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The Expression of the Mouse *Zic1*, *Zic2*, and *Zic3*

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in Body Pattern Formation

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We examined the expression of *Zic1, Zic2,* and *Zic3* genes in the mouse embryo by means of *in situ* hybridization. *Zic* genes were found as a group of genes coding for zinc finger proteins that are expressed in a restricted manner in the adult mouse cerebellum. We showed that the genes are the vertebrate homologues of *Drosophila odd-paired,* which may play an essential role in parasegmental subdivision and in visceral mesoderm development. The expression of the three *Zic* genes was first detected at gastrulation in a spatially restricted manner. At neurulation, the expression became restricted to the dorsal neural ectoderm and dorsal paraxial mesoderm. During organogenesis, the three genes were expressed in specific regions of several developing organs, including dorsal areas of the brain, spinal cord, paraxial mesenchyme, and epidermis, the marginal zone of the neural retina and distal regions of the developing limb. For all stages, significant differences in the spatial expression of *Zic1, Zic2,* and *Zic3* were observed. Furthermore, the expression of *Zic* genes in *Pax3, Wnt-1,* and *Wnt-3a* mutant embryos suggested that *Zic* genes are not primarily regulated by the three genes which were expressed in dorsal areas similar to *Zic* genes. However, in *open brain,* a mutant with severe neural tube defects, and in the *Wnt-3a* mutant mice, the expression of *Zic* genes was changed. The changed expression pattern in *Wnt-3a* mutant mice suggests that *Zic* genes in the neural tube are regulated by the factors from notochord. Our findings suggest that *Zic* genes are involved in many developmental processes. Furthermore, analysis of gene expression patterns in different mouse mutants indicated that *Zic* genes may act upstream of many known developmental regulatory genes. © 1997 Academic Press

INTRODUCTION

The coordinated process of body pattern formation in vertebrates is governed by a number of genes, the expression of which is regulated developmentally. Many of the genes which are involved in the formation of the vertebrate body are evolutionally conserved in the fruit fly *Drosophila,* in which the basic molecular mechanism of development has been thoroughly investigated (Ingham *et al.,* 1988). The genes are often grouped by similarity or a conserved structural motif. The roles of several classes of genes, such as *Hox, Pax, Wnt* gene family and *TGFβ* superfamily (reviewed by Krumlauf, 1994; Stuart *et al.,* 1993; McMahon, 1992; Kingsley, 1994; Hogan *et al.,* 1994) have been characterized.

The *Zic* gene family (Aruga *et al.,* 1994; 1996) comprises zinc finger proteins which we characterized as a structurally related group of genes expressed in a highly restricted manner in the adult mouse cerebellum. *Zic1* is expressed restrictedly in the cerebellar granule cell lineage and in the medulloblastoma, the oncogenesis of which may be involved in the cerebellar granule cell lineage (Aruga *et al.,* 1994; Yokota *et al.,* 1996).

We showed that the *Zic* genes are the vertebrate homologues of the *Drosophila* pair-rule gene, *odd-paired* (*opa*)

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(Aruga *et al.,* 1996; Benedyk *et al.,* 1994; Cimbora and Sakonju, 1995). In the *opa* mutant, segmental subdivision along the anterior–posterior axis and development of the visceral mesoderm are affected. The timely activation of the segment polarity genes, *engrailed* and *wingless,* are impaired in *opa* mutant (Benedyk *et al.,* 1994). In addition, *decapentaplegic,* of which mutants have a defective dorsoventral axis, negatively regulates *opa* expression in the visceral mesoderm (Cimbora and Sakonju, 1995). This suggests that the *opa* gene product is part of a gene network that establishes the insect body plan. We showed that the vertebrate homologues of *opa, Zic1, Zic2,* and *Zic3* genes are also expressed during mouse development and that *Zic1* is expressed largely in the neural tissue and dorsal mesenchyme whereas, in the adult, its expression is confined to the cerebellum (Aruga *et al.,* 1994, 1996). Our findings, combined with the role of the *opa* gene in *Drosophila* development, suggest an important role of *Zic* genes also during vertebrate development. However, it is necessary to know the expression pattern of every *Zic* gene as a first step in understanding their roles in development.

We compared the expression of the *Zic1, Zic2,* and *Zic3* genes in the mouse embryo by *in situ* hybridization. The three genes were expressed in partially overlapping, but distinct patterns in the developing central nervous system, dorsal paraxial mesenchyme and limb bud. Furthermore, their expression was common to the dorsal body axial structures. To understand the regulation of the dorsally restricted *Zic* genes expression, we examined the *Zic* gene expression in several mutant mice including *Pax3, Wnt-1, Wnt-3a,* and *open brain* (*opb*). Our findings suggest that a potential role of *Zic* genes is in the formation of the vertebrate body plan and that they may be regulated by the secretory factor from notochord.

MATERIALS AND METHODS

Animals. *CD1* outbred pregnant mice were purchased from NihonSLC (Shizuoka, Japan). The mice with disrupted *Wnt-1* and *Wnt-3a* genes were originally developed by McMahon *et al.* (1990) and Takada *et al.* (1994), respectively, and were kindly provided by Dr. A. P. McMahon at Harvard University. The *Splotch* (*Sp*) (*Pax3* mutant) mice were originally purchased from the Jackson Laboratory and kept in the experimental animal facilities at Tsukuba Life Science Center, RIKEN. *opb* mutant mice were originally found by Günther *et al.* (1994) and kept at the GSF-Neuherberg. Embryos were obtained after natural mating and midday of the day of a vaginal plug was considered as 0.5 days postcoitum (dpc).

In situ hybridization. The probes used in this study were as follows; *Zic1,* 690-bp *Xho*I–*Sal*I segment from the mouse *Zic1* cDNA (Aruga *et al.,* 1994); *Zic2,* 603-bp *Stu*I–*Eco*RI from mouse *Zic2* cDNA (Aruga *et al.,* 1996); *Zic3,* 435-bp *Xba*I–*Stu*I from mouse *Zic3* cDNA (Aruga *et al.,* 1996). All probes were prepared from the 3′ untranslated region of the cDNAs and did not cross-react in Northern blots (data not shown). Mouse *Pax3* and *Pax6* cDNAs were gifts from Dr. T. Saito (RIKEN). The control for each hybridization was a sense orientation riboprobe to confirm that there was no significant background staining.

Sections and whole mount preparations were hybridized *in situ* using nonradioactive digoxigenin-labeled cRNA probes. Embryo sections were hybridized essentially as described (Birren *et al.,* 1993) except 10% polyvinyl alcohol was included in the alkaline phosphatase reaction mixture. Frozen sections (20–25 μm) of paraformaldehyde-fixed embryos were processed for *in situ* hybridization. Whole mount *in situ* hybridization was performed as described in Wilkinson *et al.* (1992). Alkaline phosphatase was histochemically detected in a Purple AP Substrate (Boehringer Mannheim). The reaction times varied for each experiment. However, generally, the reaction time for the *Zic2* and *Zic3* detection required two and three times longer than that of *Zic1,* respectively. These variations of the reaction times required to detect the three genes may reflect the content of the three transcripts, since the exposure proceeds for Northern blots (Aruga *et al.,* 1996) using other probes tended to be likewise.

RESULTS

The Expression of the Zic Genes at Gastrulation and Neurulation Stages

During early primitive streak stages (7.0 dpc) (Figs. 1A–1C), transcripts of all three *Zic* genes were detected in embryonic mesoderm. In addition, the *Zic2* and *Zic3* genes were detected in ectodermal cells facing the amniotic cavity. *Zic2* was detected only in the presumptive headfold region, whereas the *Zic3* was expressed mainly in the primitive streak. The expression of both genes continued during neural tube formation. At the late primitive streak stage (7.25 dpc) (Figs. 1D–1I), *Zic1* expression in the neuroectoderm was detected in the presumptive dorsal region although expression level seemed low at this stage. The expression of all three genes in the mesoderm continued, whereas that of *Zic2* was higher in the neuroectoderm than in mesoderm. *Zic2* and *Zic3* were expressed along the rostral–caudal axis as during the early primitive streak stage. At 8.0 dpc (Figs. 2A–2C), all three *Zic* genes were expressed only in dorsal axial structures. *Zic1* expression was specific for the dorsal neural tube and dorsomedial region of the somites. *Zic2* expression was similar to that of *Zic1,* although it was also found in ventromedial somite regions. In contrast to *Zic1* and *Zic2, Zic3* expression was very low in the trunk neural tube and could only be detected in a very restricted region of the dorsal somite.

