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EXPRESSION NOTE

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Zebrafish *sox9b* is an early neural crest marker

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Abstract Sox9 is a transcription factor related to campomelic dysplasia and sex reversal in human patients. Earlier studies in zebrafish led to the identification of two homologues of mammalian *sox9*, *sox9a* and *sox9b*. The present study represents the first evaluation of expression patterns of zebrafish *sox9b* during early embryogenesis. Our analyses reveal that *sox9b* transcripts are present throughout the life-cycle of the zebrafish, but exhibit tissue-specific distribution during embryogenesis. Zygotic expression of *sox9b* occurs in the anterolateral margins and the midline of the prospective dorsal neuroectoderm during late gastrulation. During early segmentation, the transcript is expressed in pairs of longitudinal bands in the prospective midbrain, hindbrain, and trunk, which identify the cranial and trunk neural crest progenitors. Neural crest cells cease expression of *sox9b* during migration, but some of their derivatives resume *sox9b* expression. *Sox9b* can serve as a marker for neural crest precursors.

Keywords Zebrafish · sox9 · *sox9b* · *crestin* · Neural crest

Sox9 is a member of the SRY-like HMG box (SOX)-containing gene family. It plays a key role both in the development of male gonads and in the cartilage formation in mammals (Bi et al. 1999; Huang et al. 1999; Wagner et al. 1994). Mammalian Sox9 is also expressed in the brain, otic vesicle, neural tube, notochord, urogenital system, lung, and heart, suggesting diverse roles in embryonic development. Chiang et al. (2001) reported two *sox9* genes in zebrafish, *sox9a* and *sox9b*, and demonstrated that *sox9a* is expressed in the testis, while *sox9b* expression occurs in the ovaries. The transcripts of both genes are also detected in the central nervous system, some sensory organs, the developing craniofacial skeleton, and pectoral fin buds in the embryo during the pharyngula and hatching periods. However, the expression of *sox9* in the neural crest precursors has not been reported before.

We isolated a *sox9b* cDNA of 2,490 bp (GenBank accession number AY029578) during in situ screening for tissue-specific genes, and found that its first 914-bp sequence is identical to that reported by Chiang et al. (2001), but it has an additional 1,576-bp 3′ untranslated region. Using this cDNA as a probe, Northern hybridization experiments detected a non-abundant transcript of approximately 3.0 kb in embryos at the one-cell and the 30% epiboly stages when zygotic genes are not expressed (Fig. 1), suggesting that the transcript is of maternal origin. The zygotic *sox9b* gene produces a transcript of approximately 3.5 kb, which is similar in length (3,478 bp) to the contig constructed by combining the sequence of Chiang et al. (2001) and ours. The size difference between the maternal and zygotic transcripts

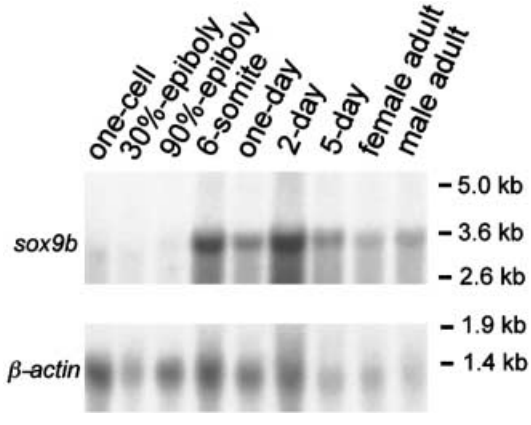

Fig. 1 Temporal expression of zebrafish *sox9b* detected by Northern blot. β-*actin* mRNA is shown in the lower panel as internal control. RNA size markers (Promega) are indicated on the right

