Development 129, 421-432 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV3503

The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*

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Accepted 24 October 2001

SUMMARY

The SOX family of transcription factors has been implicated in cell fate specification during embryogenesis. One member of this family, Sox9, has been shown to regulate both chondrogenesis and sex determination in the mouse embryo. Heterozygous mutations in Sox9 result in Campomelic Dysplasia (CD), a lethal human disorder characterized by autosomal XY sex reversal, severe skeletal malformations and several craniofacial defects. Sox9 is also expressed in neural crest progenitors but very little is known about the function of Sox9 in the neural crest. We have cloned the *Xenopus* homolog of the Sox9 gene. It is expressed maternally and accumulates shortly after gastrulation at the lateral edges of the neural plate, in the neural crest-forming region. As development proceeds, Sox9 expression persists in migrating cranial crest cells as they populate the pharyngeal arches. Depletion of Sox9 protein in developing embryos, using morpholino antisense oligos, causes a dramatic loss of neural crest progenitors and an expansion of the neural plate. Later during embryogenesis, morpholino-treated embryos have a specific loss or reduction of neural crest-derived skeletal elements, mimicking one aspect of the craniofacial defects observed in CD patients. We propose that Sox9 is an essential component of the regulatory pathway that leads to cranial neural crest formation.

Key words: Sox9, Neural crest, Craniofacial skeleton, Morpholino antisense, *Xenopus*

INTRODUCTION

The neural crest is a unique embryonic structure composed of a migratory population of multipotent cells arising at the lateral edges of the neural plate. During neurulation, the neural crest is at the dorsal most region of the neural tube, and upon neural tube closure, neural crest cells delaminate in a rostrocaudal wave and migrate throughout the embryo along specific routes. As they reach their targets, neural crest cells will contribute to various cell types, including neurons and glia of the peripheral nervous system, craniofacial skeletal elements, smooth muscle cells and melanocytes (LeDouarin and Kalcheim, 1999).

The molecular signals that control neural crest specification are not well understood. Signals emanating from the non-neural ectoderm, the paraxial mesoderm, or both, are believed to be involved in the specification of the neural crest (Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Bonstein et al., 1998; Marchant et al., 1998). There is evidence that molecules belonging to the bone morphogenetic protein (BMP) family, which are expressed by the non-neural ectoderm and the dorsal neural tube, initiate the formation of the neural crest (Liem et al., 1995; Liem et al., 1997). The neural inducers noggin and chordin act by neutralizing a BMP signal in the ectoderm (Zimmerman et al., 1996; Piccolo et al., 1996), thereby allowing neural crest to form in regions where BMPs and BMP antagonists reach an appropriate balance (Morgan and Sargent, 1997; Marchant et al., 1998; Nguyen et al., 1998). Fibroblast growth factor (FGF) signaling in conjunction with BMP inhibition can also induce neural crest fates (Mayor et al., 1997; LaBonne and Bronner Fraser, 1998). Recent studies in the mouse (Ikeya et al., 1997; Dunn et al., 2000), frog (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Bang et al., 1999; Deardorff et al., 2001) and zebrafish (Dorsky et al., 1998) have implicated the Wnt family of growth factors in neural crest formation. Ectopic expression of some Wnt family members enhances production of neural crest progenitors, and inhibition of Wnt signaling blocks neural crest formation. It is not clear whether Wnt signaling is required in the early phases of neural crest specification or later for the maintenance and/or proliferation of crest precursors.

In *Xenopus*, several transcription factors have been found to be expressed in the developing neural crest, including Slug (Mayor et al., 1995), Snail (Essex et al., 1993), Twist (Hopwood et al., 1989), Pax3 (Bang et al., 1997), Msx1 (Suzuki et al., 1997), Zic3 (Nakata et al., 1997), Zic5 (Nakata et al., 2000) and Foxd3 (Dirksen and Jamrich, 1995). While some of these have also been implicated at different levels in the regulation of

neural crest formation or migration, or both (Nakata et al., 1997; Nakata et al., 2000; LaBonne and Bronner-Fraser, 2000; Pohl and Knochel, 2001; Sasai et al., 2001), the way in which these different factors interact in the context of the developing neural crest remains to be determined.

Sox proteins comprise a large class of transcription factors related to SRY, the testis-determining factor. They are characterized by the presence of an HMG-box, a sequence specific DNA-binding domain (Pevney and Lovell-Badge, 1997; Wegner, 1999). Expression of these proteins in defined cell types during embryogenesis appears to govern cell fate determination (Kamachi et al., 2000). One member of this family, mouse Sox9, has been shown to regulate cartilage formation by binding and activating the chondrocyte specific enhancer of collagen type II (Col2a1) (Bell et al., 1997; Bi et al., 1999). Consistent with this role, Col2a1 and Sox9 are co-expressed in all chondrogenic precursors (Zhao et al., 1997; Ng et al., 1997). Heterozygous mutations of Sox9 result in Campomelic Dysplasia (CD), a lethal human disorder characterized by autosomal XY sex reversal and severe skeletal malformations (Houston et al., 1983; Wagner et al., 1994), indicating a fundamental role for Sox9 in sex determination and skeletal development (Lefebvre and de Crombrugghe, 1997). During mouse embryogenesis, Sox9 is also expressed in neural crest progenitors (Zhao et al., 1997; Ng et al., 1997), but nothing is known about the early function of Sox9 in the developing neural crest. Interestingly, individuals with CD and Sox9 heterozygous mutant mice present defects in craniofacial skeletal elements of neural crest origin, the jaw and palate (Houston et al., 1983; Wagner et al., 1994; Bi et al., 2001), suggesting that Sox9 may play an important role in the development of a subset of neural crest derivatives.

We report the cloning and functional analysis of *Xenopus* Sox9 during neural crest development. Sox9 is expressed maternally and accumulates shortly after gastrulation at the lateral edges of the neural plate, in the neural crest-forming region. In this tissue, Sox9 is spatially and temporally co-expressed with Slug, which is known to be the earliest gene activated in response to neural crest-inducing signals. Depletion of Sox9 in developing embryos, using morpholino antisense oligos (Heasman et al., 2000), causes a dramatic loss of early neural crest progenitors and a specific loss of cranial skeletal elements, indicating a requirement for Sox9 function during development of cranial neural crest derivatives.

MATERIALS AND METHODS

Constructs

Xenopus Sox9 was amplified by PCR using degenerate primers (forward, GA[T/C]CCNTT[T/C]ATGAA[G/A]ATGAC; and reverse, CANGG[C/T]TG[C/T]TCCCA[A/G]TG[C/T]TG) based on the published sequences of chicken (Healy et al., 1999) and human (Wagner et al., 1994) Sox9. Stage 17 cDNA was used as template. The 1398 bp PCR product was ligated into pGEMTeasy (Promega) and sequenced. This construct is referred as pGEMTSox9. The remaining 12 nucleotides including the ATG plus 320 bp of the 5′ UTR and the 21 nucleotides including the stop codon plus 1397 base pairs of the 3′ UTR were both subsequently amplified by RACE (Clontech) from stage 17 cDNA. Each PCR product was ligated into pGEMTeasy and sequenced. Based on the sequence information of the RACE products, primers were designed to generate two Sox9 constructs containing either the entire ORF (Sox9) or the entire ORF plus 50 bp of 5′UTR, the target sequence for Sox9-AS (Sox9+5′UTR). Each product was generated independently using stage 17 and stage 35 cDNA as template, respectively and cloned into the *Cla*I and *Xho*I sites of pCS2+ (Turner and Weintraub, 1994). Both expression plasmids were then fully sequenced and the corresponding protein monitored using an in vitro transcription/translation coupled rabbit reticulocyte lysate system (TNT, Promega, Fig. 3H). The sequence of *Xenopus* Sox9 has been deposited into GenBank (Accession Number AY035397). *Xenopus* Sox2 ORF was amplified by PCR from stage 17 cDNA using primers based on the published sequence (Mizuseki et al., 1998a) and ligated into pCS2+. Plasmid DNA (pCS2+) driving GFP (Zernicka-Goetz et al., 1996) was used as a control in the rescue experiments.