At the time of neural tube closure, the three *Zic* genes were still expressed in dorsal embryonic structures. At 9.5 dpc (Figs. 3A–3C), hybridization signals were located in the dorsal neural tube and dorsal mesoderm derivatives. *Zic1* gave the most intense signal and was detected along almost the entire rostrocaudal axis without distal tail region. In the brain, *Zic1* was intensely expressed in the dorsal midline. At the rostral end of the telencephalon, the intense staining was distributed laterally over a wider area. Hybridization of sagittal sections revealed that the rostral boundary of *Zic1* expression lay in the vicinity of the optic chiasm (Aruga *et al.,* 1994) at the boundary of the alar and basal plate (Rubenstein *et al.,* 1994; Shimamura *et al.,* 1996).

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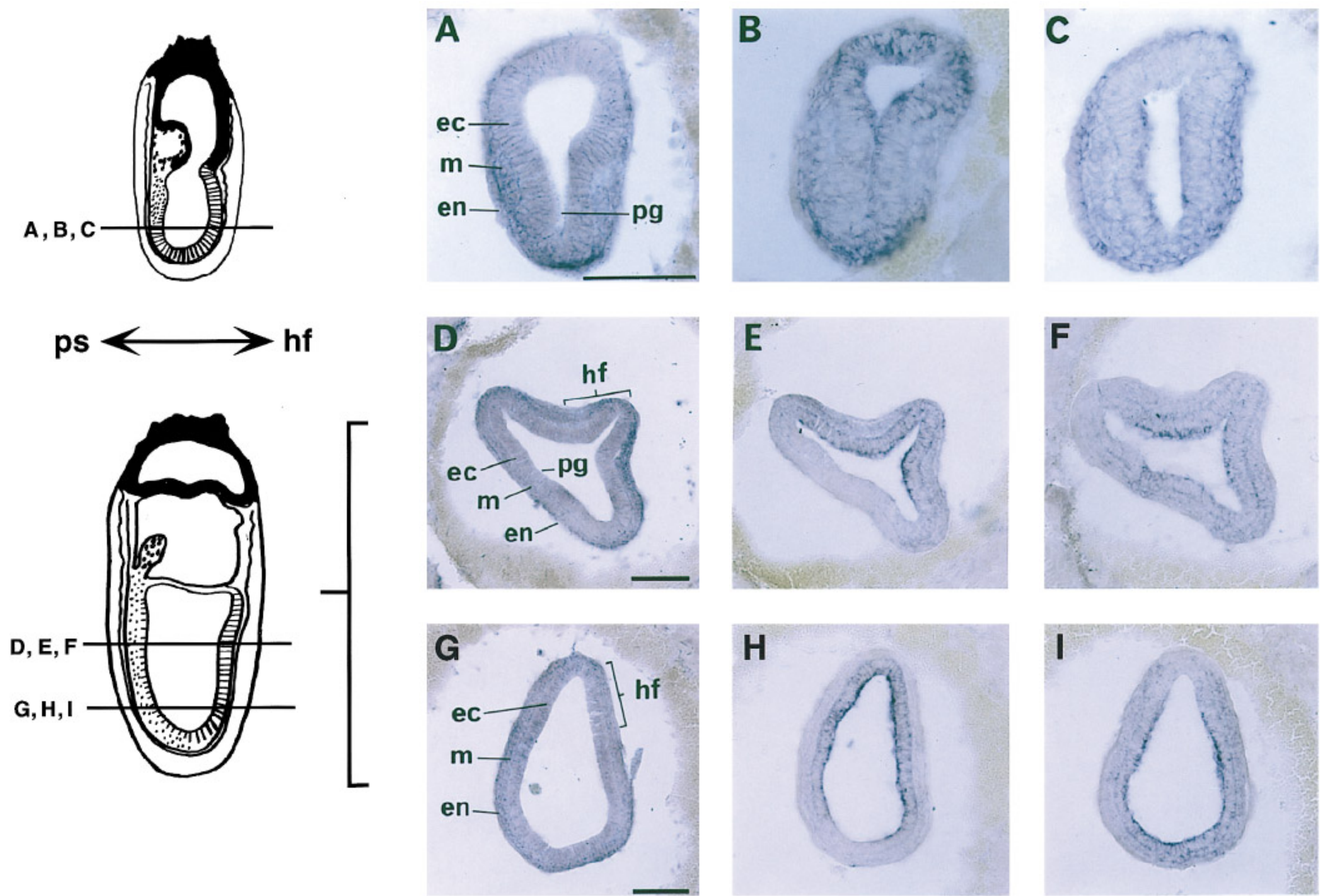

FIG. 1. Expression of *Zic* genes at primitive streak stage. Sections prepared from early- (7.0 dpc) (A–C) or late-primitive streak stage (7.25 dpc) (D–I) were hybridized *in situ* using *Zic1* (A, D, G), *Zic2* (B, E, H), and *Zic3* (C, F, I) riboprobes. The embryo has been sectioned transversely at the level indicated in the left panel (schematic drawing of parasagittal sections). ec, ectoderm; en, embryonic endoderm; hf, head fold (side); m, intraembryonic mesoderm; pg, primitive groove; ps, primitive streak side. Scale bar, 100 μm.

Along the anterior–posterior axis, there were differences in the expression of the three genes. *Zic2* and *Zic3* gave specific signals in the tail bud region which is one of the growth centers of the developing mouse. *Zic3* expression is higher in the brain and tail regions than in the trunk.

The expression in the brain region also differed among the three genes from 9.5 to 11.5 dpc (Fig. 3). *Zic1* expression was relatively restricted to the midline. In contrast to *Zic1, Zic2* and *Zic3* were found in a broader area although the strongest signals were detected in the midline like those of the *Zic1* gene.

Expression of Zic Genes during Axial Pattern Formation

The *Zic* genes showed a dorsally restricted expression pattern during later stages of development in neural tissue and dorsal paraxial mesenchyme. To further localize the transcripts along the dorsal–ventral axis, we prepared a series of transverse sections (Fig. 2).

From 8.0 to 14.5 dpc, the expression was generally restricted to the dorsal spinal cord and surrounding paraxial mesenchyme. At 8.0 dpc, expression of three genes was detected similarly in the dorsal spinal cord and dorsomedial somites except that *Zic3* expression in the spinal cord was very low. Whereas the dorsally restricted expression of *Zic* genes continued in the neural tube, mesenchymal expression gradually decreased from 12.5 dpc onward.