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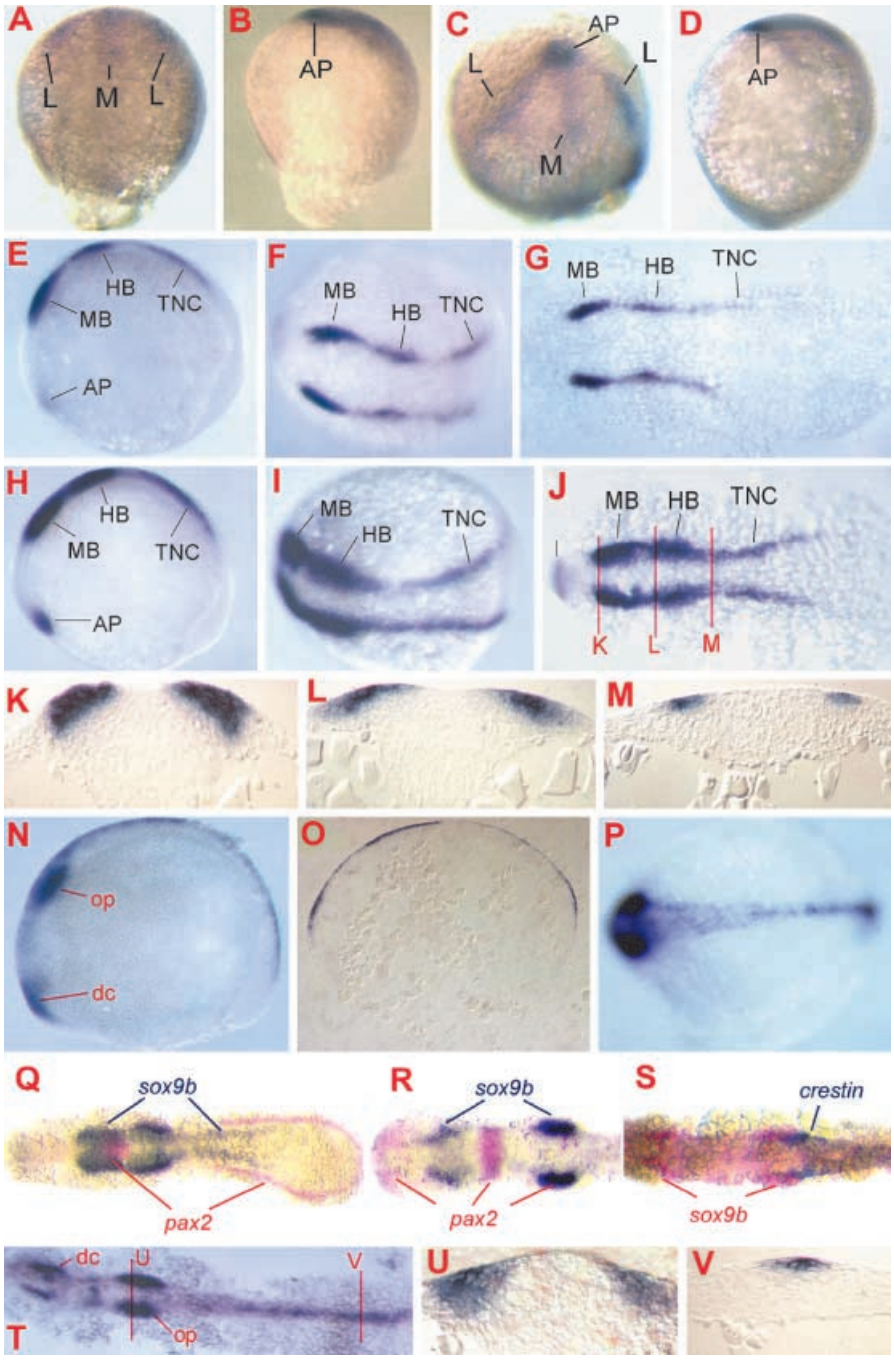

Fig. 2A–V Expression of *sox9b* during early development. **A–D** The animal pole is towards the *top*. **E–J**, **N–T** Anterior is towards the *left*. **A**, **B** Dorsal and lateral views of embryos at the 90% epiboly stage, respectively. **C**, **D** Dorsal and lateral views of embryos at bud stage, respectively. In **A–D**, letters *L* and *M* indicate the lateral and medial expression domains, respectively. *AP* indicates a omain at the anterior tip of the prospective prechordal plate. **E–G** Dorsal, lateral, and flat-mounted dorsal views of embryos at the 1-somite stage, respectively. **H–J** Dorsal, lateral, and flat-mounted dorsal views of embryos at the 6-somite stage, respectively. **K–M** Cross-sections through the positions marked in **J**. **N** Lateral view of a 10-somite embryo. **O** Sagittal section at the midline of a 10-somite embryo. **P** Dorsal view of a 10-somite embryo. **Q**, **R** Flat-mounted dorsal views of 3- and 10-somite embryos simultaneously labeled with *sox9b* (*blue*) and *pax2* (*red*) probes, respectively. **S** Flat-mounted dorsal views of a 10-somite embryo simultaneously labeled with *sox9b* (*red*) and *crestin* (*blue*) probes. **T** Flat-mounted dorsal view of a 10-somite embryo. **U**, **V** Cross-sections through the positions marked in **T**. *AP* Anterior edge of prechordal plate, *dc* diencephalon, *HB* hindbrain, *MB* midbrain, *op* otic placode, *TNC* trunk neural crest precursors