Morpholino antisense oligonucleotides

Sox9 morpholino antisense oligonucleotide (Sox9-AS), a 25-mer oligo, was designed against the 5′UTR of *Xenopus* Sox9, immediately adjacent to the initiation start site with the following base composition 5′-GCAAAAATGGGGAAAGGTAAGAAAG-3′ (Gene Tools, LLC). Doses of 1-10 ng antisense oligo were injected into single blastomeres at the two- or eight-cell stage. A control antisense oligo (Co-AS), composed of a random sequence (Gene Tools, LLC), was injected at the same concentrations.

In vitro transcription/translation and western blot analysis

The in vitro transcription/translation coupled rabbit reticulocyte lysate system (SP6-TNT, Promega) was used according to the manufacturer recommendations (Promega) in the presence of [^{35}S]methionine and resolved on a NuPAGE BIS-Tris gel (Invitrogen). For western blot analysis the in vitro transcription/translation was performed in the presence of unlabeled methionine. The gel was blotted onto nitrocellulose, incubated in the presence of a Sox9 polyclonal antibody (Bridgewater et al., 1998) at a 1:1000 dilution, washed and incubated with anti-rabbit Ig coupled to horseradish peroxidase (Amersham Pharmacia Biotech, 1:5000 dilution). The product of the reaction was revealed using the SuperSignal West Femto Maximum Sensitivity Substrate from Pierce and detected by exposure onto a BioMax film (Kodak). The specificity of the morpholino antisense oligo was determined by adding 1 μg of Co-AS or Sox9-AS to the vitro transcription/translation reaction. To analyze the activity of the morpholino antisense oligo in vivo, embryos were injected at the two-cell stage with 1 ng of Sox9 mRNA (Sox9+5′utr) in the presence of 10 ng of Sox9-AS or Co-AS. Embryos were then collected at stage 17, homogenized, resolved on a NuPAGE BIS-Tris gel, blotted onto nitrocellulose and processed for western analysis as described above. Blots were stripped according to the manufacturer recommendations (Pierce) and probed with anti-α-tubulin antibody (Sigma, 1:500 dilution).

Immunofluorescence

Embryos at the two-cell stage were injected in the animal pole with 1 ng of Sox9 mRNA and animal explants dissected at the blastula stage. At equivalent stage 12, animal explants were fixed in methanol at –80°C, embedded in Paraplast+, sectioned and processed for immunostaining. Briefly, sections were blocked in PBS plus 5% fetal calf serum and incubated successively in the presence of Sox9 polyclonal antibody (1:30 dilution), anti-rabbit Ig coupled to FITC (Jackson, 1:100 dilution) and DAPI (5 mg/ml). Sections were mounted in glycerol and observed under an epifluorescence microscope.

Northern hybridization

RNA extraction, electrophoresis, blotting and hybridization was performed as described (Taira et al., 1992; Spokony and Saint-Jeannet, 2000).

Lineage tracing and in situ hybridization

Embryos were co-injected with β-galactosidase mRNA (β-gal, 1 ng).

By stage 17 embryos were fixed in MEMFA (Harland, 1991) and successively processed for X-Gal (Promega) or Red-Gal (Research Organics) staining and in situ hybridization. Antisense FITC- or DIG-labeled probes (Genius kit, Roche) were synthesized using template cDNA encoding Nrp1 (Knecht et al., 1995), Sox2 (Mizuseki et al., 1998a), Slug (Mayor et al., 1995), Snail (Essex et al., 1993), Pax3 (Bang et al., 1997), Foxd3 (XFKH6) (Dirksen and Jamrich, 1995), Twist (Hopwood et al., 1989), xFKBP (Spokony and Saint-Jeannet, 2000), Trp2 (E. M.-F. and J.-P. S.-J. unpublished), Msx1 and Dlx3 (Feledy et al., 1999). Whole-mount in situ hybridization was performed as previously described (Harland, 1991). Sox9 in situ hybridization was performed using pGEMTSox9 construct as template. For histology, stained embryos were embedded into Paraplast+, 12 μm sections cut on a rotary microtome and briefly counterstained with Eosin.

Cartilage staining

Alcian Blue staining of stage 45 embryos was performed essentially as described (Berry et al., 1998). Briefly, embryos were fixed overnight in 10% formaldehyde, rinsed in tap water, skinned and eviscerated. Embryos were then dehydrated and stained in Alcian blue for 12 hours. After several rinses in 95% ethanol, embryos were rehydrated and macerated in 2% potassium hydroxide. Specimens were then transferred successively in 20%, 40%, 60% and 80% glycerol in 2% potassium hydroxide. The ethmoidal plate was dissected out and specimens mounted flat under a coverslip in 80% glycerol.

RESULTS

Cloning and developmental expression of a Sox gene related to murine Sox9

A PCR product corresponding to a partial sequence of *Xenopus* Sox9 was amplified from stage 17 cDNA using degenerate primers (Fig. 1A). The complete coding sequence and part of the 5′ and 3′UTRs were subsequently amplified by RACE. *Xenopus* Sox9 possesses an open reading frame encoding 477 amino acids (Fig. 1A). At the amino acid level, *Xenopus* Sox9 shares 80% identity with human Sox9 (Wagner et al., 1994), 79% identity with mouse Sox9 (Wright et al., 1995), 83% identity with chicken Sox9 (Healy et al., 1999), 85% identity with alligator Sox9 (Western et al., 1999) and 75% identity with zebrafish Sox9a (Chiang et al., 2001). When compared with *Xenopus* Sox17-α (Hudson et al., 1997), *Xenopus* Sox2 (Mizuseki et al., 1998a) and *Xenopus* SoxD (Mizuseki et al., 1998b), the overall amino acid identities drop to 32%, 31% and 25%, respectively. Based on the similarity of their HMG domains, Sox genes have been classified into seven sub groups (Wegner, 1999). Sox9 proteins fall into subgroup E that also includes Sox8 and Sox10 (Bell et al., 2000; Pusch et al., 1998).

To determine the temporal expression of Sox9, we performed northern hybridization of embryonic RNA at different stages of development (Fig. 1B). Sox9 is expressed maternally at low levels and increased expression is first detected at the neurula stage (around stage 15) and persists at least through the tadpole stage (stage 41) in the form of a major transcript around 3.0 kb.

Sox9 is expressed in the developing neural crest

To determine the spatial expression pattern of Sox9, we performed whole-mount in situ hybridization on a variety of embryonic stages. The first localized expression of Sox9 can be observed at stage 10.5-11, when transcripts are found in a superficial ring around the blastopore with signal decreasing towards the dorsal side (Fig. 2A). At stage 12 the staining decreases around the blastopore and accumulates at the lateral edges of the neural plate (Fig. 2B), the position of the prospective neural crest. This dynamic expression pattern around the blastopore and in the neural crest-forming region is reminiscent of the expression of another transcription factor, maker of the neural crest in *Xenopus*, Snail (Essex et al., 1993; Linker et al., 2000).