In the spinal cord, *Zic1* expression was restricted to the dorsal third at 10.5 dpc. However, the position of the ventral boundary of expression was different from that of *Pax3* gene (Goulding *et al.,* 1991) (Figs. 2E and 2H). *Zic2* and *Zic3* were expressed in a narrower dorsal region of the spinal cord. The expression of *Zic3* in the spinal cord until 10.5 dpc was almost negligible and slightly detectable at 12.5 dpc. In 14.5 dpc embryos, *Zic1* and *Zic2* were expressed in the medial

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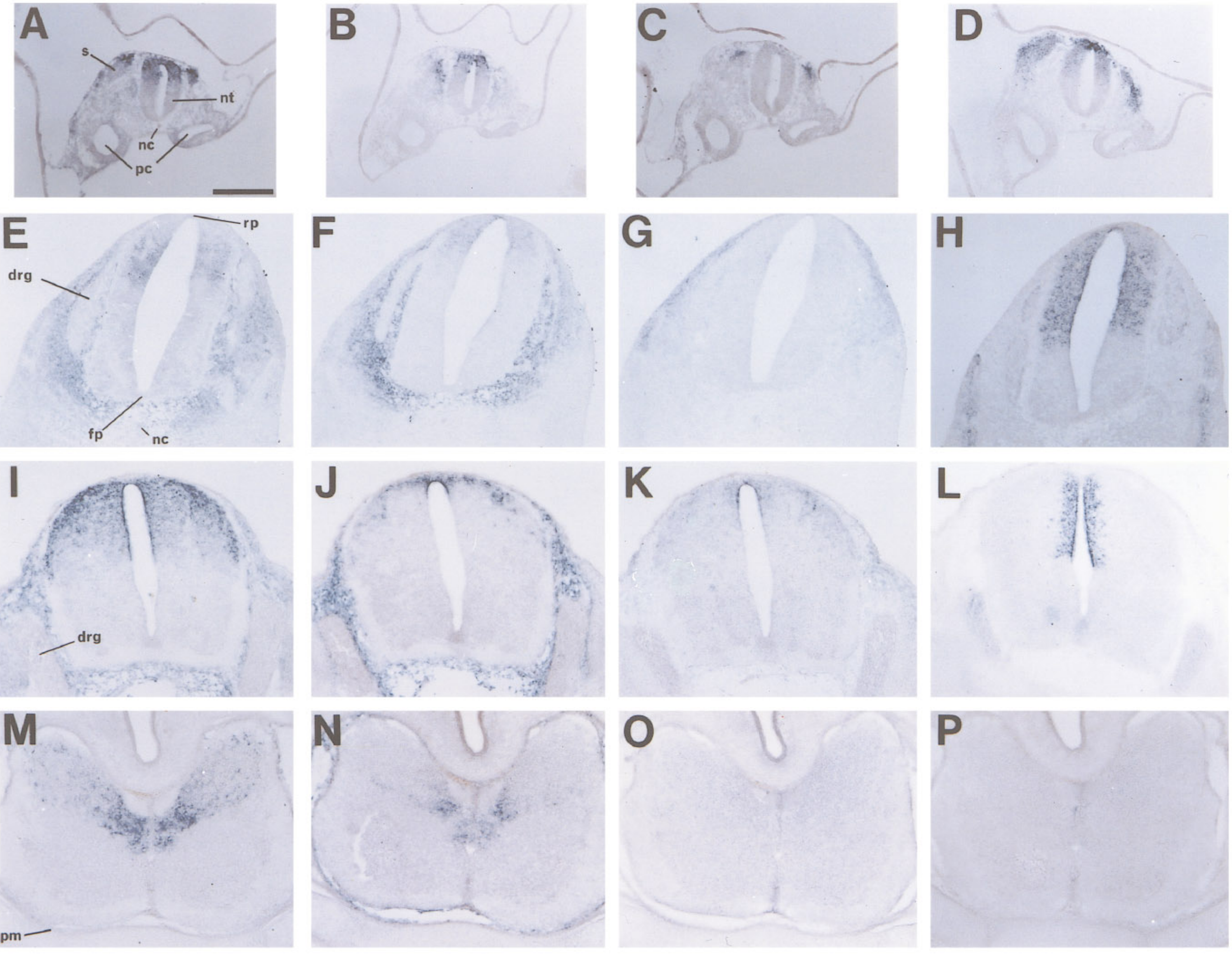
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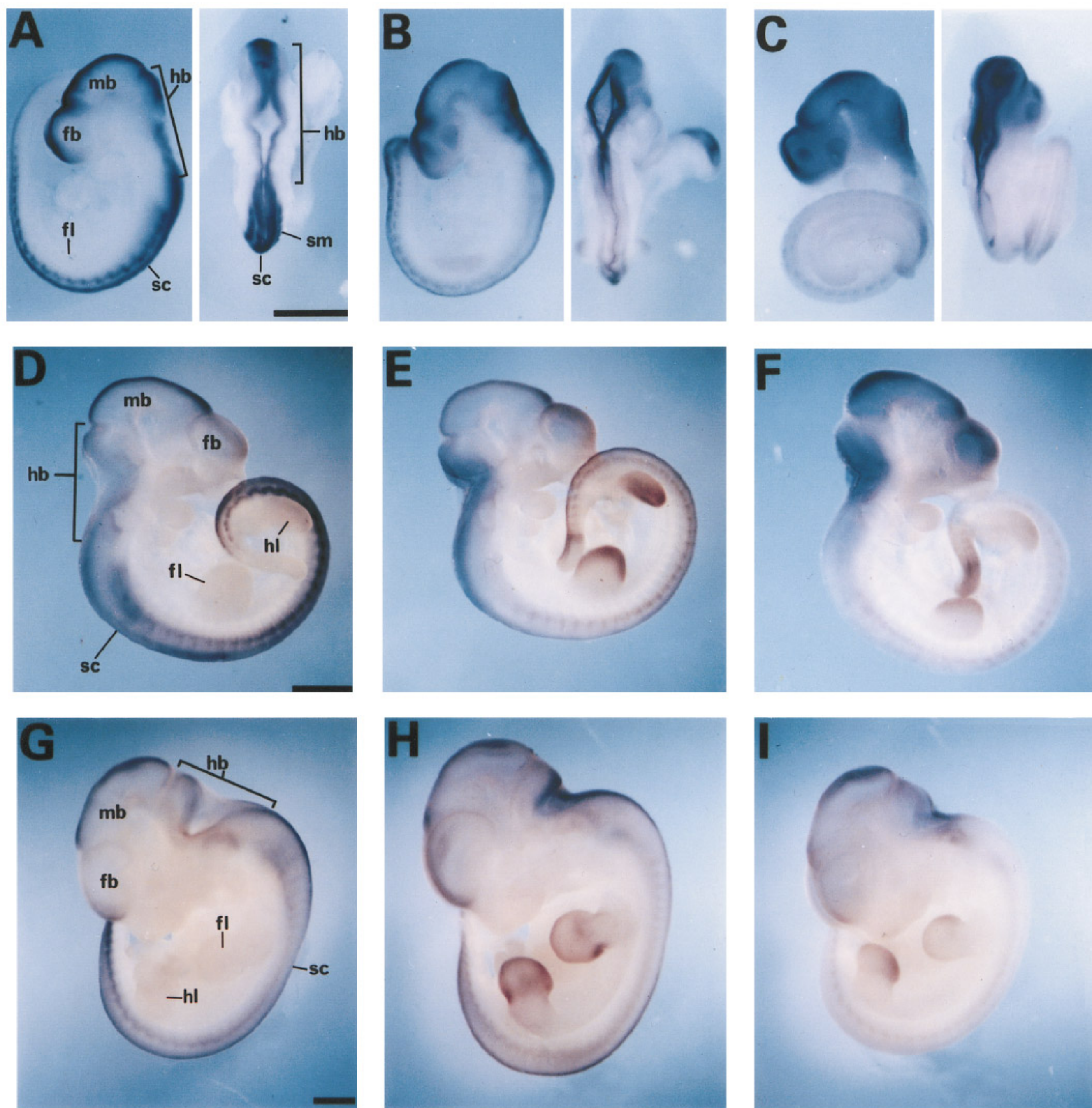

FIG. 3. Whole mount *in situ* hybridization. Embryos at 9.5 (A–C), 10.5 (D–F), and 11.5 dpc (G–I) were hybridized with *Zic1* (A, D, G), *Zic2* (B, E, H), and *Zic3* (C, F, I). In A, B, and C, left panels are lateral views and right panels are dorsal views. fb, forebrain; fl, forelimb; hb, hindbrain; hl, hindlimb; mb, midbrain; sc, spinal cord; sm, somites. Scale bar, 1 mm.

FIG. 2. Dorsoventral expression of *Zic* genes in a series of transverse sections through the caudal region of cervical spinal cord. *Zic1* (A, E, I, M), *Zic2* (B, F, J, N), *Zic3* (C, G, K, O), and *Pax3* (D, H, L, P) expression was examined at 8.0 (A–D), 10.5 (E–H), 12.5 (I–L), and 14.5 dpc (M–P). drg, dorsal root ganglion; fp, floor plate; nc, notochord; nt, neural tube; pc, pericardio-peritoneal canal; pm, primitive meninx; rp, roof plate; s, somites. Scale bar, 200 μm.

