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Paraxial-Fated Mesoderm Is Required for Neural Crest Induction in *Xenopus* Embryos

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Neural crest induction is thought to occur by a two-step process. Axially fated mesoderm induces neural plate, which is then recruited to neural crest by nonneural epidermal ectoderm at the neural plate border. This model suggests a rather indirect role for mesoderm in inducing neural crest. We extensively examined the role of mesoderm in neural crest induction by determining which types of mesoderm induce neural crest cells in *Xenopus* embryos. We found that noggin-dorsalized ventral marginal zone (VMZ) explants differentiate as melanocytes in the absence of axial mesoderm. Dorsalized VMZ is also a potent inducer of melanocytes when juxtaposed to animal cap ectoderm in recombinant explants. Dorsalized VMZ is analogous to the dorsal–lateral marginal zone (DLMZ) region of the embryo. Neural crest-inducing activities of gastrula stage DLMZ and dorsal marginal zone (DMZ) were also compared in recombinant explants. DLMZ was a stronger inducer of neural crest than was DMZ; DLMZ induced high levels of XSlug expression and melanocyte formation in recombinants, whereas DMZ weakly induced neural crest. In whole embryos lacking DLMZ, XSlug expression and melanocyte formation were significantly reduced; in contrast, no significant reduction of XSlug expression or melanocyte formation was seen in embryos lacking a DMZ. These results suggest that paraxial-fated mesoderm plays a central role in neural crest formation by inducing a novel type of lateral neural plate. This lateral neural plate is then recruited to neural crest by adjacent nonneural epidermal ectoderm. © 1998 Academic Press

Key Words: Xenopus laevis; **paraxial mesoderm; neural crest induction; melanocytes; neural induction.**

INTRODUCTION

In *Xenopus* embryos, the central nervous system forms by a multistepped inductive process (reviewed in Doniach, 1993; Harland, 1994; Kelley and Melton, 1995). The most dorsal mesoderm (Spemann organizer) is thought to induce neuroectoderm in adjacent ectoderm cells, thus distinguishing these cells from the nonneural ectoderm. After this initial neural induction, which is of an anterior nature, patterning becomes specified along the anteroposterior axis of the developing neural plate (reviewed in Doniach, 1993). The inductive signals regulating anteroposterior axis formation in the neural plate may be derived from inductive events occurring in the plane of contact between presumptive neural plate and dorsal mesoderm (vertical signals), or these inductive signals may also emanate from the underlying involuted mesoderm to the adjacent overlying presumptive neural plate (horizontal signals) (Doniach, 1993).

A number of secreted molecules which are expressed in Spemann's organizer and can induce anterior neural tissue have been found, such as noggin, follistatin, and chordin (Lamb *et al.,* 1994; Hemmati-Brivanlou *et al.,* 1994; Sasai *et al.,* 1994). While these molecules are structurally diverse, they all have one common target, they antagonize the activity of BMP-4[2] (Wilson and Hemmati-Brivanlou, 1995; Sasai *et al.,* 1995; Re'em-Kalma *et al.,* 1995; Zimmerman *et al.,* 1996; Piccolo *et al.,* 1996). Antagonism of BMP-4 signaling in ectoderm suffices to convert epidermally fated nonneural ectoderm to neural fates (Wilson and Hemmati-Brivanlou, 1995). Recently, bFGF was shown to act as a caudalizing agent which converts anterior-fated neural tissue to more posterior fates (Kenkgaku and Okamoto, 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995). This combination of BMP-4 inhibition and FGF signaling may establish anteroposterior polarity in the neural plate (reviewed in Doniach, 1995).

The next step in nervous system demarcation is the in-

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[2] Abbreviations used: VMZ, ventral marginal zone; DLMZ, dorsal–lateral marginal zone; DMZ, dorsal marginal zone; AC, animal cap; BMP-4, bone morphogenetic protein 4; DNR, dominant-negative receptor.

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duction of dorsoventral polarity within the developing spinal cord. The notochord plays a central role in regulating this dorsoventral polarity. Signals emanating from the notochord induce ventral structures such as floor plate and motor neurons, while inhibiting formation of dorsal markers (reviewed in Jessell and Dodd, 1993). The Sonic Hedgehog protein, which is secreted from the notochord, is likely the inducer of ventral neural tube (reviewed in Smith, 1994).

Less is known about the signals regulating dorsal neural tube and neural crest cell formation. Neural crest tissue is derived from ectodermal cells that are not included in the developing dorsal neural tube or epidermis. These cells, which originate at the edge of the neural plate, give rise to a wide range of tissues, such as pigmented melanocytes, peripheral nervous system, adrenal glands, heart smooth muscle, and craniofacial cartilage/bone. While much information has accumulated about neural crest migration in vertebrates, little is known about the early inductive processes which instruct a cell to become neural crest. Experiments with recombinant chick and frog explants have shown that when neural plate cells are juxtaposed to nonneural epidermal ectoderm, the neural plate cells are redirected to neural crest fates (Selleck and Bronner-Fraser, 1995, 1996; Dickinson *et al.,* 1995; Mancilla and Mayor, 1996). It has also been reported that BMP molecules secreted from the epidermal ectoderm can redirect neural plate cells to dorsal cell fates such as neural crest (Liem *et al.,* 1995). These experiments support a two-step model of neural crest induction (reviewed in Kelley and Melton, 1995; Selleck and Bronner-Fraser, 1996). In the first inductive step, the axial mesoderm (Spemann's organizer) fated for notochord induces neural plate in the adjacent ectoderm. This neural plate then receives a lateral signal from the nonneural ectoderm which converts cells at the neural-plate epidermal border to neural crest. This model suggests a rather passive role for mesoderm in neural crest induction; the initial induction of neural plate by the axial mesoderm is the only mesoderm/ectoderm interaction required for neural crest induction.