Sox9 expression is dramatically increased in the neural crest-forming regions around stage 14, in both the medial (red arrowhead) and the lateral (red arrow) neural crest (Fig. 2C,D). This expression pattern is identical to that described for Snail (Essex et al., 1993) and Slug (Mayor et al., 1995). Slug expression at stage 14 is shown for comparison (Fig. 2E,F). As early as stage 13/14, Sox9 is also detected in the sensory layer of the ectoderm (not shown), as a bilateral patch of cells immediately adjacent to the lateral crest and corresponding to the prospective otic placode (yellow arrows in Fig. 2C,D). Sox9 expression in the otic placode/vesicle will persist throughout embryogenesis. Tissue sections of stage 14 embryos highlight the medial (arrowheads) and lateral (arrows) neural crest expression of Sox9 (Fig. 2G). As the neural tube closes, Sox9 remains strongly expressed in both components of the neural crest (Fig. 2H). At the early tailbud stage (stage 23), neural crest cells start to migrate in the cranial region and strong expression of Sox9 is detected in individual streams of migrating crest cells (Fig. 2I) and at the dorsal midline in the trunk neural crest (Fig. 2J). Additional domains of expression include the genital ridges (Fig. 2I,J, green arrows), the developing eye (Fig. 2I,K, blue arrows), the nasal placode (Fig. 2K, purple arrows) and the prospective pineal gland (Fig. 2K, orange arrow). Around stage 25, Sox9 expression is downregulated in the trunk neural crest but persists in the cranial crest cells as they populate the pharyngeal arches, the otic placode, the developing eye, the genital ridges and also the notochord (Fig. 2L-O). By stage 31, Sox9 is still strongly expressed in the pharyngeal arches (Fig. 2P).

Early on, Sox9 expression in the neural crest-forming regions is spatially and temporally identical to that of Slug and Snail, known to be the earliest genes activated in response to neural crest-inducing signals in *Xenopus*. Furthermore, Sox9 expression is maintained as neural crest cells migrate in the cranial region and invade the pharyngeal arches. These observations suggest that Sox9 may play an important role in the formation and development of cranial neural crest cells.

Sox9-AS-injected embryos lack neural folds

To investigate Sox9 function during early neural crest development, we performed loss-of-function studies using morpholino antisense oligonucleotides (Summerton and Weller, 1997; Heasman et al., 2000). A Sox9 morpholino antisense oligo (Sox9-AS) was designed against the 5′UTR of *Xenopus* Sox9, directly upstream of the translation start site. Embryos at the two-cell stage were injected in one blastomere with 10 ng of Sox9-AS or control oligo (Co-AS, composed of a random sequence) together with mRNA encoding the lineage tracer β-galactosidase. At the early neurula stage, Co-AS-injected embryos appeared to develop normally (Fig. 3D-F), while Sox9-AS-injected embryos failed to form a distinct

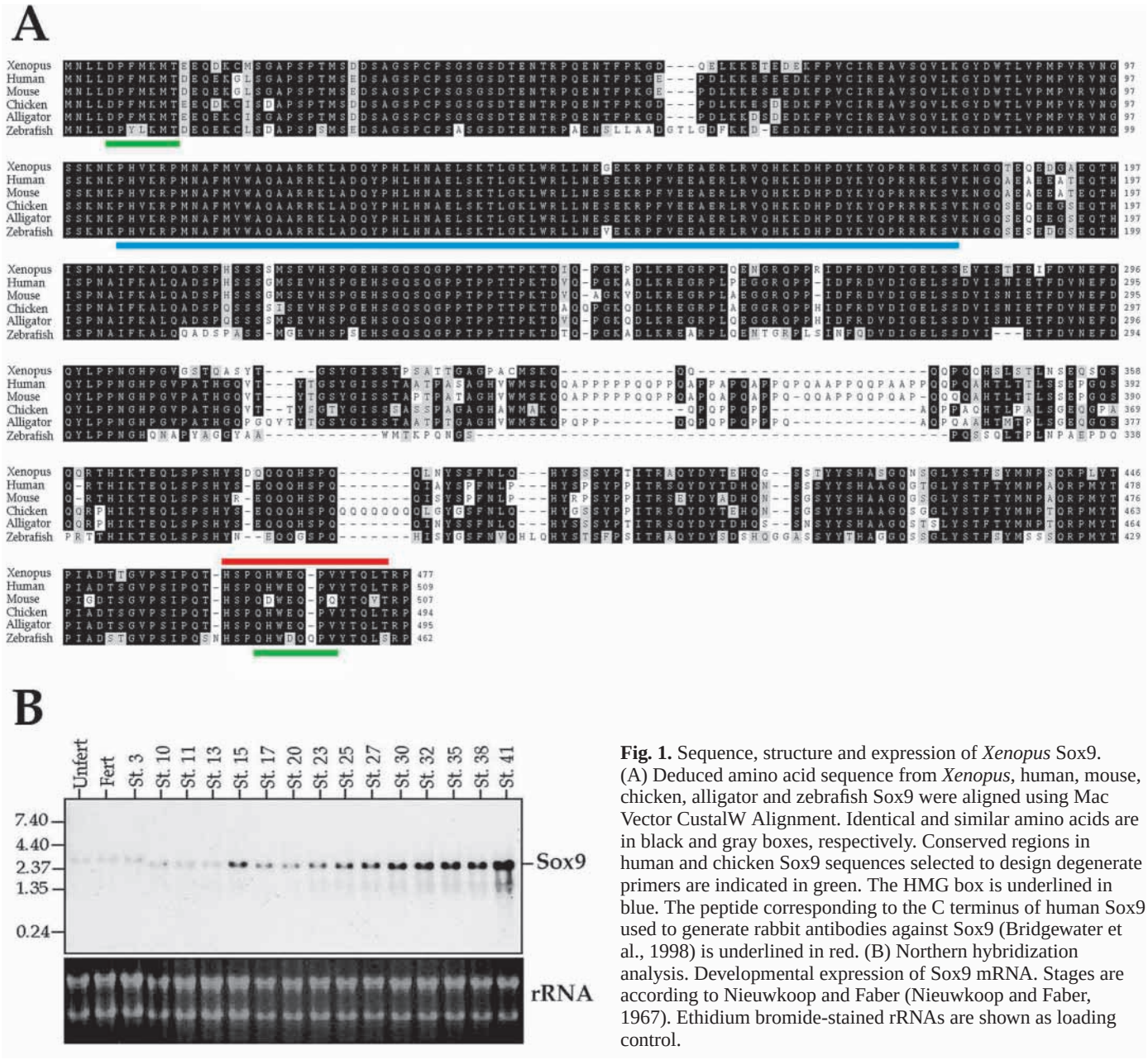

Fig. 1. Sequence, structure and expression of *Xenopus* Sox9. (A) Deduced amino acid sequence from *Xenopus*, human, mouse, chicken, alligator and zebrafish Sox9 were aligned using Mac Vector CustalW Alignment. Identical and similar amino acids are in black and gray boxes, respectively. Conserved regions in human and chicken Sox9 sequences selected to design degenerate primers are indicated in green. The HMG box is underlined in blue. The peptide corresponding to the C terminus of human Sox9 used to generate rabbit antibodies against Sox9 (Bridgewater et al., 1998) is underlined in red. (B) Northern hybridization analysis. Developmental expression of Sox9 mRNA. Stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Ethidium bromide-stained rRNAs are shown as loading control.

neural fold on the injected side (Fig. 3A-C). Transverse sections through Sox9-AS-injected embryos confirmed the absence of a well-defined neural fold (Fig. 3B,C) when compared with Co-AS-injected embryos (Fig. 3E,F). Importantly, surrounding tissues (somites and notochord) appeared unaffected in Sox9-AS-injected embryos (Fig. 3C). The neural crest-forming region (neural fold) appears to be primarily affected in these embryos (Fig. 3G), consistent with Sox9 expression in this tissue.

Sox9-AS specifically blocks translation of Sox9 mRNA

To verify the specificity of Sox9-AS, we generated two constructs that encompass the entire open reading frame of Sox9 (Sox9) with one of the constructs also containing 50 nucleotides of 5′UTR (Sox9+5′UTR). The 50 bp of 5′UTR includes the 28 bp target sequence for Sox9-AS. Using a Sox9-specific antibody (Fig. 1A) (Bridgewater et al., 1998), we performed western blot analysis of in vitro transcription/translation products directed by each of the Sox9 cDNAs or by another Sox family member, *Xenopus* Sox2. The anti-Sox9 antibody recognizes only the major product of Sox9 reactions (Fig. 4A). Addition of Sox9-AS oligo to the reaction caused a dramatic reduction in the amount of Sox9 protein produced by Sox9+5′UTR construct, but failed to alter the level of Sox9 protein generated from the Sox9 construct that lacked the 5′UTR (Fig. 4B). Addition of a nonspecific control antisense oligo (Co-AS) had no effect on the translation of Sox9 mRNA derived from either construct (Fig. 4B). This result establishes that Sox9-AS specifically blocks the synthesis of Sox9 protein.