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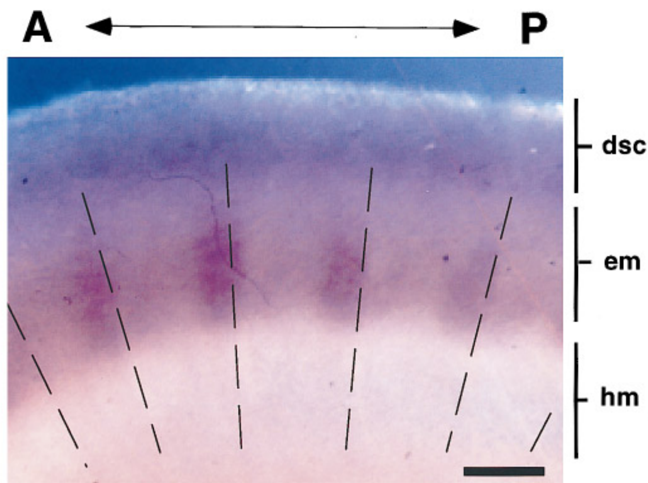

FIG. 4. *Zic1* expression in the somite moieties. Whole mount *in situ* hybridization was performed on 10.5 dpc embryo using *Zic1* probe. Lateral view of the somites in thoracic region. The borders of each somite are indicated by broken lines. Anterior (A) and posterior (P) sides are indicated above the panel. dsc, dorsal spinal cord; em, epaxial mesoderm; hm, hypaxial mesoderm. Scale bar, 100 μm.

region of the spinal cord. At later stages, the signals gradually decreased in the spinal cord and only weak expression could be detected in the adult spinal cord (data not shown). In comparison to *Pax3,* which was expressed restrictedly in the ventricular layer of the spinal cord, *Zic* genes were detected in both the ventricular and mantle layer of the spinal cord. In dorsal root ganglia, which are derived from neural crest cells, only weak *Zic* expression was observed.

At 10.5 dpc, *Zic1* and *Zic2* expression was detected strongly in the entire perineural mesenchyme (which is derived from sclerotome), moderately in the dorsal dermomyotome, and weakly in the myotome (Figs. 2 and 3). In contrast, *Zic3* expression was restricted to dorsal dermomyotome regions and subepidermal dorsal mesenchyme (Fig. 2G). Furthermore, a segmented pattern of *Zic1* was observed in trunk somites at 10.5 dpc (Fig. 3D). *Zic1*-positive stripes were localized in the posterior-most moiety in a somite and extended across somite borders into a small anterior stripe within the next caudal somite (Fig. 4). This expression was dorsally restricted with a sharp boundary to hypaxial body regions. Slightly later (11.0 dpc), *Zic1* expression was observed throughout the dorsal somite with no recognizable differences between anterior and posterior somite regions (data not shown). After 12.5 dpc, the expression of the three *Zic* genes in the mesenchymal tissue decreased. Only *Zic2* expression persisted in the primitive meninx at 14.5 dpc (Fig. 2N).

Expression of Zic Genes in the Developing Limb

Zic2 and *Zic3* were predominantly expressed in developing limbs. Since the limb is an ideal model of the vertebrate pattern formation (Tickle, 1995), we examined *Zic* gene expression in the developing limb (Figs. 3 and 5)

First, we detected *Zic2* expression in the mesodermal tissue of the distal end of forelimb buds at 9.5 dpc, at the earliest stages of limb bud formation (Fig. 3B). At 10.5 dpc (Fig. 3E), the expression patterns in fore- and hindlimb buds were similar in spite of the difference due to the different developmental stage. *Zic2* expression was restricted to the distal mesodermal tissue beneath the apical ectodermal ridge (AER) (Fig. 5C). Thereafter, *Zic2* expression diffused widely in the limb (11.5 dpc; Fig. 5H). However, the expression was more intense in the lateral margin and in the anterior and posterior necrotic zones. After the precartilaginous mesenchymal condensation appeared, *Zic2* expression was detected in the condensed areas, whereas expression in the marginal regions disappeared (Fig. 5K). At 13.5 dpc, *Zic2* was expressed in precartilaginous condensations in the proximal region of the autopodium but not in areas where chondrogenesis has occurred (Fig. 5N).

Zic3 expression was also, initially, detected at 9.5 dpc. Regions of *Zic3* expression were located close to *Zic2*-positive domains, but more anterior (Figs. 2F, 5E, and 5F). Neither gene was detected in the AER. At 13.5 dpc, *Zic3* was expressed in more peripheral zones of the limb mesenchyme and presumptive muscle (Figs. 5L and 5O). *Zic2* and *Zic3* expression appeared to be complementary in the mesenchyme at this stage, as seen in the paraxial mesoderm, too. Later, the expression of *Zic2* and *Zic3* decreased. At 14.5 dpc, the *Zic3* expression was in the marginal mesenchyme and the basilar bone (data not shown).

Expression of Zic Genes in the Developing Eye

The three *Zic* genes were characteristically expressed in the developing eye. As a control, the expression of *Pax6* (Walter *et al.,* 1991), which is restrictedly expressed in the developing eye, is shown in adjacent sections (Figs. 6D, 6H, 6L, and 6P). We detected the expression of the three *Zic*

FIG. 5. Expression of *Zic* genes in the developing limb. *In situ* hybridization was performed on whole mount preparations (A, C, E, G, H, I) or frozen sections (B, D, F, J, K, L, M, N, O) from 10.5 (A–F), 11.5 (G–I), 12.5 (J–L), and 13.5 dpc (M–O) embryos using *Zic1* (A, B, G, J, M), *Zic2* (C, D, H, K, N), and *Zic3* (E, F, I, L, O). In J, K, and L, the right panels are magnified from the left panels (×2.5). J–L are from sections vertical to the dorsoventral axis of the limb, M–O are from parallel sections. c, calcaneus; drg, dorsal root ganglion; fl, forelimb; nt, neural tube; pc, precartilaginous mesodermal condensation; pm, premuscle mass; t, tarsus. Scale bar, 500 μm except the right panels of J, K, L in which the bar is 200 μm.

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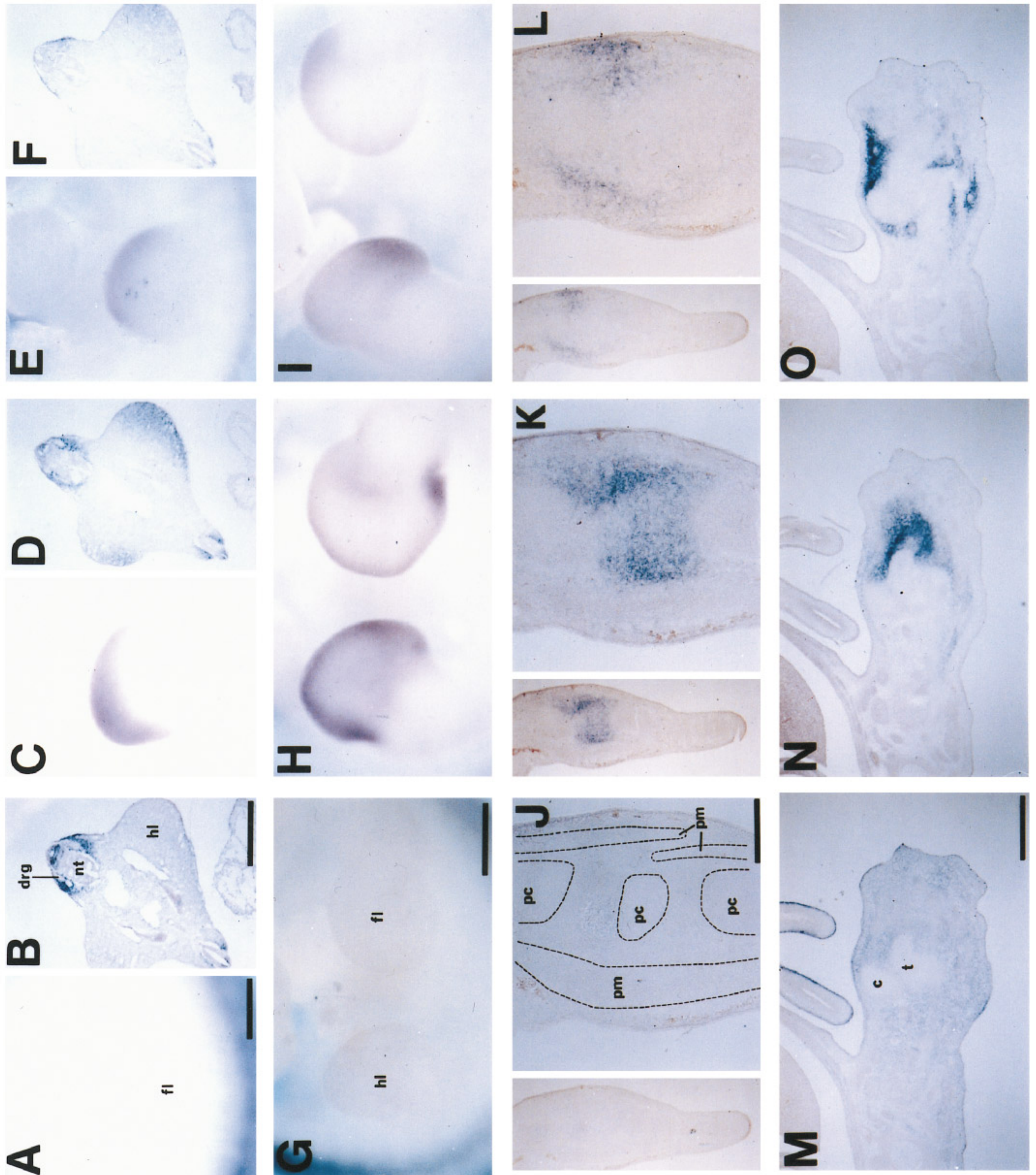

