner-Fraser, 1999), very similar to the *Sox9* knock-out mice (Akiyama et al., 2004; Barrionuevo et al., unpublished). *Krox20* is specifically expressed in the uneven rhombomeres R3 and R5 that do not generate CNCCs and is essential for their identity (Voiculescu et al., 2001). A potential function for Krox20 could be to repress *Sox9* expression in the future rhombomeres R3 and R5, so that these rhombomeres cannot generate neural crest cells.

Apart from their essential functions in the formation of neural crest cells, Wnt and Fgf signaling pathways have been

implicated in the specification of the otic placode in mouse, chicken, *Xenopus* and zebrafish embryos (Ladher et al., 2000; Maroon et al., 2002; Liu et al., 2003; Wright and Mansour, 2003). In *Xenopus*, *Sox9* expression in the otic placode is regulated by both the Wnt/beta-catenin and Fgf signaling pathway and is maintained by Dlx3 (Saint-Germain et al., 2004). Similarly, *Sox9* expression in the otic placode in zebrafish is dependent (*sox9a*) on or is up-regulated (*sox9b*) by Fgf3/Fgf8 and *sox9a/sox9b* and *dlx3b/dlx4b* cross-regulate their expression (Liu et al., 2003). In mice and in humans, Dlx proteins have also been implicated in inner ear development. *Dlx5*^{-/-} mice show inner ear defects (Acampora et al., 1999), and mutations in human *DLX3* result in hearing problems (Price et al., 1998). The presence of highly conserved binding sites for Tcf/Lef1, Ets and Dlx within enhancer E3 suggests that the regulation of *Sox9* by these factors is direct and evolutionarily conserved up to human and mouse.

In conclusion, our study provides another example of the validity of the comparative genomics approach for the identification of conserved and functional regulatory elements located at considerable distances from the gene they control. That the *long-range SOX9* enhancers we have characterized only account for a subset of the complex spatiotemporal expression pattern of this developmental gene is not too surprising. Future work will need to identify still elusive enhancers such as those responsible for *Sox9* expression during chondrogenesis and testogenesis. Nevertheless, the *cis*-regulatory elements presented and discussed here will already provide an entry point to the characterization of some of the transcription factors controlling the expression of *Sox9*, allowing for a better definition of the regulatory network that specifies cells/organs such as cranial neural crest cells, the otic placode and glial cells in humans or mice.

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