We were not able to detect endogenous Sox9 protein using this antibody. Nevertheless, Sox9 protein could be visualized by indirect immunofluorescence in the nucleus of the cells

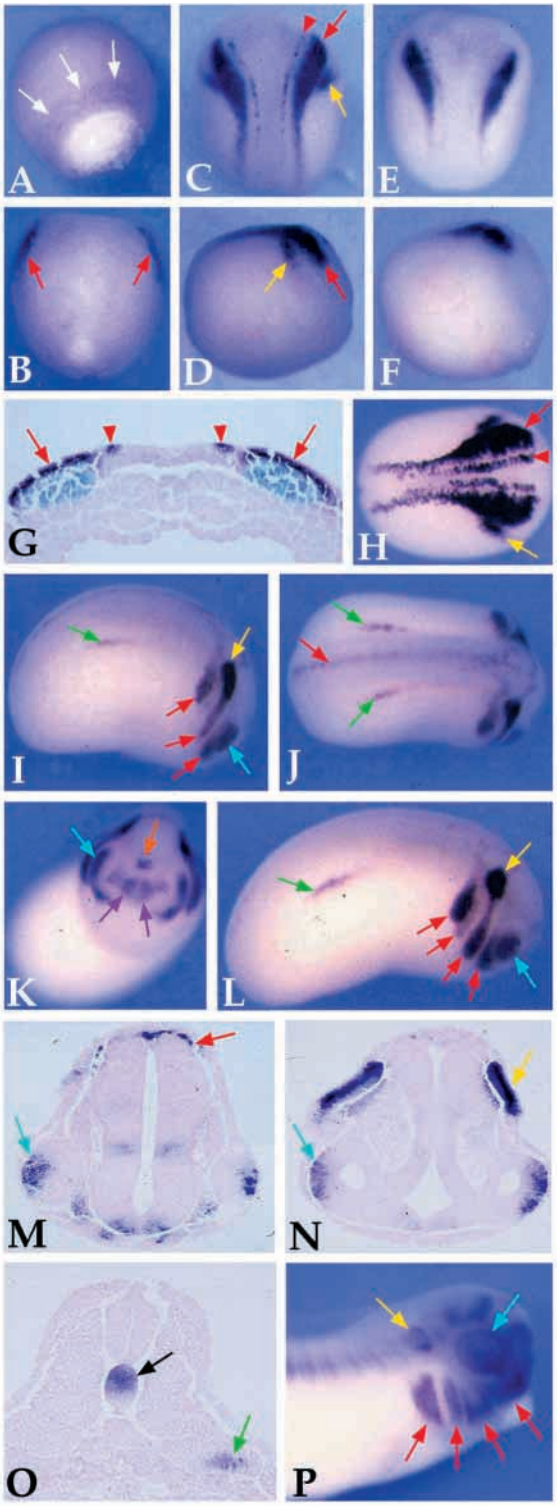

Fig. 2. Developmental expression of Sox9 by whole-mount in situ hybridization. (A) Sox9 expression at the gastrula stage is found in a superficial ring around the blastopore (white arrows); lateral view. (B) Sox9 RNA is detected at the lateral edges of the neural plate (arrows) in a stage 12 embryo. Dorsal view, anterior towards the top. (C,D) Sox9 expression at stage 14 is in the neural crest (red arrow and arrowhead) and in the presumptive otic placode (yellow arrow). (E,F) Slug expression is shown for comparison. (C,E) are dorsal views, anterior towards the top; (D,F) are lateral views, anterior towards the right. (G) Transverse section of a stage 14 embryo. Sox9 expression is restricted to the medial (red arrowheads) and lateral (red arrows) neural crest. (H) Stage 16 embryo, dorsal view, anterior towards right. As the neural tube closes, Sox9 remains strongly expressed in both components of the neural crest (red arrow/arrowhead) and in the otic placode (yellow arrow) (I,J) Stage 23 embryos, (I) lateral view and (J) dorsal view. Genital ridge (green arrows), optic vesicle (blue arrows), otic placode (yellow arrow). (K,L) Stage 25 embryo. (K) In this frontal view, Sox9 is found in the nasal pits (purple arrows) and the prospective pineal gland (orange arrow). (L) Lateral view, anterior towards right. Sox9 is detected in the four streams of cranial neural crest (red arrows), otic placode (yellow arrows), genital ridge (green arrows) and the developing eye (blue arrows in K,L). (M-O) Transverse sections of a stage 25 embryo. (M) In this section, at the level of the forebrain, Sox9 is detected in the developing eye (blue arrow), dorsally in migrating neural crest cells (red arrow) and in discrete domains in the brain. (N) More posteriorly, Sox9 is strongly expressed in the developing otic placode (yellow arrow). (O) In the trunk region Sox9 is restricted to the genital ridge (green arrow) and the notochord (black arrow). (P) At stage 32, Sox9 expression is in the pharyngeal arches (red arrows), the otic vesicle (yellow arrow) and in restricted regions of the brain.

derived from animal explants injected with Sox9 mRNA (Fig. 4C) indicating that upon overexpression, Sox9 protein can enter and accumulate in the nucleus consistent with its predicted function as a transcription factor. Moreover, by western blot analysis of extracts derived from embryo co-injected with Sox9+5′UTR mRNA and 10 ng of Sox9-AS we demonstrated that Sox9-AS prevents the accumulation of Sox9 protein in vivo (Fig. 4D), further demonstrating the specificity of this antisense oligo in the developing embryo.

Sox9-AS prevents Slug expression in a dose-dependent manner

The transcription factor Slug (Mayor et al., 1995) is known to be the earliest gene activated in response to neural crest-inducing signals. Therefore, we analyzed the expression pattern of Slug in Sox9-AS-injected embryos. Upon injection of 10 ng of Sox9-AS into one blastomere at the two-cell stage, 94% of the embryos (n=158) showed a reduction (Fig. 5B) or a complete loss (Fig. 5C) of Slug expression on the injected side at stage 17. The percentage of affected embryos dropped to 61%, 35% and 18% for lower doses of Sox9-AS, 5 ng (n=112), 2 ng (n=130) and 1 ng (n=111) respectively (Fig. 5D), indicating that Sox9-AS blocks Slug expression in a dose-dependent manner. During normal development Slug is first detected in the neural crest forming region at the late gastrula stage (stage 12) (Mayor et al., 1995). Therefore, we asked whether Sox9-AS would prevent the early onset of Slug expression. Injection of 10 ng of Sox9-AS completely blocked Slug expression at stage 12 in 98% of the embryos analyzed (n=82, Fig. 5E). This result suggests that Sox9 function is required at the neural plate border at the initial stages of neural crest formation.