may be due to either alternative splicing or the use of different promoters. However, this remains to be confirmed in future experiments. Like Chiang et al. (2001), we also detected the *sox9b* transcript in both male and female adults, indicating that *sox9b* mRNA is continuously maintained throughout the life-cycle of zebrafish.

To examine the spatiotemporal expression pattern of *sox9b*, we performed whole-mount in situ hybridization using *sox9b* cDNA sequence that we cloned. The transcripts were initially detected by this method at 90% epiboly. At this stage, we observed three faint, short bands (one at the midline and two at the lateral margins of the prospective neuroectoderm on the anterior dorsal side) that joined at the anterior tip of the neuroectoderm and radiated toward the vegetal pole of the embryo (Fig. 2 A, B). This expression pattern was more obvious at the bud stage, when the bands increased in length (Fig. 2C, D). Shortly after initiation of segmentation, the transcripts were distributed to the anterior edge of the prechordal plate (AP domain) and the two symmetric lateral bands, while the previous medial band was no longer visible (Fig. 2E–G). The region between the AP domain and the anterior borders of the lateral bands was devoid of *sox9b* transcripts. This expression pattern was maintained dur-

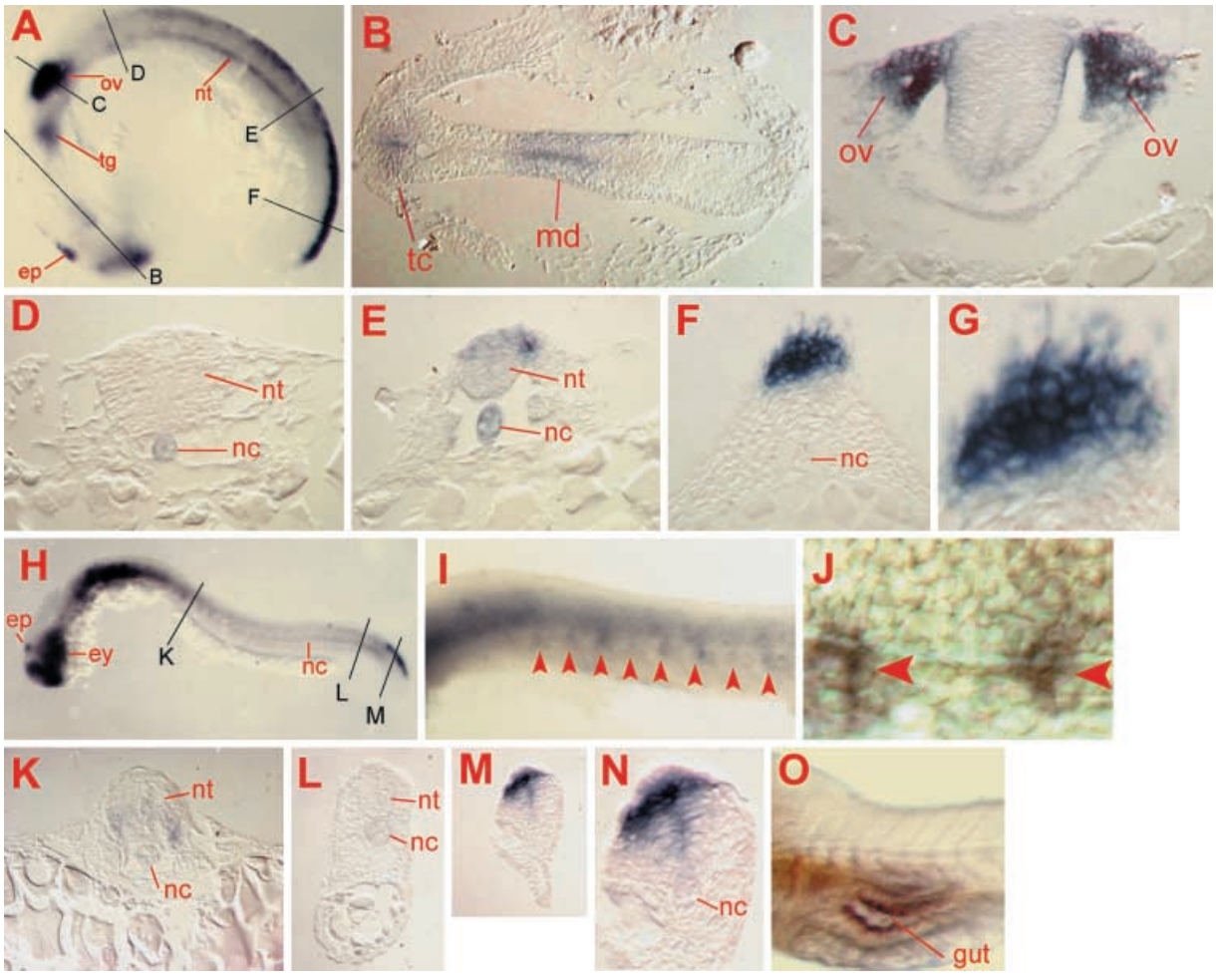

Fig. 3A–O Expression of *sox9b* during late segmentation and pharyngula periods. **A** Lateral view of an 18-somite embryo. **B–F** Sections at positions marked in **A**. **G** Higher magnification of **F**, showing *sox9b* expression in the developing dorsal median fin fold. **H** Lateral view of an embryo at 28 hpf. **I** Lateral view of anterior trunk at 28 hpf, showing stained migratory neural crest cells indicated by *arrowheads*. **J** Normarski image of single migrating cells in **I** at higher magnification. **K–M** Cross-sections through an embryo at positions marked in **H**. **N** Higher magnification of **M**, showing *sox9b* expression in the proximal part of the dorsal median fin. **O** Lateral view of a 5-day embryo, showing *sox9b* expression in the intestine. *ep* Epiphysis, *ey* eye, *md* midbrain, *nc* notochord, *nt* neural tube, *ov* otic vesicle, *pf* pectoral fin, *tg* trigeminal ganglion