Thus, little is known about the role of mesoderm in regulating neural crest induction in the ectoderm. Some studies suggested that dorsal mesoderm participates in neural crest induction (Raven and Kloos, 1945; Mayor *et al.,* 1995), but these experiments did not clearly distinguish between paraxial (fated for muscle) and axial mesoderm (fated for notochord) as neural crest inducers. In contrast, another study has suggested that ventrally fated mesoderm is the most efficient inducer of neural crest-derived cells such as melanocytes (Mitani and Okamato, 1991).

No study has conclusively determined which type of mesoderm is required for induction of neural crest in nonneural ectodermal cells. In this study, we have utilized recombinant explants made between gastrula stage mesoderm and naive animal cap ectoderm to extensively examine the role of mesoderm in regulating neural crest cell induction in *Xenopus* embryos. Our results suggest an active role for paraxial mesoderm and not axial or ventral mesoderm in the induction of neural crest in the developing *Xenopus* embryo.

MATERIALS AND METHODS

Xenopus Embryos, Explants, and Inducing Factors

Ovulation, *in vitro* fertilization, embryo culture, and dissections were carried out as described (Frank and Harland, 1992). Embryos were staged according to Nieuwkoop and Faber (1967). Recombinant explants were dissected using an eyebrow knife and grown in 1× Steinberg's medium (Peng, 1991) and gentamycin (50 μg/ml). Ventral marginal zone (VMZ) pieces (45–60° in size) were removed at stages 10+ to 10.25, when a pigment line is formed at the dorsal lip by involuting bottle cells, thus enabling identification of presumptive dorsal and ventral sides. A 45–60° piece of dorsal marginal zone (DMZ) was dissected at stages 10.25–10.5. For removal of stage 11.25 DMZs, stage 10+ to 10.25 embryos were marked at the blastopore lip with Nile blue and allowed to develop until stage 11.25, when a 45° piece of the organizer was removed. Dorsal–lateral marginal zone (DLMZ) represents a 45–60° piece of the marginal zone taken approximately 30–90° lateral to the dorsal midline. Animal caps (ACs) were removed at blastula stages 8–9.5. Recombinants were juxtaposed to one another with watchmaker's forceps and grown to the different stages as described in the text. Marginal zone explants used as inducer tissues were made from homozygous albino embryos in which albino eggs were fertilized with sperm from an albino male, thus no background of melanocytes was visually detected. ACs were usually derived from heterozygous albino embryos in which albino eggs were fertilized with sperm from a pigmented male. This facilitated easy visual detection of melanocytes on a light background. Occasionally, lightly pigmented ACs were used (see text). LiCl treatment was carried out at the 64-cell stage. Embryos were immersed in 0.3 M LiCl in $\frac{1}{3}\times$ modified Ringers for 12 min. The embryos used in the experiments had a dorsal–anterior index (Kao and Elinson, 1988) of >8.5.

Noggin was prepared from CHO-B3 cell line-conditioned medium (Lamb *et al.,* 1994). Noggin-treated ($\frac{1}{4}\times$ CHO-B3 conditioned medium) VMZ explants were grown in 1× strength low calcium–magnesium modified Ringer's (LCMR; Stewart and Gerhart, 1990) and gentamycin from stage 10+ to 10.25 until stage 12.5 to 13; at this stage, explants were transferred to fresh 1× Steinberg's medium lacking noggin for further growth. Control explants were grown in 1× LCMR with $\frac{1}{4}\times$ conditioned medium from the non-noggin-producing parental CHO cell line.

RNA Microinjection

Capped synthetic *Xenopus* BMP-4 dominant-negative receptor RNA (Graff *et al.,* 1994) was prepared according to Smith and Harland (1991). RNA (0.2–0.4 ng) was injected marginally into embryos at the one-cell stage.

Northern Blot Analysis, in Situ Hybridization, and Lineage Tracing

RNA was extracted from embryos and prepared for Northern blot analysis as described (Frank and Harland, 1992). Electrophoresis, probe preparation, filter hybridization, and exposure were performed as described (Re'em-Kalma *et al.,* 1995). Quantitation was performed on a Fuji phosphoimaging system. EF1α was used as a

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positive standard for comparing levels of RNA loaded per well at any given stage (Krieg *et al.*, 1989). Relative expression in explants and whole embryos was calculated in the following manner: the integrated numerical value for a gene to be examined was divided by the integral value of EF1α. This number was set at 1 for whole embryos to define a correction factor for an individual experiment. This correction factor could then be applied for other samples analyzed on the same filter. Values above 1 are interpreted to indicate that a transcript is enriched in these samples relative to whole embryo mRNA. Whole-mount *in situ* hybridization was carried out with digoxigenin-labeled probes (Hemmati-Brivanlou *et al.*, 1990a; Harland, 1991; Knecht *et al.*, 1995). For lineage tracing analysis, one-cell-stage albino embryos were injected in the animal hemisphere with RNA encoding β-galactosidase (β-gal; Smith and Harland, 1991). Recombinant explants were made using ACs from β-gal-injected albino embryos and DLMZs or DMZs from uninjected albino embryos. At midneurula stages, embryos were fixed in MEMFA (0.1 M Mops, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 20 min, washed three times with PBS (phosphate-buffered saline), and then added to a β-gal reaction mix containing the red staining reagent 6-chloro-3-indolyl-β-D-galactopyranoside. After staining, embryos were washed three times in PBS and refixed for another hour. Embryos then underwent whole-mount *in situ* hybridization with the XSlug probe.