Sox9-AS blocks formation of neural crest progenitors and expands neural tissues

To further characterize the phenotype of Sox9-depleted embryos we examined the expression of five additional neural

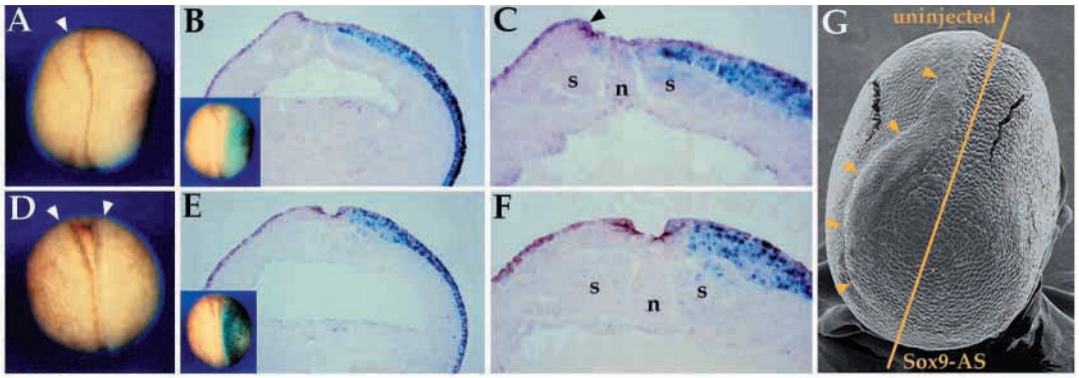

Fig. 3. Sox9-AS prevents neural fold formation. Embryos were injected in one blastomere at the two-cell stage with 10 ng of Sox9-AS (A-C) or 10 ng of Co-AS (D-F) and analyzed at stage 17. RNA encoding the lineage tracer β-galactosidase was co-injected to identify the injected side (blue), shown on the right in all panels. (B,C) Transverse section of the embryo presented in A shows that the neural fold is missing on the injected side (blue staining) when compared with the uninjected side (arrowhead). (E,F) Transverse sections of the embryo presented in D shows that the neural fold is unaffected on the injected side (blue staining). n, notochord; s, somite. Insets in B,E depict whole embryo β-galactosidase staining (blue) of the specimens presented in A,D, respectively. (G) Scanning electron micrograph of a Sox9-AS-injected embryo at the early neurula stage. Note the absence of the neural fold in the injected side when compared with the uninjected control side (arrowheads).

crest markers, Twist (Hopwood et al., 1989), Snail (Essex et al., 1993), Pax3 (Bang et al., 1997), Msx1 (Su et al., 1991a) and Foxd3 (Dirksen and Jamrich, 1995) and two neural plate markers, Nrp1 (Knecht et al., 1995) and Sox2 (Mizuseki et al., 1998a). All five neural crest markers were either severally reduced or eliminated upon injection of 10 ng of Sox9-AS (Fig. 6A-B; Table 1). In these experiments, lateral and medial neural crest appeared to be affected to a similar extent (Fig. 6A, Snail expression). At stage 23, Twist is expressed in the migrating cranial neural crest and Sox9-AS-injected embryos failed to show any Twist-positive cells on the injected side (Fig. 6A, Twist). In contrast to these observations, the pan-neural markers (Nrp1 and Sox2) were significantly expanded in Sox9-AS-injected embryos (Fig. 6A-B; Table 1). Injection of a control oligo (Co-AS) at the same concentration had no effect on expression of any of these markers. Axial mesoderm was unaffected in Sox9-AS-injected embryos (Table 1) as determined by the expression of the notochord marker xFKBP (Spokony and Saint-Jeannet, 2000). These results demonstrate that Sox9 depletion leads to a specific loss of neural crest progenitors and to an expansion of neural plate, thus indicating a fundamental requirement for Sox9 function at the neural plate border for the generation of neural crest fate.

The phenotype of Sox9-depleted embryos can be rescued by restoring Sox9 expression

We next asked whether the phenotype of Sox9-depleted embryos could be rescued by restoring Sox9 expression. In these experiments, one animal blastomere was injected on the dorsal side at the eight-cell stage in order to restrict the manipulation of Sox9 expression to a limited region of the embryo (Fig. 7A). Overexpression of Sox9 by injection of a plasmid lacking the Sox9-AS recognition site (Sox9) had no effect on Slug expression (Fig. 7B,C), or could led to a mild expansion of the Slug expression domain in 10% of injected embryos (not shown). Co-injection of Sox9 (100 pg) and Sox9-AS (5 ng) in one blastomere at the eight-cell stage restored bilateral Slug expression in more than 50% of the embryos when compared with embryos that received injection of Sox9-AS alone (Fig. 7B,C). Co-injection of a control plasmid DNA driving GFP (100 pg) with Sox9-AS failed to rescue bilateral Slug expression (Fig. 7B,C). These results indicate that Sox9

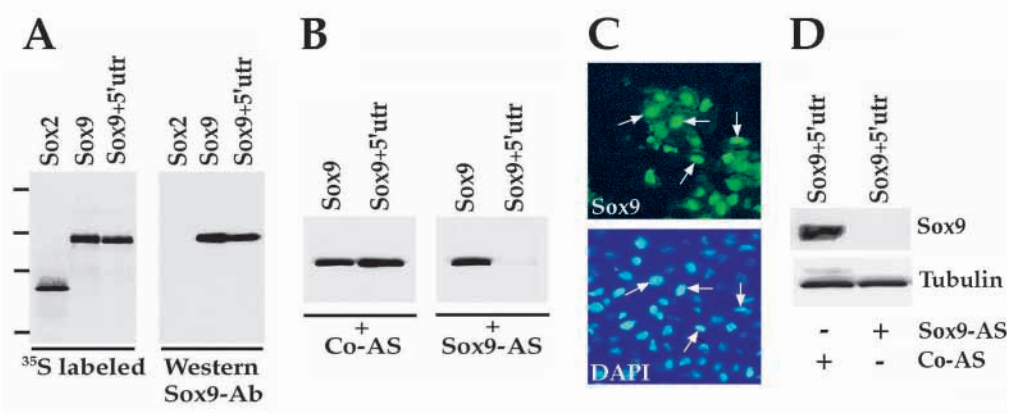

Fig. 4. Analysis of Sox9-AS specificity. (A) A Sox9 specific antibody recognizes only the major product of Sox9 in vitro translation reactions. (B) Western blot of Sox9 in vitro translated products. Sox9-AS blocked translation of Sox9 mRNA containing the 5′UTR target sequence (Sox9+5′UTR), but did not affect translation of Sox9 mRNA lacking the target sequence (Sox9). A nonspecific control oligo (Co-AS) had no effect on the translation of Sox9 mRNA derived from either construct. (C) Sox9 protein can be visualized by indirect immunofluorescence (green) in the nucleus (arrows) of the cells derived from animal explants injected with 1 ng of Sox9 mRNA at the two-cell stage. DAPI (blue) staining is shown for nucleus identification. (D) In vivo depletion of Sox9 protein. Detection of Sox9 protein in extracts from stage 17 embryos co-injected at the two-cell stage with 1 ng Sox9 mRNA (Sox9+5′UTR) and 10 ng of Co-AS or Sox9-AS. Tubulin is presented as a loading control.

Table 1. Altered expression of neural crest and neural plate markers in embryos injected at the two-cell stage with 10 ng of Sox9-AS or Co-AS

			Percentage of embryos		
Injection	Probe	*n*	Unaffected	Expanded	Reduced
Sox9-AS	Slug	158	6	0	94
Co-AS	Slug	91	93	3	4
Sox9-AS	Snail	70	10	0	90
Co-AS	Snail	42	98	0	2
Sox9-AS	Foxd3	36	11	0	89
Co-AS	Foxd3	22	100	0	0
Sox9-AS	Pax3	53	6	6	88
Co-AS	Pax3	37	92	0	8
Sox9-AS	Twist	32	6	0	94
Co-AS	Twist	31	100	0	0
Sox9-AS	Msx1	54	9	0	91
Co-AS	Msx1	71	96	4	0
Sox9-AS	Nrp1	39	21	61	18
Co-AS	Nrp1	32	84	0	16
Sox9-AS	Sox2	53	19	81	0
Co-AS	Sox2	38	97	0	3
Sox9-AS	xFKBP	24	100	0	0
Co-AS	xFKBP	15	100	0	0

expression can specifically rescue the effect of the depletion mediated by Sox9-AS.

Sox9-depleted embryos develop an abnormal craniofacial skeleton.