ing the 2- to 6-somite period (Fig. 2H–J). Each of the two lateral bands contained three recognizable domains along the anterior-posterior axis. As indicated by double staining with pax2 (Fig. 2Q) and subsequent cell movements, these domains should describe prospective midbrain (MB), hindbrain (HB), and trunk neural crest origins (Fig. 2E–J), respectively. The *sox9b*-negative area between the anterior edge of the prechordal plate and the MB domain presumably identified the future diencephalon region.

The s*ox9b*-expressing cells in the prospective MB and HB migrated towards the midline during formation of the neural keel, and ultimately settled dorsally on either side of the neural keel by the end of this phase. Interestingly, *sox9b* expression in the prospective midbrain was downregulated during the migration, as confirmed by very weak staining at the 10-somite (Fig. 2T) and later stages. Expression in the prospective hindbrain was also downregulated during the 8- to 18-somite period, except in the otic primordium (Fig. 2O–T and Fig. 3A). We speculate that *sox9b*-expressing cells in the lateral domain of the prospective MB and HB are cranial neural crest precursors that cease to express *sox9b* upon migration from their initial positions.

At the 10-somite stage, the AP domain no longer expresses *sox9b*, which makes it difficult to determine the definitive fate of the cells within this domain. However, a new pair of lateral bands appeared at this stage in the prospective forebrain region, immediately posterior to the AP domain (Fig. 2N, R–T). The cells in this new location may specify cell lineages other than neural crest cells (e.g., telencephalon and epiphysis). Around the 16-somite stage, yet another new pair of patches appeared on either side of the HB and anterior to the otic vesicle, which may denote the trigeminal ganglion (Fig. 3A).