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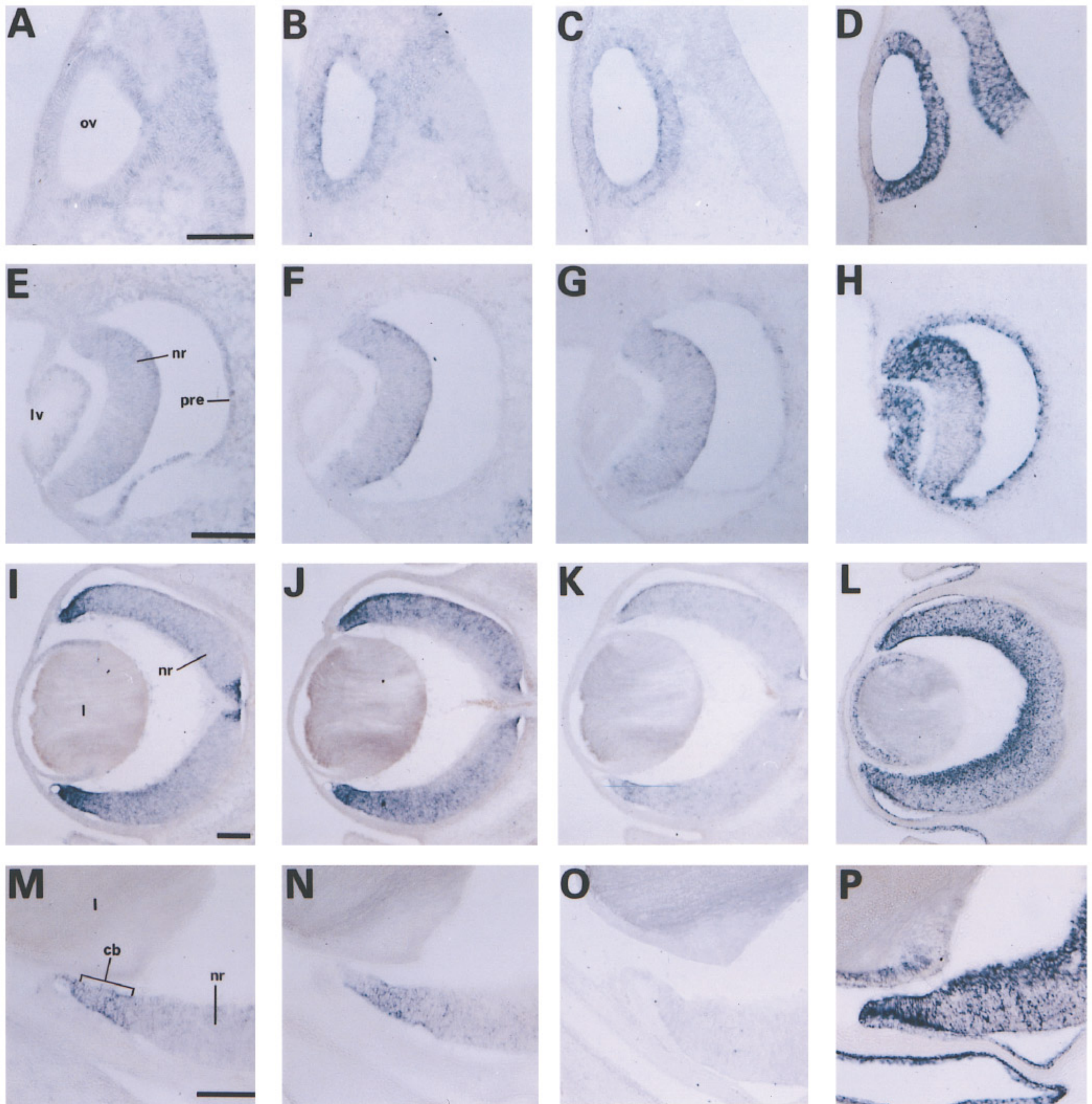

FIG. 6. Expression of *Zic* genes in the developing eye. A developmental series of sections through the eye (9.5 (A–D), 10.5 (E–H), 13.5 (I–L), and 16.0 dpc (M–P)) were hybridized with *Zic1* (A, E, I, M), *Zic2* (B, F, J, N), *Zic3* (C, G, K, O), and *Pax6* (D, H, L, P) probes. cb, ciliary body; l, lens; lv, lens vesicle; nr, neural retina; ov, optic vesicle; pre, pigmented retinal epithelium. Scale bar, 100 μm.

genes at 9.5 dpc (Figs. 6A–6C). All three genes were expressed in the optic vesicle and in the optic stalk (data not shown). At 10.5 dpc (Figs. 6E–6G), *Zic1* expression was detected both in the inner neural layer, outer pigmented epithelial layer, and inner surface layer of the lens vesicle. *Zic2* and *Zic3* were expressed only in the inner neural layer. During the formation of the eye, all three genes were mainly expressed in the neural retina but not in the lens. This was in contrast to the expression of *Pax6,* which was detected both in the developing neural retina and lens (Figs. 6D and

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6H). At 13.5 dpc, the inner neuroblastic layer expressed high levels of *Zic1,* whereas *Zic2* was more strongly expressed in the outer neuroblastic layer (Figs. 6I and 6J).

After 10.5 dpc, the expression of the three genes generally converged into the marginal zone of the neural retina. Later, at 16.5 dpc, the *Zic1* and *Zic2* expression in the neural retina was highly restricted to a region, referred to as the preciliary domain (Monaghan *et al.,* 1991).

Zic Genes in the Neural Tube Defect Mice

In order to understand the regulatory mechanism involved in *Zic* genes expression in dorsal embryonic regions, we examined the expression of *Zic1* and *Zic2* genes in several mutant mouse lines that exhibit abnormalities of the dorsal neural tube. We chose these mutants based on the expression pattern of the mutated gene and their phenotypes.