RESULTS

Melanocytes and Neural Markers Are Induced in Noggin-Dorsalized VMZ Explants

VMZ explants were removed from gastrula stage embryos (stage 10+ to 10.25) and dorsalized by noggin. Noggin-treated VMZs were cultured until stage 39–41 to score for melanocytes; in parallel, RNA was isolated for Northern analysis at neurula to tailbud stages. Noggin-dorsalized VMZ explants differentiated high levels of melanocytes in comparison to control VMZ explants (Figs. 1A and 1B). By Northern analysis of dorsalized VMZ explants in comparison to untreated controls (Fig. 1C), we detected elevated expression of general neural markers such as NCAM, XIF-3, and nrp-1 (Kintner and Melton, 1987; Sharpe *et al.*, 1989; Knecht *et al.*, 1995) and neuron-specific markers such as Hoxb9 and n-tubulin (Wright *et al.*, 1990; Richter *et al.*, 1988), but not the XAG-1 cement gland marker (Sive *et al.*, 1989). Dorsalized VMZs expressed the muscle-specific actin and XMyoD genes (Smith *et al.*, 1993). Epidermal cytokeratin was moderately downregulated in dorsalized VMZs. In AC ectoderm, noggin induces anterior neural tissue and cement gland structures (Lamb *et al.*, 1994); melanocytes are not detected (personal observation). Hoxb9 and n-tubulin are also not expressed in noggin-neuralized ACs (Lamb *et al.*, 1994). Thus the induction of neural crest-derived cells (melanocytes) and neuroblast markers (n-tubulin and Hoxb9) by noggin in VMZs is a unique phenomenon which differs from the anterior induction observed in noggin-neuralized ACs.

Dorsalized VMZs Induce Melanocytes in Naive AC Ectoderm

VMZ explants contain an epithelial layer of mesoectoderm tissue. It is possible that noggin-dorsalized mesoderm (dorsal–lateral mesoderm fated for muscle) is sending a secondary inducing signal to the overlying ectoderm in the VMZ and converting it into neural crest; thus noggin itself is not the direct inducer of neural crest in the VMZ. Therefore, it may be possible to induce melanocytes in naive ectoderm in the absence of noggin. Two sets of experiments were designed to address this question. In the first experimental set, gastrula stage VMZs were noggin-treated until early neurula stages; noggin was removed from the medium, and explants were washed and grown for 2 additional hours. At this time, dorsalized VMZs were bisected (exposing the internal mesoderm) and recombined with blastula-stage ACs. Recombinants were grown to tadpole stages to score for melanocytes. Injection of the BMP-4 dominant-negative receptor (DNR) mRNA has the same effect as noggin in dorsalizing VMZs, since noggin dorsalizes the VMZ by inhibiting endogenous BMP-4 activity (Graff *et al.*, 1994; Re'em-Kalma *et al.*, 1995; Zimmerman *et al.*, 1996). Therefore, in the second experimental set, VMZs taken from embryos injected with the BMP-4 DNR mRNA were recombined with blastula stage ACs.

In both experimental systems, the inducing tissue is taken from a homozygous albino embryo (Fig. 2A, see Materials and Methods). The induced AC ectoderm is usually taken from a heterozygous albino (Fig. 2A). Therefore, black melanocyte cells exclusively induced in the naive AC ectoderm can be visually detected with ease on the white albino background. VMZs dorsalized by noggin (compare Figs. 2B and 2C) or by the BMP-4 DNR (Figs. 2D and 2E) induced melanocytes in a nearly identical manner; 75% of the dorsalized VMZ–AC recombinant explants formed melanocytes (Table 1) versus 14% in the control VMZ–AC explants (Table 1). Melanocyte-positive control VMZ–AC explants apparently underwent autodorsalization to muscle, since melanocytes were only detected in control VMZ–AC recombinant explants expressing muscle actin mRNA (not shown). We have observed this autodorsalization phenomenon in 5–15% of the untreated VMZ explants in any given experiment (personal observation). Thus, dorsalized VMZ, but not noggin, is a potent inducer of melanocytes in juxtaposed AC tissue.

Paraxial-Fated Mesoderm Is a Strong Inducer of Neural Crest in Contrast to Axial-Fated Mesoderm

Dorsalized VMZ is analogous to the normal dorsal–lateral marginal zone (DLMZ–paraxial mesoderm) region of the embryo which is fated to make muscle. Models of neural crest induction suggest that the main inducer of neural crest is the dorsal marginal zone–Spemann organizer (DMZ–axial mesoderm) region which is fated to make notochord. To clarify this point, we compared the neural crest-inducing activities of gastrula stage DLMZ and DMZ tissues. In these experiments, DLMZs or DMZs from homozygous albino embryos were recombined with heterozygous albino blastula stage ACs and scored for melanocytes at tadpole stages. Melanocytes were observed in 72% of DLMZ–AC recombinant explants (Fig. 3A, Table 1). In con-