Sox9 is highly expressed in the migrating cranial neural crest and persists in the pharyngeal arches at least up to stage 32, while in the trunk region, Sox9 expression is downregulated around stage 25. Based on this expression pattern, we decided to analyze whether cranial neural crest cells were preferentially affected in Sox9-depleted embryos. To this end, 5 ng of Sox9-AS was injected in one dorsal animal blastomere at the eight-cell stage. When co-injected β-gal was detected in the cranial region, embryos failed to develop well defined pharyngeal arches (Fig. 8A,B). This result is consistent with an early loss of cranial crest progenitors, as alteration in the normal emigration of the cranial neural crest cells is known to result in abnormal morphogenesis of the pharyngeal arches (Graham and Smith, 2001). However, when β-gal was detected in the trunk regions, pigment cell precursors appeared to develop normally at the dorsal midline, as determined by the expression of a Trp2 specific probe (not shown).

We further analyzed the pharyngeal arch phenotype by documenting the development of crest-derived skeletal elements in each pharyngeal arch. The pattern of neural crest derivatives in the cranial skeleton of the *Xenopus* embryo (Fig. 8C,D) has been established by Sadaghiani and Thiebaud

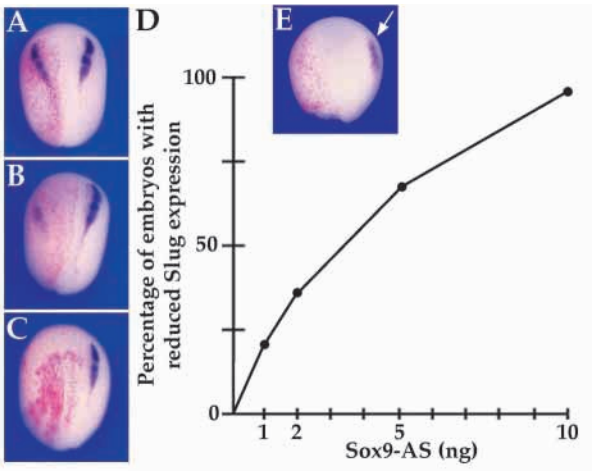

Fig. 5. Sox9-AS prevents Slug expression in a dose-dependent manner. Embryos were injected in one blastomere at the two-cell stage with different concentration of Sox9-AS and analyzed for Slug expression by whole-mount in situ hybridization at stage 17. (A-C) Representative embryos illustrating unperturbed Slug expression (A) and partial (B) or complete (C) loss of Slug on the injected side. Dorsal view, anterior is at the top. RNA encoding the lineage tracer β-galactosidase was co-injected with Sox9-AS to identify the injected side (red staining); the left side in all panels. (D) Quantification of Slug in situ hybridization results. (E) Early onset of Slug expression (arrow) at the late gastrula stage (stage 12) is blocked in embryos injected with 10 ng of Sox9-AS. Dorsal view, anterior is at the top, injected side (red staining) is to the left.

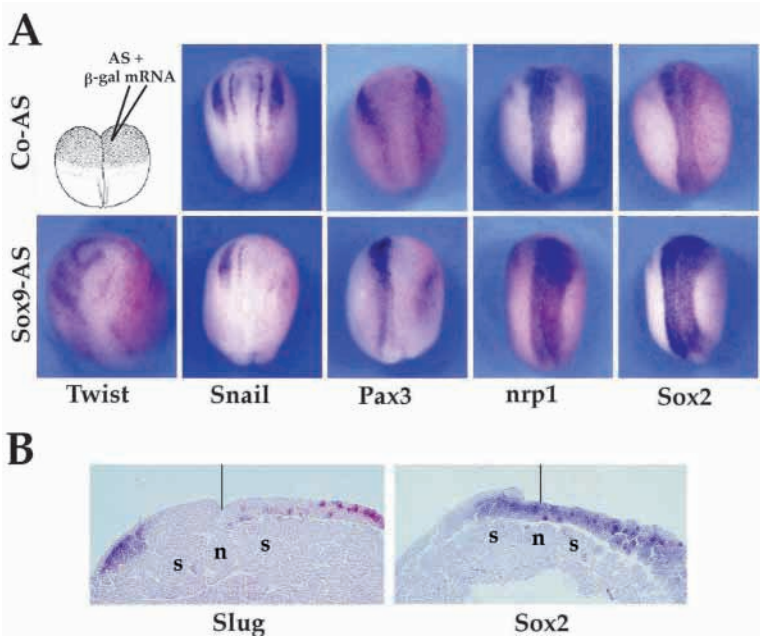

Fig. 6. Sox9 depletion leads to a loss of neural crest progenitors and an expansion of neural tissues. (A) Embryos were injected in one blastomere at the two-cell stage with 10 ng of Sox9-AS or Co-AS and analyzed by whole-mount in situ hybridization at stage 17-19 (Snail, Pax3, Nrp1 and Sox2) or stage 23 (Twist). Dorsal view, anterior is at the top. RNA encoding the lineage tracer β-galactosidase was co-injected to identify the injected side (red staining), the right side in all panels. Upon injection of Sox9-AS, expression of the neural crest markers Twist, Snail and Pax3 are greatly reduced, while expression of the pan-neural marker Nrp1 and Sox2 is expanded. Expression of the same markers in Co-AS-injected embryos is presented for comparison. (B) Tissue sections of representative Sox9-AS-injected embryos stained for Slug or Sox2 expression. Injected side is on the right. n, notochord; s, somite.

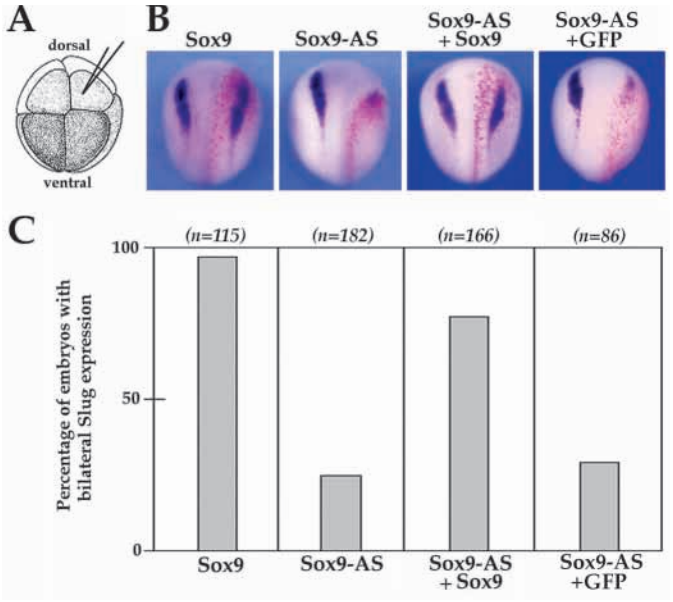

Fig. 7. The phenotype of Sox9-depleted embryos can be rescued by restoring Sox9 expression. (A) Rescue experiments were performed by injection of an animal dorsal blastomere at the eight-cell stage. (B) Representative case of Slug whole-mount in situ hybridization of stage 17 embryos injected with 100 pg of Sox9 plasmid (Sox9) or 5 ng of Sox9-AS (Sox9-AS), or a combination of both (Sox9-AS+Sox9) or a combination of Sox9-AS and 100 pg of a control GFP plasmid (Sox9-AS+GFP). RNA encoding the lineage tracer β-galactosidase was co-injected to identify the injected side (red staining), right side in all cases. Dorsal view, anterior is at the top. (C) Quantification of the in situ hybridization results. *n*, number of cases analyzed.