Pax3 mutant. The expression of the *Zic* genes in the trunk region was similar to that of *Pax3* at 8.0 dpc (Figs. 2A–2D). *Pax3* is expressed in the dorsal spinal cord and dermomyotome and the impairment of *Pax3* causes severe deformity in the dorsal structure and often neural tube closure defects (Epstein *et al.,* 1991) as found in the homozygote of the naturally occurring *Sp* (*Pax3* null) mutation. We examined *Zic* gene expression in *Sp/Sp* mutant mice at 13.5 dpc (Figs. 7A–7D). Overall, the expression of all three *Zic* genes was not affected in *Sp/Sp* embryos. Even in the limb bud, where muscle tissue does not form in *Sp/Sp* embryos, *Zic* genes expression was not significantly changed (data not shown).

Wnt-1 mutant. The mouse homozygous for the mutated *Wnt-1* allele lacks midbrain and rostral metencephalon (McMahon and Bradley, 1990). *Wnt-1* expression extends along the dorsal midline of the midbrain and spinal cord where *Zic* genes are also expressed. When *Zic* genes expressions were examined in the *Wnt-1* mutant mice and compared to their wild-type littermate, there is almost no change in the expression pattern of the *Zic1* and *Zic2* genes (Figs. 7E–7H). Even in the midbrain–hindbrain region, where a part was lost in the mutant mice, there were no significant changes in expression, except for those correlated with anatomical abnormalities.

Open brain. Homozygous mutant embryos are characterized by severe defects in the developing neural tube and epaxial musculature (Günther *et al.,* 1994; Spörle *et al.,* 1996). In most severely affected regions of the spinal cord, the neural tube exhibits a circular shape, the formation of a roof plate is not observed and, instead, a region of differentiated cells is detected in dorsal-most regions (Günther *et al.,* 1994; T.G., R.S., K.S., unpublished data). Analysis of roof plate markers like *Wnt-1* and *Wnt-3a* at Day 13.5 demonstrated the loss of the roof plate and adjacent regions in *opb* mutant embryos (Fig. 7L and data not shown). Expression of *Pax3* in the dorsal spinal cord did not extend to the dorsal margin as in wild-type embryos but revealed an inner layer of *Pax3*-positive cells and a *Pax3*-negative layer at the dorsal margin (Fig. 7K). Expression of *Zic1* and *Zic2* was also altered in *opb* homozygous embryos (Fig. 7I and 7J). At Day 12.5, *Zic2* exhibited two different dorsal expression domains in the wild-type spinal cord: a dorsomedial and two adjacent dorsolateral stripes, separated by *Zic2*-negative stripes (Fig. 2J). In *opb* mutant embryos, however, only a single dorsal band of *Zic2* expressing cells could be detected at the dorsal margin (Fig. 7J). In addition, *Zic1* expression in *opb* embryos was restricted to a narrower dorsal region when compared to wild-type embryos.

Wnt-3a mutant. *Wnt-3a* gene is expressed in the primitive streak at egg cylinder stage and later in the dorsal CNS. *Wnt-3a* mutant mice lack caudal somites, have a disrupted notochord, and show CNS dysmorphology (Takada *et al.,* 1994). The degeneration of the notochord is more severe in the caudal region (Fig. 8G) than in the rostral region (Fig. 8A) and the expression of the *Sonic hedgehog* gene was not detected in the presumptive notochord region in the caudal region (Yoshikawa *et al.,* unpublished data). There is a pair of additional (secondary) neural tubes collaterally which partly fused in the midline (Figs. 8A, 8D, and 8G) (see also Discussion). *In situ* hybridizations to transverse sections showed that there is no obvious change in the expression of the *Zic* genes in the rostral regions where the notochord exists (Figs. 8B and 8C). On the other hand, *Zic* genes expression were changed in the caudal region in the absence of notochord (Figs. 8H and 8I). Namely, the expression was not dorsally restricted and could be detected also in the ventral half of the primary neural tube. Interestingly, the expression of *Zic1, Zic2* could be detected also in the secondary neural tube. In the secondary neural tube, they are expressed evenly without the notochord (Figs. 8H and 8I), whereas, in the presence of notochord, their expression was restricted dorsolaterally (Figs. 8E and 8F) or entirely disappeared (Figs. 8B and 8C). Thus, the dorsally restricted expression of *Zic* seemed to be related with the presence of notochord. Furthermore, the dorsal restriction is consistent with the presence of *Sonic hedgehog* expression as determined by *in situ* hybridization (data not shown). These findings suggest that *Zic1* and *Zic2* genes could be negatively regulated by the factors, possibly the *Sonic hedgehog,* from the notochord.

DISCUSSION

Comparison of the Expression of the Three Zic Genes

We compared the expression of the three mouse *Zic* genes (Table 1). Generally, the three genes are expressed in the dorsal structure of the embryo. From 8.0 to 16.5 dpc, all three genes were expressed in the dorsal one-third of the spinal cord and dorsal mesoderm-derived structure. Although the expression along the dorsal–ventral and the medial–lateral axes was quite similar among the three genes, the expression profile along the anterior–posterior axis

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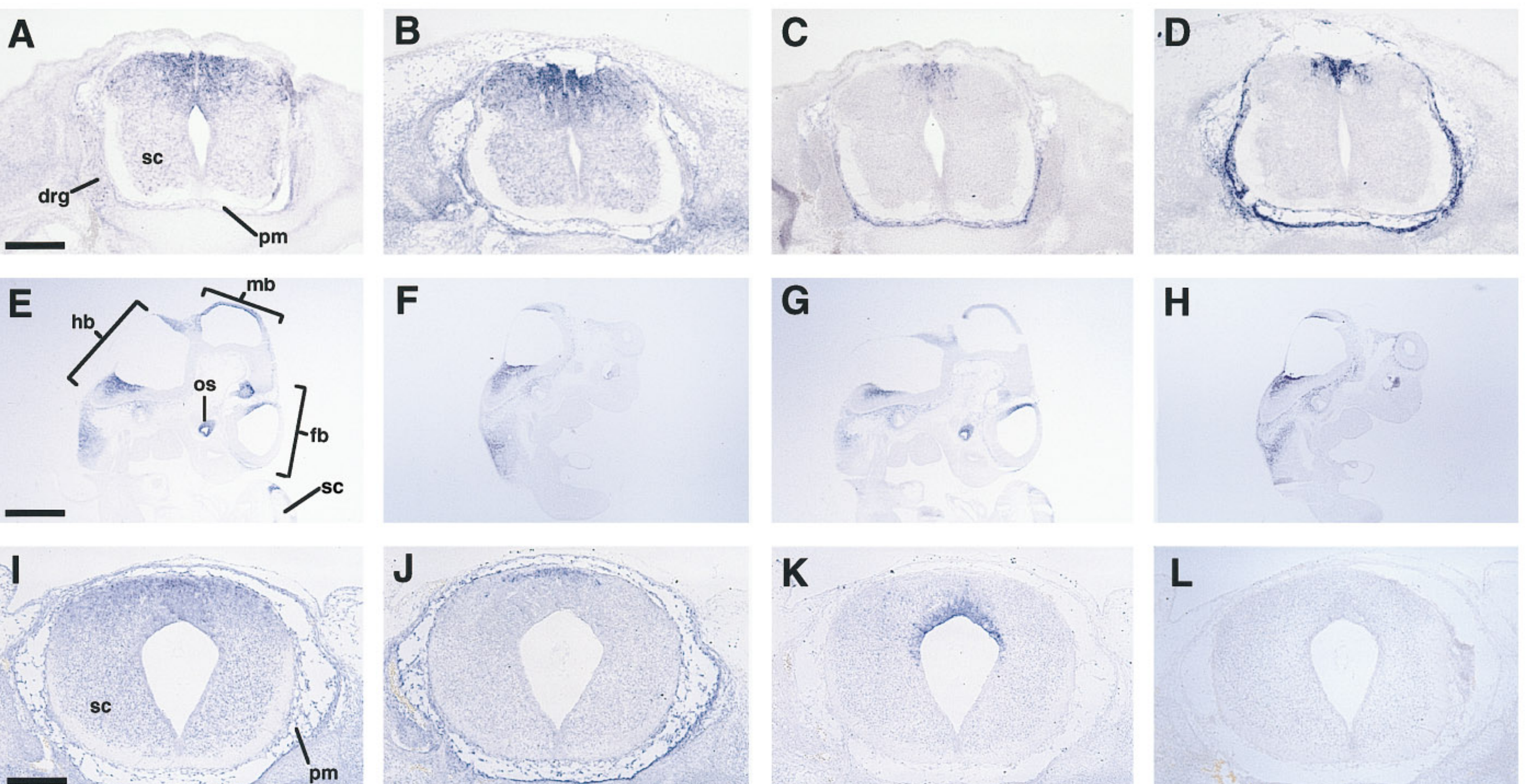