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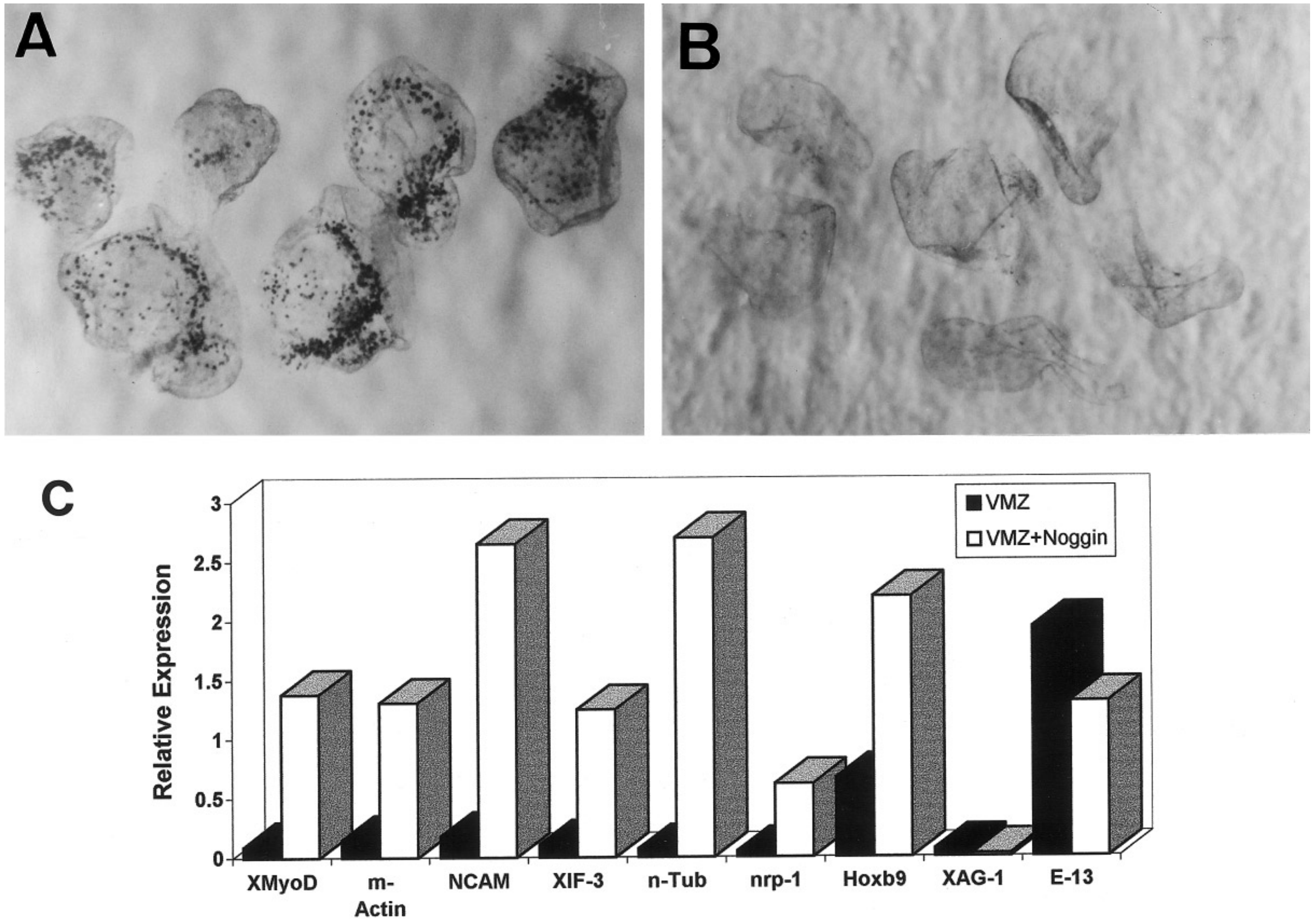

FIG. 1. Melanocytes and neural markers are induced in noggin-dorsalized VMZ explants. In a representative experiment, 36 VMZ explants were removed from stage 10+ to 10.25 embryos. Eighteen explants were noggin-treated until stage 12.5–13 and the 18 remaining explants served as controls. All explants were grown to stage 41 and scored for melanocytes. Approximately 75% of the noggin-treated VMZs made melanocytes in comparison to 5% of the controls. Embryos were fixed in MEMFA according to Hemmati-Brivanlou *et al.* (1990a) and cleared in 2:1 benzyl benzoate:benzyl alcohol before photography. (A) Six representative noggin-dorsalized VMZ explants. (B) Six representative control VMZ explants. (C) Induction of neural markers in dorsalized VMZ explants. Eighteen VMZ explants were removed from embryos at stage 10+ to 10.25. Nine explants were noggin-treated until stage 12.5–13, and 9 untreated explants served as controls. Explants were grown to several stages (20, 26, 36, and 41), and total RNA was isolated for Northern analysis. Total RNA was also isolated from pools of five whole embryos. All 9 explants and one embryo equivalent of RNA were loaded per well. Filters were sequentially hybridized with *Xenopus* cDNA probes for muscle-specific actin (m-actin), XMyoD, NCAM, nrp-1, XIF-3, n-tubulin, Hoxb9, XAG-1, E13 (epidermal cytokeratin), and EF1α. The results from stage 26 explants are shown; similar results (not shown) were observed at stages 20, 36, and 41. All data were quantitated as described under Materials and Methods.

trast, DMZ–AC recombinants rarely made melanocytes, which were detected in only 15% of the explants (Fig. 3B, Table 1). There was also a striking quantitative difference between melanocyte-positive DLMZ–AC and DMZ–AC recombinant explants. Over 75% of the melanocyte-positive DLMZ–AC explants made large patches of melanocytes (Fig. 3A). In contrast less than 25% of the melanocyte-positive DMZ-AC explants made large patches of melanocytes; most of these explants contained a sparse pattern of melanocyte cells (not shown). The DMZ–AC explants which made low levels of melanocytes also expressed intermediate levels of muscle actin mRNA (not shown). Dissection error or blastopore formation 5–10° left–right of the dorsal midline (Keller, 1991) could account for some DMZs inducing melanocytes. This inductive potential is also not dependent on the anteroposterior nature of the DMZ, since a more posterior organizer (stage 11.25 DMZ) did not induce melanocytes in ACs (Table 1).

The XSlug gene was shown to be the earliest marker of presumptive neural crest cells in *Xenopus* embryos (Mayor *et al.*, 1995). By Northern analysis, we examined expression of XSlug mRNA in neurula-stage recombinant explants. XSlug expression was induced 10-fold higher in DLMZ–AC recombinant explants versus DMZ–AC recombinant

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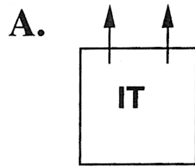
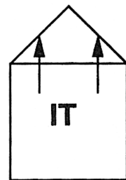
A.

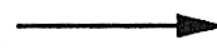


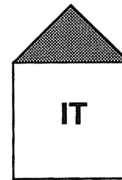
Inducing tissue (IT) is from a homzygous albino embryo.

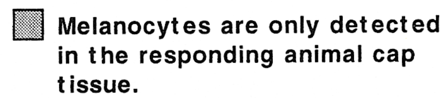
Responding animal cap tissue is derived from an albino egg that was fertilized with sperm from a pigmented male.