The two lateral bands representing trunk neural crest progenitors also migrated toward the dorsal midline during neuralation, and get closer to each other due to cell convergence (Fig. 2G, J, P). They initially fused in the

middle region of the anterior-posterior axis, around the 8-somite stage. The fusion progressed in the posterior direction and was completed in the 10-somite stage, when the labeled cells were positioned atop the neural keel (Fig. 2N–P, V). At this stage, the *sox9b* expression domains in the posterior HB and the dorsal midline of the trunk overlapped those of the *crestin* gene (Fig. 2S), a marker of premigratory and migrating neural crest cells (Luo et al. 2001; Rubinstein et al. 2000).

After migration to the top of the neural keel, premigratory trunk neural crest cells generally segregate from the dorsal neuroepithelium, and migrate away by either a medial or a lateral pathway. Unlike *crestin* that is expressed in migrating neural crest cells (Luo et al. 2001; Rubinstein et al. 2000), *sox9b* expression is switched off soon after the cells leave the dorsal midline. We observed that the staining intensity for *sox9b* decreased initially in the anterior part of the trunk at the 10-somite stage (Fig. 2P, T).

The expressin of *sox9b* occurred at a high level in a region of the posterior dorsal midline at 18-somite and later stages (Fig. 3A). Cross-sectioning through this region revealed that the transcript was clearly expressed in the forming dorsal median fin fold, and its expression in distal cells was weaker (Fig. 3F, G), suggesting that some *sox9b*-positive neural crest cells at the caudal dorsal midline differentiate into fin ectomesenchyme precursors. During the pharyngula period, only a short region of the caudal-most dorsal midline sustained the expression (Fig. 3M, N). It is likely that the outgrowth of the dorsal fin occurs in an anterior-posterior sequence, and expression of *sox9b* is downregulated during fin growth.

Interestingly, *sox9b* was re-expressed in some other neural crest-derived tissues. For example, *sox9b* transcripts were detected in some migratory trunk neural crest cells of the anterior trunk during the early pharyngula period (Fig. 3I, J), and in the intestine on day 5 (Fig. 3O). Craniofacial cartilages and pectoral fin rudiments, which are derivatives of cranial neural crest, were also found to express *sox9b* (data not shown; Chiang et al. 2001). We also detected *sox9b* expression in other neuronal and non-neuronal tissues during the pharyngula period. The details can be found in Chiang et al. (2001).

In summary, we show that zebrafish *sox9b* is an early marker for both cranial and trunk neural crest precursors. The timing and pattern of *sox9b* expression resemble that of *fkd6*, an early neural crest marker used currently (Odenthal and Nusslein-Volhard 1998). However, the early expression domains of *sox9b* include the prospective MB, whereas *fkd6* expression is not detected in the neural crest precursors within the MB. The initial expression of another neural crest marker *crestin*, when compared with *sox9b*, occurs later and more posteriorly. Recently, it has been shown that *Xenopus Sox9* is also expressed in the neural crest-forming region during early gastrulation, and morpholino-induced knockdown of Sox9 causes a significant loss of neural crest progenitors (Spokony et al. 2002). This implies that *sox9* may be commonly involved in the formation and differentiation of the neural crest in vertebrates.

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References

Bi W, Deng JM, Zhang Z, Behringer RR, Crombrugghe B de (1999) Sox9 is required for cartilage formation. Nat Genet 22:85–89

Chiang EF, Pai CI, Wyatt M, Yan YL, Postlethwait J, Chung E (2001) Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. Dev Biol 231:149–163

Huang B, Wang S, Ning Y, Lamb AN, Bartley J (1999) Autosomal XX sex reversal caused by duplication of SOX9. Am J Med Genet 87:349–353

Luo R, An M, Arduini BL, Henion PD (2001) Specific pan-neural crest expression of zebrafish Crestin throughout embryonic development. Dev Dyn 220:169–174

Odenthal J, Nusslein-Volhard C (1998) Fork head domain genes in zebrafish. Dev Genes Evol 208:245–258

Rubinstein AL, Lee D, Luo R, Henion PD, Halpern ME (2000) Genes dependent on zebrafish cyclops function identified by AFLP differential gene expression screen. Genesis 26:86–97

Spokony RF, Aoki Y, Saint-Germain N, Magner-Fink E, Saint-Jeannet JP (2002) The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*. Development 129:421–432

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, et al (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 79:1111–1120