(Sadaghiani and Thiebaud, 1987). Briefly, in the first pharyngeal arch, neural crest cells contribute to the upper (quadrate, Qu) and lower (Meckel's, Me) jaws; in the second arch they contribute to the cerathoyal (Ce) cartilage, while in the third and fourth arches, neural crest cells contribute to the anterior and posterior regions of the branchial/gills (Br) cartilage, respectively. In five independent experiments, embryos were co-injected with 3 ng of Sox9-AS or Co-AS and β-gal mRNA in one dorsal animal blastomere at the eight-cell stage. At stage 17, a subset of the embryos were fixed, stained for β-gal and analyzed by in situ hybridization for Slug expression. The remaining embryos were allowed to develop up to stage 45, processed successively for β-gal and Alcian Blue staining for skeletal analysis. The gross morphology of Sox9-AS-injected embryos at stage 45 revealed an overall reduction of cranial structures on the injected side (Fig. 8G,H; red staining) when compared with Co-AS-injected embryos (Fig. 8E,F; red staining). Alcian Blue staining revealed that all affected embryos presented a complete loss of Meckel's cartilage (Fig. 8J,K) while branchial and cerathoyal cartilages were more mildly affected, showing different levels of reduction as illustrated in Fig. 8J,K. Interestingly, both the infrarostral (In) and basihyal (Ba) cartilage, which are

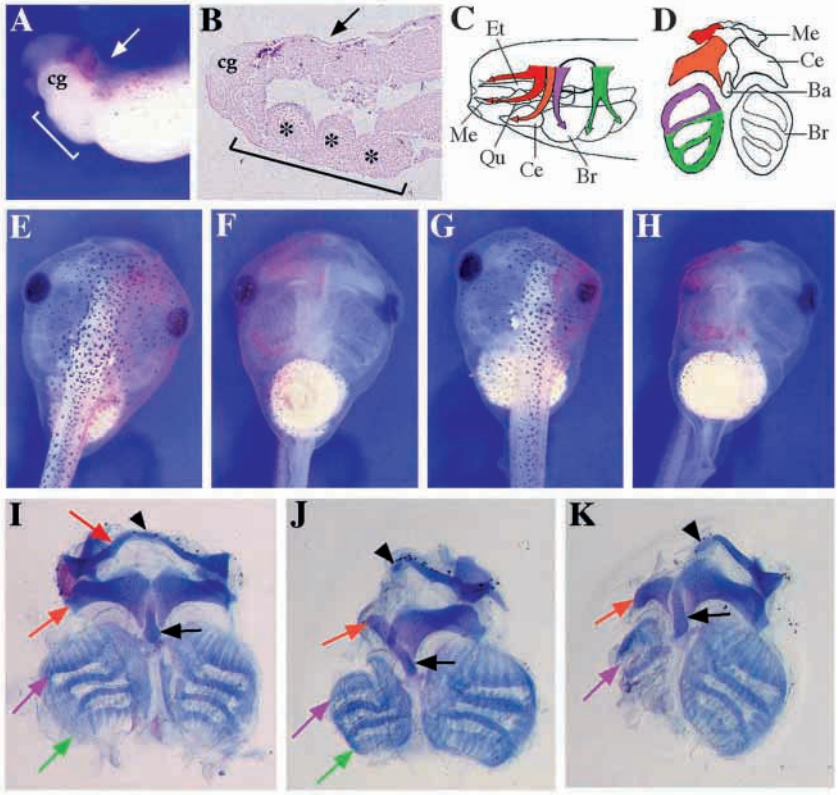

Fig. 8. Sox9-depleted embryos develop abnormal pharyngeal arches and altered pattern of skeletal elements. (A) Ventral view of a tailbud stage embryo injected with 5 ng of Sox9-AS and RNA encoding the lineage tracer β-galactosidase in one blastomere at the eight-cell stage. The bracket indicates the pharyngeal arches on the uninjected side. On the injected side (arrow, red β-galactosidase staining), the pharyngeal arches are missing. (B) Longitudinal section of an embryo similar to the one presented in A. Note the absence of well-defined pharyngeal arches on the injected side (arrow) when compared with the control side (bracket). The asterisks indicate individual pharyngeal arch. cg, cement gland. Migration (C) and contribution to cranial skeletal elements (D) of individual neural crest segments. Modified from Sadaghiani and Thiebaud (Sadaghiani and Thiebaud, 1987). Meckel's (Me), quadrate (Qu), ethmoid (Et), cerathoyal (Ce), basihyal (Ba) and branchial/gills (Br) cartilages. Embryos were co-injected in one blastomere on the dorsal side at the eight-cell stage with 3 ng of Co-AS (E,F) or 3 ng of Sox9-AS (G,H) and RNA encoding the lineage tracer β-galactosidase. At stage 45, tadpoles were fixed and stained for β-galactosidase to identify the injected side (red staining), right side in all cases. Dorsal (E,G) and ventral (F,H) views indicate an overall reduction of the cranial structures in Sox9-AS-injected embryos (G,H). (I-K) Flat-mounts of Alcian Blue stained skeletal structures from Stage 45 tadpoles. Injected side is on the left. (I) Normal pattern of skeletal elements in a Co-AS-injected embryo. (J,K) Sox9-AS-injected embryos presenting different levels of skeletal defects including loss of Meckel's cartilage and reduction of ceratohyal and branchial cartilages. Color-coded arrows indicate the origin of each skeletal element according to D. The mesoderm-derived basihyal (black arrows) and infrarostral (black arrowheads) cartilages are unaffected in Sox9-AS-injected embryo (J,K).

Table 2. Altered patterns of neural crest progenitors (stage 17) and cranial skeletal derivatives (stage 45) in embryos injected at the eight-cell stage with 3 ng of antisense oligos (Sox9-AS or Co-AS) or a combination of Sox9-AS and 100 pg of Sox9 plasmid (Sox9-AS+Sox9)

	Stage 17			Stage 45		
		Slug expression			Cranial skeleton	
Injection	*n*	Normal	Reduced	*n*	Normal	Reduced
Sox9-AS	78	47%	53%	120	52%	48%
Sox9-AS+Sox9	49	78%	22%	53	91%	9%
Co-AS	41	98%	2%	53	100%	0%

mesodermal derivatives, were unaffected in these embryos. The altered pattern of cranial cartilage observed in Sox9-depleted embryos was efficiently rescued by co-injection of 100 pg of Sox9 plasmid (Table 2). There is a good correlation between the percentage of injected embryos that showed abnormal skeletal development and the number of sibling embryos that demonstrated reduced Slug expression at an earlier stage (Table 2). While cranial skeletal elements were severely affected in Sox9-AS-injected embryos, the general pattern of pigment cell differentiation was largely unperturbed in these embryos (Fig. 8G), suggesting that Sox9 is primarily involved in the development of cranial neural crest derivatives.

DISCUSSION

In this study, we report the cloning of the *Xenopus* Sox9 gene, which is expressed in the neural crest-forming region predicting an important function during early development of the neural crest. Using a morpholino antisense oligo (Heasman et al., 2000) that specifically interferes with the production of Sox9 protein, we generated embryos exhibiting a severe and specific loss of neural crest progenitors. Later in embryogenesis, this loss of progenitors correlated with a number of defects in skeletal elements derived from the cranial neural crest. These results strongly argue that Sox9 is an essential component of the signaling cascade that leads to neural crest specification and reflect the existence of a Sox9-dependent pathway required for cranial neural crest formation in the developing embryo.

At least nine Sox genes have been identified in *Xenopus*: Sox17-α and -β (Hudson et al., 1997); xSox12 (Komatsu et al., 1996); xSox7 (Shiozawa et al., 1996); XLS13A and 13B (Hiraoka et al., 1997); Sox2 (Misuzeki et al., 1998a); Sox3 (Penzel et al., 1997; Kayano et al., 1997; Sakai et al., 1997); and SoxD (Misuzeki et al., 1998b). Among these, Sox2, Sox3 and SoxD are expressed in overlapping domains in the dorsal ectoderm at the gastrula stage and remain confined to the early neural plate and developing neural tube throughout development (Misuzeki et al., 1998a; Penzel et al., 1997; Misuzeki et al., 1998b). While SoxD is believed to control induction of anterior neural tissue by attenuation of BMP4 signaling (Misuzeki et al., 1998b), Sox2 appears to act as a competence factor by conferring neural ectoderm responsiveness to FGF, a candidate posteriorizing signal (Kishi et al., 2000). Loss-of-function for SoxD and Sox2, by overexpression of truncated Sox proteins (which lack most of the DNA binding domain), resulted in a broad inhibition of neural as well as neural crest markers (Mizuseki et al., 1998b; Kishi et al., 2000; Sasai, 2001). Sox proteins are known to regulate their target genes through interaction with cell-specific partner factors (Kamachi et al., 2000). Therefore, we cannot exclude the possibility that these dominant negative proteins interfered broadly with the function of other Sox family members (including Sox9) by competing for similar partner molecules. By contrast, Sox9 loss-of-function, using morpholino antisense oligos, resulted in a strict loss of neural crest progenitors associated with an enlargement of the neural plate.