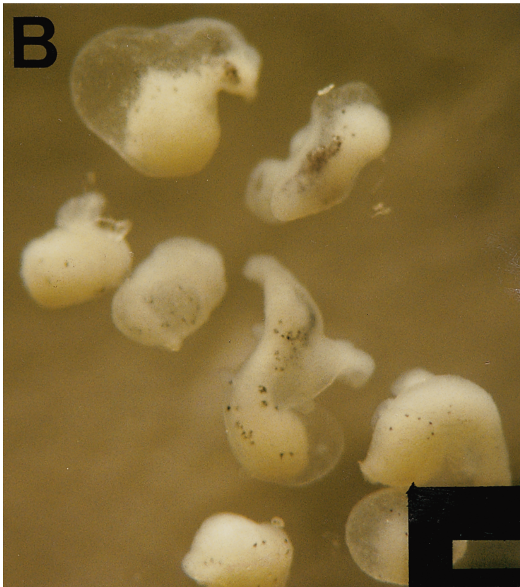


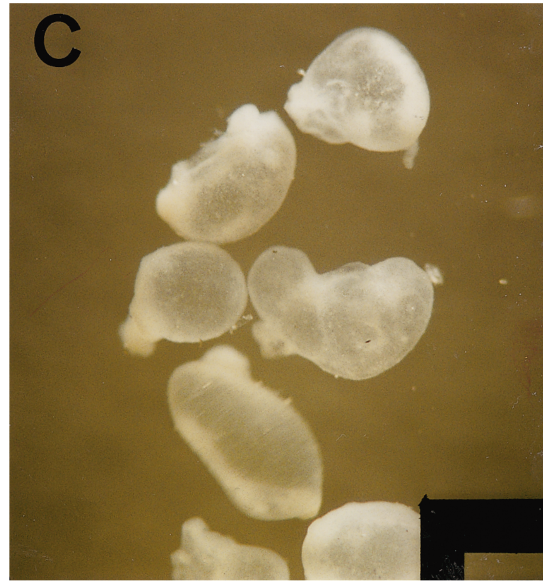
Grown to stages 39 - 41

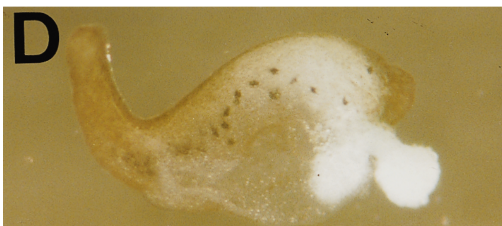












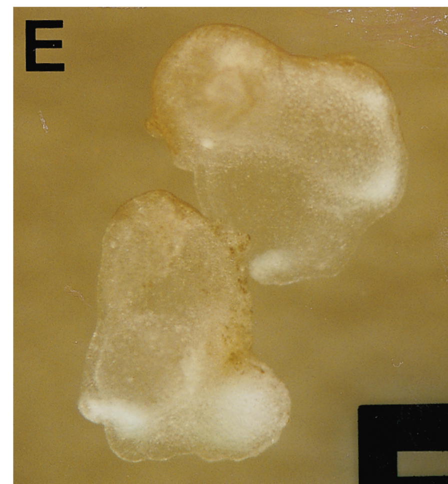


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TABLE 1
Induction of Melanocytes by Mesoderm

Embryos and recombinant explants	Melanocyte positive	% Melanocyte positive
VMZ noggin-AC	12/16	75
VMZ BMP4 DNR-AC	24/32	75
VMZ-AC	7/50	14
DLMZ-AC	70/98	72
DMZ-AC	13/88	15
DMZ-AC (st. 11.25)	0/18	0
Embryos (-DLMZ)	9/32	28
Embryos (-DMZ)	32/32	100

explants (Fig. 3C). The DLMZ–AC recombinant expressed over 5-fold higher XSlug levels than background levels in the nonrecombined DLMZ (Fig. 3C). Lineage tracing analysis was performed to show that XSlug expression in recombinant explants is AC specific. The explants were made using ACs derived from albino embryos injected with RNA encoding β-galactosidase (Smith and Harland, 1991) and DLMZs from uninjected albino embryos (Materials and Methods). By *in situ* hybridization, we determined that the XSlug expression in recombinant explants was like melanocyte induction, AC specific. The purple alkaline phosphatase staining from XSlug hybridization was always detected in the red β-gal-stained animal cap cells, but not in the unstained DLMZ (Fig. 3D).

Expression of the cranial neural crest marker, Xtwi (Hopwood *et al.,* 1989), was also stimulated 4-fold in DLMZ–AC neurula-stage explants in comparison to DLMZs alone or DMZ–AC explants as determined by Northern analysis (not shown). XAG-1, an anterior cement gland marker was only expressed in DMZ–AC recombinants (Fig. 3C) and was stimulated about 18-fold versus background levels in the nonrecombined DMZ (Fig. 3C). While both the DMZ and the DLMZ differed as inducers of XSlug and XAG-1, they both induced panneural markers such as nrp-1 (Fig. 3C) and NCAM (not shown) at strikingly equal levels; nrp-1 was stimulated about 3-fold in recombinant explants versus background levels in the nonrecombined DMZ and DLMZ (Fig. 3C). As a control for dissection accuracy, mesoderm markers were also examined in late neurula DLMZs and DMZs. The DLMZ expressed muscle actin but the DMZ did not (Figs. 3C). The DMZ, however, expressed high levels of the notochord-specific collagen type II (Amaya *et al.,* 1993) mRNA (not shown). These results suggest that paraxial-fated mesoderm is a strong inducer of neural crest cells in naive AC ectoderm and that axial fated mesoderm is a weak neural crest inducer.