FIG. 7. Expression of Zic1 and Zic2 genes in the neural tube defect mutant mice. Transverse sections through forelimb level of a 13.5 dpc Sp/Sp (B, D), control littermate of Sp/Sp (A, C), opb/opb (I–L), sagittal sections of 11.5 dpc Wnt-1 mutant mice (F, H) and control littermates of Wnt-1 mutant mice (E, G) were hybridized with Zic1 (A, B, E, F, I), Zic2 (C, D, G, H, J), Pax3 (K), and Wnt-1 (L) probes. drg, dorsal root ganglion; fb, forebrain; hb, hindbrain; mb, midbrain; os, optic stalk; pm, primitive meninx; sc, spinal cord. Scale bar, 200 μm (A–D, I–L) or 1 mm (E–H).

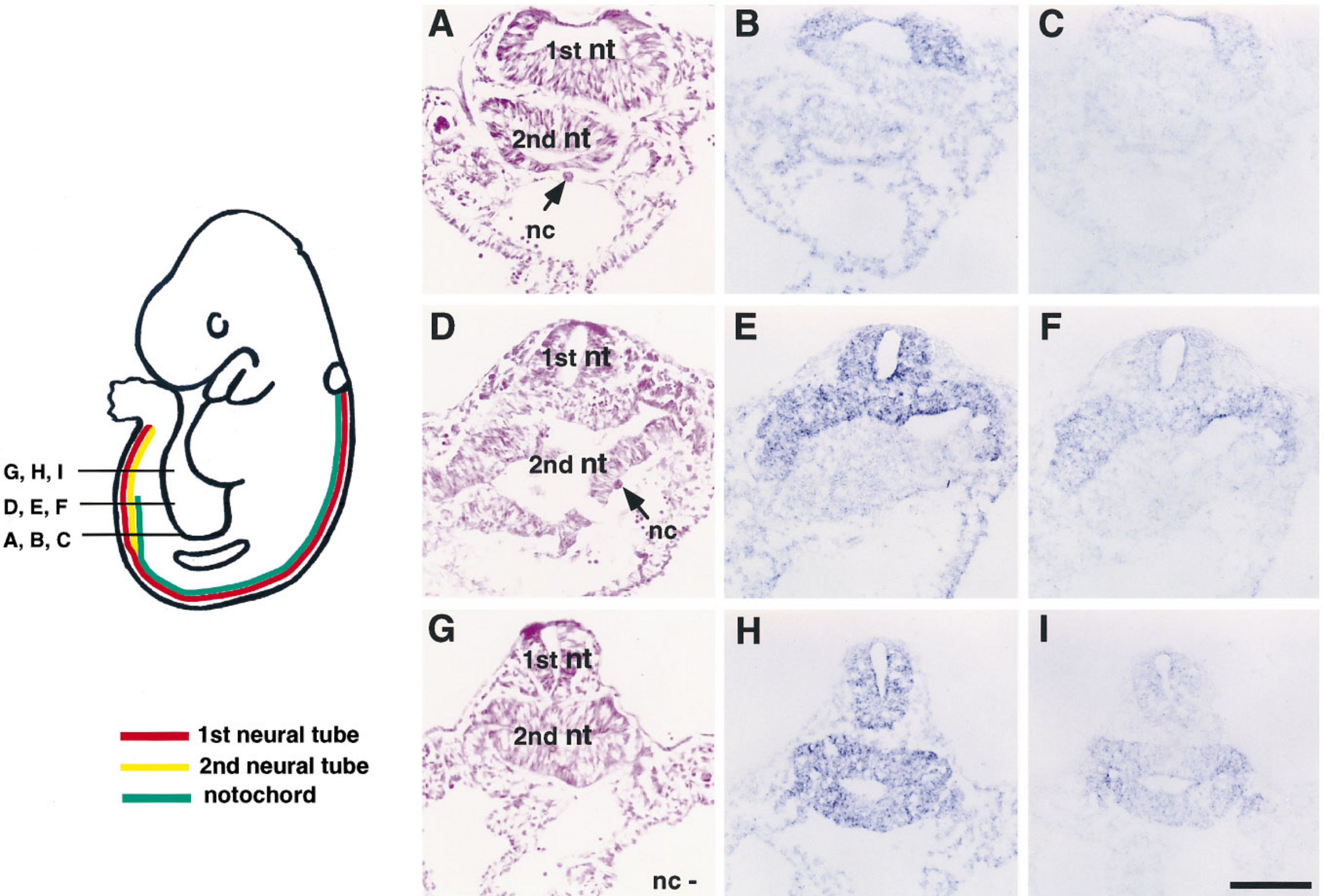

FIG. 8. Expression of Zic1 and Zic2 genes in the caudal region of the 9.5 dpc Wnt-3a mutant mice. The tranverse sections through the caudal trunk region shown in left schematic drawing were hybridized with Zic1 (B, E, H), Zic2 (C, F, I) probes or stained with Haematoxylin and Eosin (A, D, G). Note that sections A, B, C, D, E, and F contain a notochord, whereas G, H, I do not. Sonic hedgehog expression was detected strongly in the level of A, B, C, weakly in the level of D, E, F at the notochord and the neural tube near the notochord, but was not detected in the level of G, H, I by in situ hybridization (data not shown). nc, notochord; 1st nt, authentic (primary) neural tube; 2nd nt, additional (secondary) neural tube. Scale bar, 100 μm.

TABLE 1
Comparative Summary of the Expression of the Three *Zic* Genes in the 9–10 or 14–16 dpc Embryos

Days postcoitum	Tissues	*Zic1*	*Zic2*	*Zic3*
9–10	CNS			
	Forebrain	++[a]	++[a]	+++[a]
	Midbrain	++[a]	++[a]	++[a]
	Hindbrain	+[a]	++[a]	+++[a]
	Spinal cord	+++[a]	++[a]	+[a]
	Eye			
	Optic vecicle	+	+	+
	Optic stalk	++	++	+
	Somite			
	Dermomyotome	+++	++	+++
	Screlotome	+++	+++	−
	Limb			
	Progressive zone	−	+++[a]	++[a]
14–16	CNS			
	Forebrain			
	Olfactory region			
	Olfactory bulb	+++[a]	−	+[a]
	Olfactory epithelium	+	−	−
	Thalamus	+++[a]	+++[a]	+++[a]
	Others	−	−	−
	Midbrain	+++	+++	+++
	Hindbrain			
	Cerebellum	+++	++	+++
	Others	−	−	−
	Spinal cord	+++[a]	+++[a]	−
	Eye			
	Neural retina			
	Outer layer	+++	++	+
	Inner layer	++	+++	+
	Precilialy body	+++	+++	−
	Others	−	−	−
	Primitive meninx	−	++	−
	Limb mesenchyme			
	Premuscle	−	−	+++
	Precartilage	−	+	−
	Others	++	−	−

[a] With distinct spatial distributions.

seemed to be varied. *Zic1* is relatively evenly distributed along the anterior–posterior axis, but no expression was detected in the tail. In contrast, *Zic2* and *Zic3* were highly expressed in the head and tail bud, but weakly in the trunk region. These findings suggested that the three genes could have complementary roles along the anterior–posterior axis. Differences between *Zic1* and (*Zic2* and *Zic3*) were also found in the neuroectoderm formation at primitive streak stage. Both *Zic2* and *Zic3* are expressed in the primitive ectoderm, whereas *Zic1* was not. It was detected after mesoderm and neuroectoderm formation. The induction of *Zic1* expression seems to be closely associated with mesoderm and neuroectoderm formation.