Current models for neural crest induction suggests that graded amounts of BMP signaling could be responsible for generating both neural and neural crest fates (LaBonne and Bronner-Fraser, 1999). The expansion of the neural plate tissue in Sox9-depleted embryos could reflect the inability of the embryo to regulate proper levels of BMP signaling in the neural folds. Thus, Sox9 activity would be required at the neural plate border to confer competence to respond to BMP signal. This type of activity would be reminiscent of the requirement for Sox2 in the neural ectoderm to confer responsiveness to FGF signaling (Kishi et al., 2000). Several Wnt molecules have been shown to regulate neural crest formation in *Xenopus* (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Bang et al., 1999; Deardorff et al., 2001). Therefore, Sox9 activity could also be required in the neural folds to regulate response to Wnt signaling, as previously proposed for other Sox family members during axis formation (Zorn et al., 1999).

What is the fate of Sox9-depleted cells that fail to contribute to the neural crest lineage? The expansion of the neural plate in Sox9-AS-injected embryos suggests that Sox9-depleted cells might be incorporated into the neuroepithelium. Consistent with this view, Sox9-depleted cells expressing the lineage tracer β-gal were found among Sox2- and Nrp1-positive cells, suggesting that they may have adopted a neural plate fate (Fig. 6B). Additionally, β-gal-positive cells were also detected in the non-neural ectoderm, indicating a possible epidermal fate. Nevertheless, we cannot exclude the possibility that some Sox9-depleted cells are eliminated by cell death. A more detailed analysis of this was prohibited by the high level of apoptosis normally occurring during early neurogenesis in *Xenopus* (data not shown) (Hensey and Gautier, 1998).

One of the well-characterized target genes of Sox9 during chondrocyte differentiation is the *Col2a1* gene, which encodes type II collagen (Bell et al., 1997). Sox9 expression parallels that of Col2a1 during chondrogenesis (Ng et al., 1997; Zhao et al., 1997), and analysis of mouse chimeras using $Sox9^{-/-}$ ES cells shows that $Sox9^{-/-}$ cells are excluded from cartilage tissues and are unable to express Col2a1 (Bi et al., 1999). Because type II collagen is not expressed in *Xenopus* neural crest forming region (Su et al., 1991b), it is unlikely to be a target gene for Sox9 in this tissue. However, genes expressed in the neural crest-forming region, and downregulated in Sox9-depleted embryos, are likely to represent downstream targets of Sox9. These genes include Slug, Snail, Twist, Pax3, Msx1 and Foxd3. However, it is not clear at this time whether any of these genes are directly under the control of Sox9 or are positioned further downstream in the regulatory pathway that leads to neural crest formation.

Injection of Slug antisense mRNA in *Xenopus* resulted in an inhibition of neural crest migration, and caused a broad range of defects in cranial and trunk crest derivatives (Carl et al., 1999). Interestingly, in these embryos, early expression of Snail and Twist was not affected, consistent with a role of Slug in crest migration rather than specification (Carl et al., 1999). More recently, using a hormone-inducible inhibitory mutant of Slug (that interfere with Snail and Slug function), it has been proposed that Slug/Snail are in fact required for both neural crest formation and its subsequent migration (LaBonne and Bronner-Fraser, 2000). In Sox9-AS-injected embryos, Twist expression is completely eliminated. If neural crest cell migration rather than specification had been affected in Sox9-depleted embryos, one would expect to find accumulation of Twist-positive cells adjacent to the hindbrain at the tailbud stage, but this is not the case (Fig. 6A). This observation, together with the absence of expression of a variety of early neural crest markers, strongly indicates that Sox9 function is required for neural crest specification. However, because neural crest progenitors failed to form in Sox9-depleted embryos, it is difficult to rule out a later role for Sox9 in neural crest cell migration.

Sporadic autosomal dominant mutations in one Sox9 allele result in Campomelic Dysplasia (CD), a lethal human disorder characterized by XY sex reversal and severe skeletal malformations (Houston et al., 1983; Wagner et al., 1994). Individuals with CD also have defects in craniofacial skeletal elements of neural crest origin, the jaw and palate (Houston et al., 1983; Wagner et al., 1994). Similarly, Sox9 heterozygous mutant mice present major skeletal defects including a shortened jaw and cleft palate (Bi et al., 2001), consistent with early Sox9 expression in the neural crest-forming region (Zhao et al., 1997; Ng et al., 1997). Nevertheless, as Sox9 homozygous mutant embryos die midway through gestation (R. R. Behringer, personal communication), it is difficult to determine whether specific subsets of cranial crest derivatives are affected in these embryos. In *Xenopus*, Sox9 depletion leads to embryos with severe defects in a subset of cranial skeletal elements derived from the neural crest, including a complete loss of Meckel's cartilage. Meckel's cartilage originates from the first pharyngeal arch and makes a major contribution to the lower jaw. There is a striking similarity between the craniofacial phenotype observed in *Xenopus* Sox9-depleted embryos (missing jaws) and one aspect of the craniofacial defects described in individual with CD or Sox9 mutant mice (shortened jaws). Interestingly, neural crest-derived pigment cells form normally in the trunk region of Sox9-depleted embryos, suggesting that development of trunk crest derived pigment cells are not dependent on Sox9 activity. However, as pigment cell precursors continue to develop until fairly late during embryogenesis (LeDouarin and Kalcheim, 1999), we cannot exclude the possibility that by the time of their emergence, Sox9-AS may have lost its activity. Altogether, our results indicate a preferential requirement for Sox9 function in the development of some cranial neural crest derivatives.

During mouse embryogenesis, another Sox family member, Sox10, is expressed in the cells of the neural crest at the time of their emergence and is detected transiently in enteric neurons and melanoblasts. Sox10 persists in the peripheral nervous system into adulthood where it seems to be confined to the peripheral glia (Kuhlbrodt et al., 1998; Pusch et al., 1998; Britsch et al., 2001). Mutations in one Sox10 allele have been found in individuals that suffer from congenital aganglionic megacolon (Hirschsprung disease) associated with a combination of pigmentation defects and deafness (Waardenburg syndrome) (Pingault et al., 1998). In the spontaneous mouse mutant *Dom*, Sox10 carries a frameshift mutation. As a result, *Dom* mice suffer from a loss of neurons and glia cells in the peripheral nervous system, an absence of enteric neurons and pigmentation defects (Southard-Smith et al., 1998). Interestingly, humans and mice that carry mutations in Sox10 suffer from defects in trunk crest derivatives (enteric neurons, pigment cells and dorsal root ganglia), while cranial crest-derived structures (craniofacial cartilages and bones) appear largely unaffected, suggesting a preferential requirement for Sox10 function in the development of trunk neural crest derivatives.

Given the remarkable parallel between the activity of Sox9 in the cranial neural crest and Sox10 function in the trunk neural crest, we propose that during embryogenesis a limited number of Sox proteins, differentially expressed in the developing neural crest, are required for the specification and differentiation of subsets of neural crest derivatives as they emerge along the anteroposterior axis.

This paper is dedicated to Dr Anne-Marie Duprat on her retirement. The authors thank Dan Kessler, Peter Klein and Trish Labosky for discussions and critical reading of the manuscript. We are grateful to Benoit de Crombrugghe for the anti-Sox9 antibody and Richard Behringer for unpublished information. We thank Anne Bang, Dan Kessler and Tom Sargent for plasmids and Jim Ferris for technical assistance in scanning electron microscopy. This work was supported by grants from the Whitehall Foundation, Johnson & Johnson and the March of Dimes.

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