Neural Crest Formation Is Reduced in Whole Embryos Lacking Paraxial Mesoderm

We examined melanocyte formation and XSlug expression in whole embryos lacking either axial or paraxial mesoderm. In these experiments, both DLMZs were or the DMZ was removed by dissection from embryos at stage 10.25–10.5 (Fig. 4A). Embryos developed until stage 39–41 (Fig. 4B) and were scored for melanocytes. DMZ removal from embryos caused anterior-truncated embryos (reduced eyes and cement gland) containing large numbers of trunk melanocytes (Fig. 4D; Table 1). In all embryos examined, neural crest-derived melanocytes were detected; we never observed an embryo lacking DMZ that did not make melanocytes. Embryos lacking DLMZ formed fairly normal heads about two-thirds of the time (eyes and cement gland), but lacked melanocytes along the reduced body axis (Fig. 4C). Only 28% of these embryos made melanocytes (Table 1); melanocytes were present at reduced levels and usually on only one side of the embryo. These melanocyte-positive embryos also twitched, suggesting that despite microsurgical removal, some DLMZ-fated muscle cells remained on one side of the embryo (personal observation). In neurula-stage embryos lacking a DLMZ, XSlug expression is reduced about 2.5-fold and muscle actin expression is reduced about 4.5-fold in comparison to control embryos or embryos lacking a DMZ (Fig. 5A). Nrp-1 expression was not altered significantly in any of the manipulated embryos (Fig. 5A). By whole-mount *in situ* hybridization, we also examined spatial expression of XSlug in perturbed neurula stage embryos. Embryos lacking a DMZ usually have two sharp regions of XSlug expression (Fig. 5C), which appear analogous to the

FIG. 2. Dorsalized VMZ induces melanocytes in naive animal cap ectoderm. (A) Experimental strategy: VMZs were taken from homozygous albino embryos and recombined with AC ectoderm from heterozygous albino embryos (see Materials and Methods) or AC ectoderm taken from lightly pigmented embryos. This ensures that melanocytes detected in the recombinant explant AC are exclusively a result of induction. The use of heterozygous albino or lightly pigmented ACs enables easy visualization of black melanocytes on a light background. In a routine experiment 18 recombinant explants were compared between each group. In none of the experiments did ACs ever spontaneously differentiate as melanocytes. (B) VMZs were noggin-treated, washed, and recombined with ACs as described in the text. Seven representative recombinant explants (stages 39–41) are shown. (C) Control VMZs that underwent a parallel treatment in the absence of noggin were recombined with ACs as described in the text. Seven representative recombinant explants (stages 39–41) are shown. (D) Homozygous albino embryos were injected with 0.2–0.4 ng of BMP-4 DNR RNA. VMZs were removed at stage 10.25 and recombined with ACs taken from lightly pigmented embryos. Explants were grown to stages 39–41. Melanocytes are observed in trunk-shaped explants (top) as well as cylindrical shaped explants (bottom). (E) Control VMZs were removed at stage 10.25 and recombined with ACs derived from lightly pigmented embryos. Explants were grown to stages 39–41. These explants elongated less than those shown in Fig. 4D and did not differentiate melanocytes or make muscle (not shown).

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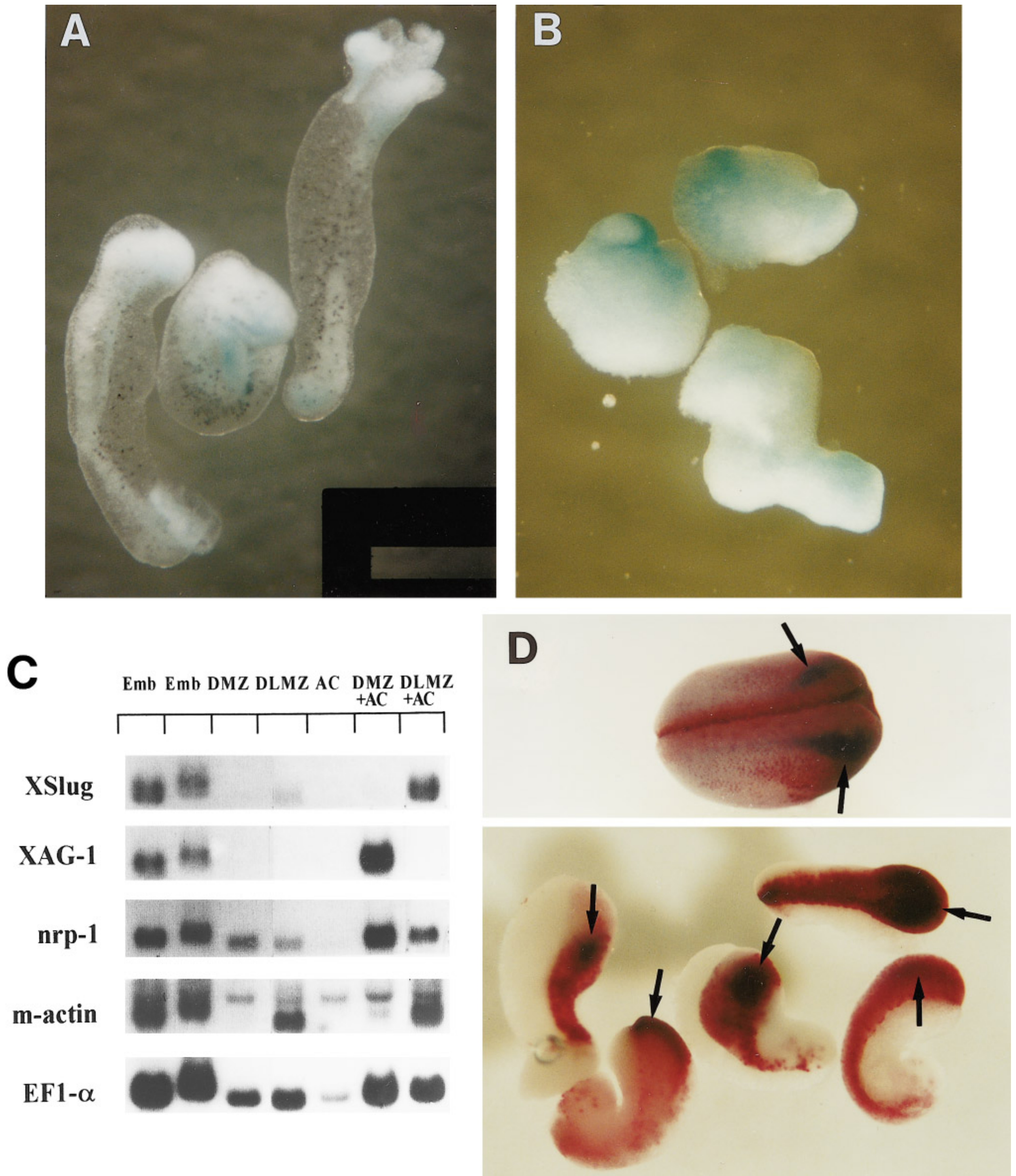