Another novel observation was the expression of the *Zic2* and *Zic3* genes in the limb and presomitic mesoderm. The regions may be the growth centers that exert an important role in vertebrate morphogenesis. The expression pattern of *Zic* genes in the developing limb suggest that *Zic* genes could be involved in pattern formation of limb buds. Here, *Zic2* and *Zic3* were expressed in the distal mesenchyme of the limb and, later, in the mesenchyme at the proximal region of the autopodia. At the early stage (9.5 to 10.5 dpc), the region includes the progress zone, where cells proliferate rapidly in an undifferentiated state. Therefore, *Zic2* and *Zic3* genes may be involved in pattern formation along the distal–proximal axis of the limb. Taken together with the observations that other mammalian homologues of pair-rule genes, such as *evx1* (*even-skipped* homologue) and *prx1*

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(*paired* homologue) are expressed in the distal mesenchyme of the limb bud (Dush and Martin, 1992; Nohno *et al.,* 1993), a group of vertebrate homologues of Drosophila pair-rule genes seems to be in a similar regulatory cascade.

Thus, basically, the three *Zic* genes seemed to be expressed in highly overlapped region. Combining their close structural resemblance (Aruga *et al.,* 1996), roles of the three genes may be similar to each other. However, particularly in the growth center of the mesoderm and in the neuroectoderm formation, the expression profiles suggested that they play different roles.

The Regulation of the Zic Gene in the Vertebrate Body Pattern Formation

We showed that all three genes were expressed in a restricted manner in the central nervous system and highly specifically in the cerebella at adult stage (Aruga *et al.,* 1994, 1996). *Zic1* expression persists in cells of the cerebellar granule cell lineage. Furthermore, the *Zic1* was expressed in the medulloblastoma, the oncogenesis of which may be involved in cells of cerebellar granule cell lineage, whereas it is not expressed in other brain-derived tumors (Yokota *et al.,* 1996). Based on these findings, we speculate that one potential role of the *Zic1* gene is in neural cell fate determination and in the maintenance of the differentiated properties of mature neurons.

This study indicated, however, another potential role of *Zic* genes in body formation. *Zic* genes are expressed in the dorsal area of the developing CNS, paraxial mesoderm, and epidermis. In addition, spatially restricted expression was also identified in the developing limb and eye. These findings suggested that *Zic* genes function in body pattern formation at several sites.

It has been suggested that the dorsal–ventral specification of the spinal cord is influenced by secretory factors from adjacent organs, namely the notochord and dorsal epidermis (Yamada *et al.,* 1991; Dickinson *et al.,* 1995). The expression pattern of the *Zic* genes in the caudal region of the *Wnt-3a* mutant mice without notochord suggests that they are regulated in a similar manner to *Pax3* and *Pax6,* which was shown to be regulated by factors from notochord (Golding *et al.,* 1993). The *Zic1, Zic2* are expressed evenly along the dorsal–ventral axis in the authentic neural tube. Furthermore, the two genes are also expressed evenly along the medial–lateral axis in the secondary neural tube, which may be formed through involution from the primitive ectoderm (Takada *et al.,* unpublished observation), whereas the *Wnt-1* is expressed in the distal side (lateral end of the secondary neural tubes) (Takada *et al.,* 1994). Thus, the dorsally restricted expression of *Zic* genes may be regulated by the factor from notochord. It is essential to reveal that the regulatory factors, such as *Sonic Hedgehog,* is actually involved in the regulation of *Zic* genes, since they are shown to be involved in dorsoventral patterning of the neural tube (Marti *et al.,* 1995; Roelink *et al.,* 1995). In addition to the factor from notochord, the factor from surface ectoderm (Liem *et al.,* 1995) should be examined since it is possible that the factors from two origins act cooperatively.

Analysis of several marker genes, including the three *Zic* genes, revealed characteristic changes within dorsal spinal cord regions of homozygous *opb* mutant embryos: the roof plate and neighboring regions did not become specified properly and, instead, a region of differentiated cells that exhibit dorsolateral characteristics, formed in dorsal-most regions of the mutant spinal cord. The absence of dorsomedial regions (roof plate and adjacent regions) was reflected by the loss of *Wnt-1* and *Wnt-3a,* and a narrower domain of *Zic1* gene expression. *Pax3* expression revealed that the dorsal *opb* spinal cord develops an inner layer of undifferentiated cells and an outer layer of differentiated cells. The changes in *Zic2* expression could thus be explained in the following way: in wild-type embryos, the dorsomedial *Zic2* expression domain overlaps with *Wnt-1-* and *Wnt-3a*-positive regions. These regions are absent in *opb* mutants and, as a consequence, the *Zic2*-positive, dorsomedial expression domain is lost. Similarly, the cells that are present in the adjacent, *Zic2*-negative stripes in wild-type embryos must be absent. This results in the presence of a continuous band of *Zic2*-positive cells which is observed directly underneath the dorsal margin. This *Zic2*-positive band should thus represent differentiating neurons that are normally developing in more lateral regions.

Our observations show that *Zic2* represents a valuable marker gene for dorsal spinal cord regions (differentiating and undifferentiated cells) and that, at 13.5 dpc of mouse development, *Zic2* specifically labels a subpopulation of sensory neurons in the dorsal spinal cord.

Comparison with Drosophila Opa Gene

Drosophila opa, a homologue of the vertebrate *Zic,* is expressed in the visceral mesoderm and in a subset of cells in the neurogenic region (Cimbora and Sakonju, 1995). Compared with *opa* expression in *Drosophila* embryo, *Zic* expression seemed to be analogous if the dorsoventral axis is inverted between insects and vertebrates (Arendt and Nübler-Jung, 1994). Furthermore, the visceral mesoderm is interrupted at many positions along the anterior–posterior axis (Cimbora and Sakonju, 1995) in the *opa* mutant. As in vertebrate *BMP4* and *Drosophila decapentaplegic* (Winnier *et al.,* 1995), in which not only the expression is inverted along the dorsoventral axis but also the similarity between the *Drosophila* and mammalian genes extends to the functional level (Padgett *et al.,* 1993; Sampath *et al.,* 1993), *Zic* genes may be involved in the development of the dorsal mesoderm.

It is attractive to consider the relationship of the *opa* with other *Drosophila* genes whose vertebrate homologues were already identified. The *Drosophila opa* gene regulates the timely activation of *wingless* in the segmental subdivision process (Benedyk *et al.,* 1994). *Decapentaplegic* regulates *opa* expression in visceral mesoderm formation (Cimbora and Sakonju, 1995). The mammalian homologues of both

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genes, *Wnt-1, Wnt-3a, BMP2,* and *BMP4,* are expressed and have significant roles in mouse development. We speculated that they may interact with the *Zic* genes.

The expression of *Wnt-1* gene is restricted to the dorsal midline of the mouse embryo (Shackleford and Varmus, 1987). This study revealed that the region overlaps that where *Zic* genes are expressed. *BMP2* and *BMP4* are involved in the ventral mesoderm development in *Xenopus laevis* and in the mouse (reviewed by Hogan *et al.,* 1994; Kingsley *et al.,* 1994). The *BMP4* transcripts are located in mesoderm around the hindgut and foregut and in the ventral lateral mesoderm from 8.5 to 9.0 dpc (Jones *et al.,* 1991; Winnier *et al.,* 1995). These are exclusive to the dorsally restricted regions of the *Zic* genes at the same developmental stage. Although the localization of *BMP4* protein is not clear yet, we infer that the interaction between *Zic* genes and *BMP2* and *BMP4* genes may exist during the axial mesoderm differentiation. In addition, the *BMP2* and *BMP4* genes are also expressed in the limb bud (reviewed in Hogan *et al.,* 1994) where *Zic2* and *Zic3* are expressed.

In summary, the expression patterns of *Zic* genes suggest that they are involved in body pattern formation at multiple sites as is the case in the *Drosophila opa* gene. Whether or not the genetic interactions observed in *Drosophila* are evolutionally conserved, should be further examined in the sites where *Zic* genes are expressed. The analyses of model animals with a loss or gain of function of these genes, including *Zic,* would be helpful in revealing such interactions and definite role of *Zic* genes.

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