FIG. 3. Paraxial-fated mesoderm is a strong inducer of neural crest cells. Stage 10.25–10.5 DLMZs and DMZs were removed from homozygous albino embryos and recombined with blastula stage ACs. DLMZs, DMZs, and ACs were also removed in parallel as controls. In a routine experiment, 18 explants from each group were scored for melanocytes at stage 41, and 9 explants from each group were grown until stage 17 for Northern analysis. (A) Three representative DLMZ–AC recombinant explants are shown. (B) Three representative DMZ–AC recombinant explants are shown. (C) Northern analysis of recombinant DLMZ and DMZ explants. Nine explant equivalents and one embryo equivalent (from a pool of five control embryos) of RNA were loaded per well. Filters were sequentially hybridized with *Xenopus*

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neural folds region of the normal embryo (Fig. 5B). Embryos lacking a DLMZ have a more diffuse and less intense XSlug expression pattern, which is usually found in one neural-fold-like region of the embryo (Fig. 5D). Perturbed and normal embryos make neural plate in a similar spatial manner; large patches of nrp-1-expressing cells were detected in the neural plate (not shown).

XSlug expression was also examined in embryos dorsally radialized by LiCl treatment at the 64-cell stage. LiCl-hyperdorsalized embryos have an expanded DMZ region and a reduced DLMZ region (Kao and Elinson, 1988). Hyperdorsalized embryos expressed about threefold less XSlug and muscle actin mRNAs than normal embryos (Fig. 5A); nrp-1 expression was not altered in these embryos (Fig. 5A).

These results show that paraxial-fated mesoderm is required for normal neural crest formation in whole embryos and that axial-fated mesoderm is an inefficient inducer of neural crest-derived cells. As previously described, neural crest induction is generally thought to take place by a two-step pathway (Fig. 6A). Our results suggest at least two variations to this existing model. The paraxial-fated mesoderm may either induce a novel type of lateral neural plate or modify a preexisting neural plate that was induced by the organizer. This novel neural plate is then recruited to neural crest by adjacent nonneural epidermal ectoderm (Fig. 6B). Alternatively, paraxial mesoderm may directly induce neural crest in the overlying neural folds region (Fig. 6C).

DISCUSSION

Noggin-Dorsalized Ventral Marginal Zone Differentiates Melanocytes and Neuroblasts

Our data show that paraxial-fated dorsal–lateral mesoderm is a strong inducer of neural crest cells. We initially observed formation of melanocytes and induction of neuroblast-specific markers in VMZs dorsalized by noggin (Fig. 1). Unlike ACs neuralized by noggin, dorsalized VMZs do not express anterior cement gland markers such as XAG-1 but express neuron-specific markers such as n-tubulin and Hoxb9 (Fig. 1C); in addition, we never observe formation of melanocytes in noggin-neuralized ACs. Thus, the noggin-triggered neural induction in the VMZ appears to differ from that of the noggin-neuralized AC. Since the VMZ contains cells of noninvoluting epidermal ectoderm, it is possible that noggin induces unique neural tissue in this ventral ectoderm. However, this seems unlikely, because studies comparing noggin-treated UV-ventralized ACs (ventral ectoderm) with normal ACs showed that both of them were induced to anterior neural tissue (Knecht *et al.,* 1995). We have also never seen induction of melanocytes in noggin-treated UV ACs (unpublished observations).

Dorsalized Ventral Marginal Zone Induces Melanocytes in Naive Ectoderm

It is possible that the dorsalized mesoderm is secondarily inducing neural tissue in the overlying ventral ectoderm in the VMZ explants. To address this question, recombinant explants were made between naive AC ectoderm and VMZs dorsalized by either noggin or a BMP-4 dominant-negative receptor. In both cases (Table 1), the dorsalized VMZs induce melanocytes in 75% of the juxtaposed ACs, in comparison to 14% in the control VMZ–AC recombinants (these 14% have undergone autodorsalization, see Results). These results show that noggin is not directly involved in inducing neural crest cells; it likely acts by dorsalizing ventral mesoderm into a strong inducer of neural crest. A combination of bFGF and noggin in ACs leads to activation of the early neural crest marker XSlug (Mayor *et al.,* 1995). Quite possibly a similar secondary induction was observed in these experiments; bFGF induced ventral mesoderm in the ACs and noggin dorsalized these caps to paraxial mesoderm. Noggin can dorsalize FGF-treated ACs to muscle (Umbhauer *et al.,* 1995) and this dorsalized mesoderm could have induced remaining ectoderm cells in the AC to neural crest.

Mitani and Okamoto (1991) claim that the VMZ is the best inducer of melanocytes. In these studies, VMZ and ectoderm explants were dispersed, remixed, and cocultured on plastic tissue culture dishes. It is quite possible that the cell dispersal and growth conditions caused the VMZs to dorsalize to DLMZs by disrupting BMP-4 signaling, since AC ectoderm can autoneuralize after cell dispersion as a result of inhibited BMP-4 signaling (Wilson and Hemmati-Brivanlou, 1995). This point is not clarified in their work and thus their data are difficult to interpret. However, our data show that undispersed VMZs cannot induce melanocytes in ectoderm unless they have been dorsalized by noggin or the BMP dominant-negative receptor (Fig. 2).

Paraxial-Fated Mesoderm Is a Strong Inducer of Neural Crest Cells—Axial-Fated Mesoderm Is a Weak Inducer

Since dorsalized VMZ is analogous to the embryonic DLMZ, we examined whether normal DLMZ is a strong inducer of neural crest cells. We compared the neural crest-inducing ability of the DLMZ versus the DMZ, since the

cDNA probes for XSlug, XAG-1, nrp-1, muscle-specific actin (m-actin), and EF1α. On all filters, the muscle-specific actin is the lower band; the upper band is cross-hybridization to cytoskeletal actin. (D) Whole-mount *in situ* hybridization with XSlug was performed on mid-neurula-stage embryos and explants (see Materials and Methods). Top: XSlug expression in a mid-neurula-stage embryo injected with β-gal, dorsal view. Bottom: DLMZ–AC recombinant explants in which AC cells were injected with β-gal; the arrows show the purple alkaline phosphatase staining on the red β-gal background.

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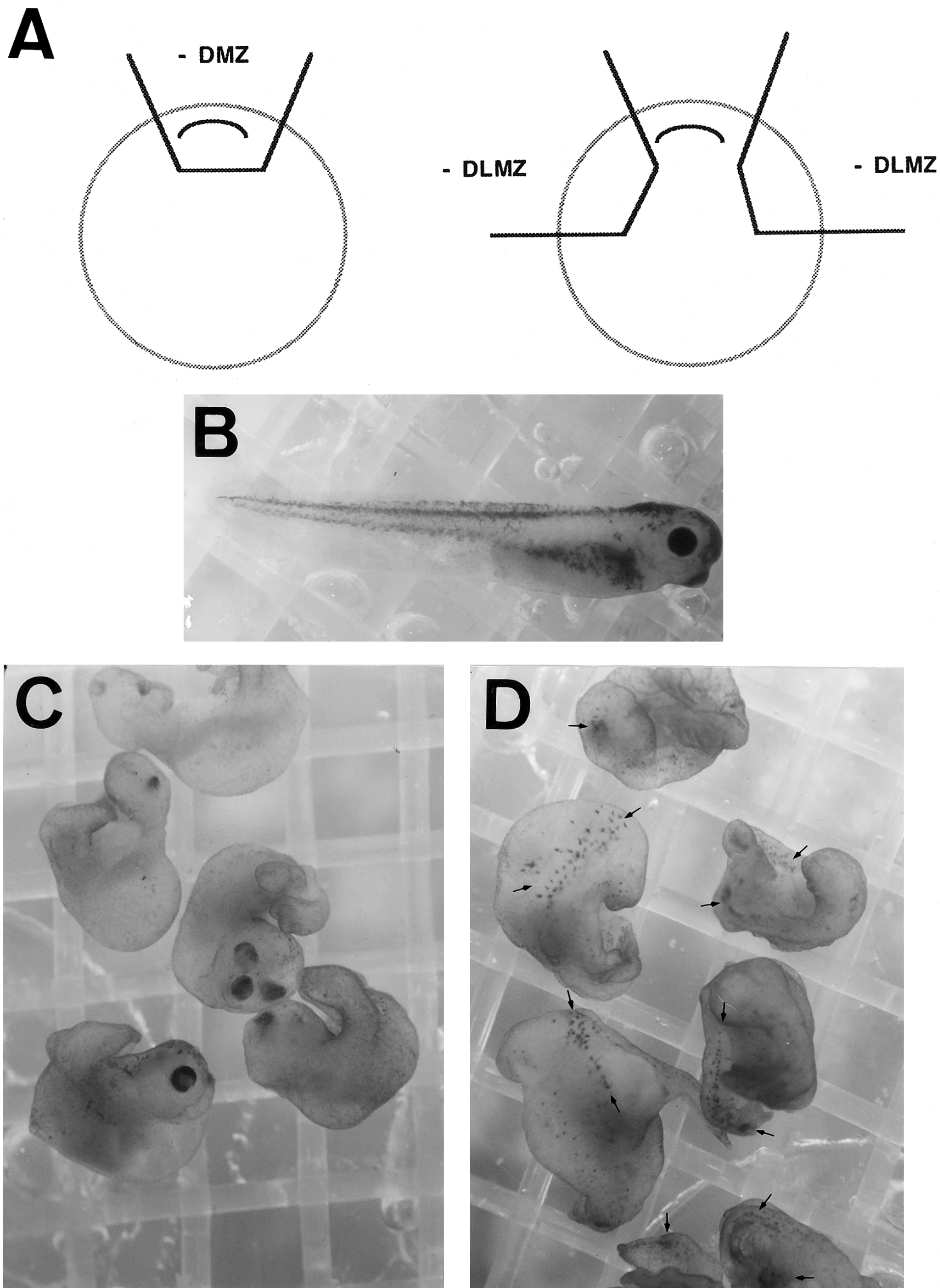

FIG. 4. Melanocyte formation is inhibited in embryos lacking paraxial mesoderm. (A) Embryos were dissected at stage 10.25–10.5. Either a 60° piece of the DMZ or both 60° pieces of the DLMZ were removed from nine embryos. Embryos were scored for melanocyte formation at stage 39–41. (B) A lateral view of a control embryo. (C) Embryos lacking a DLMZ region make heads and shortened trunks but lack melanocytes. (D) Embryos lacking a DMZ region make reduced amounts of anterior structures (eyes and cement glands) but contain large patches of trunk melanocytes (arrows mark patches of melanocytes).

DMZ is thought to be the main neural plate/crest inducer. Early gastrula-stage DLMZ induces melanocytes in juxtaposed ACs in 72% of the recombinant explants; in contrast, the DMZ induces melanocytes in only 15% of the recombinant explants (Table 1). Melanocyte induction is not dependent on the anteroposterior nature of the organizer, since a more posterior organizer (st. 11.25 DMZ) also did not induce melanocytes (Table 1). XSlug expression was compared in DLMZ–AC versus DMZ–AC explants, since XSlug is the earliest known panneural crest-specific marker (Mayor *et*

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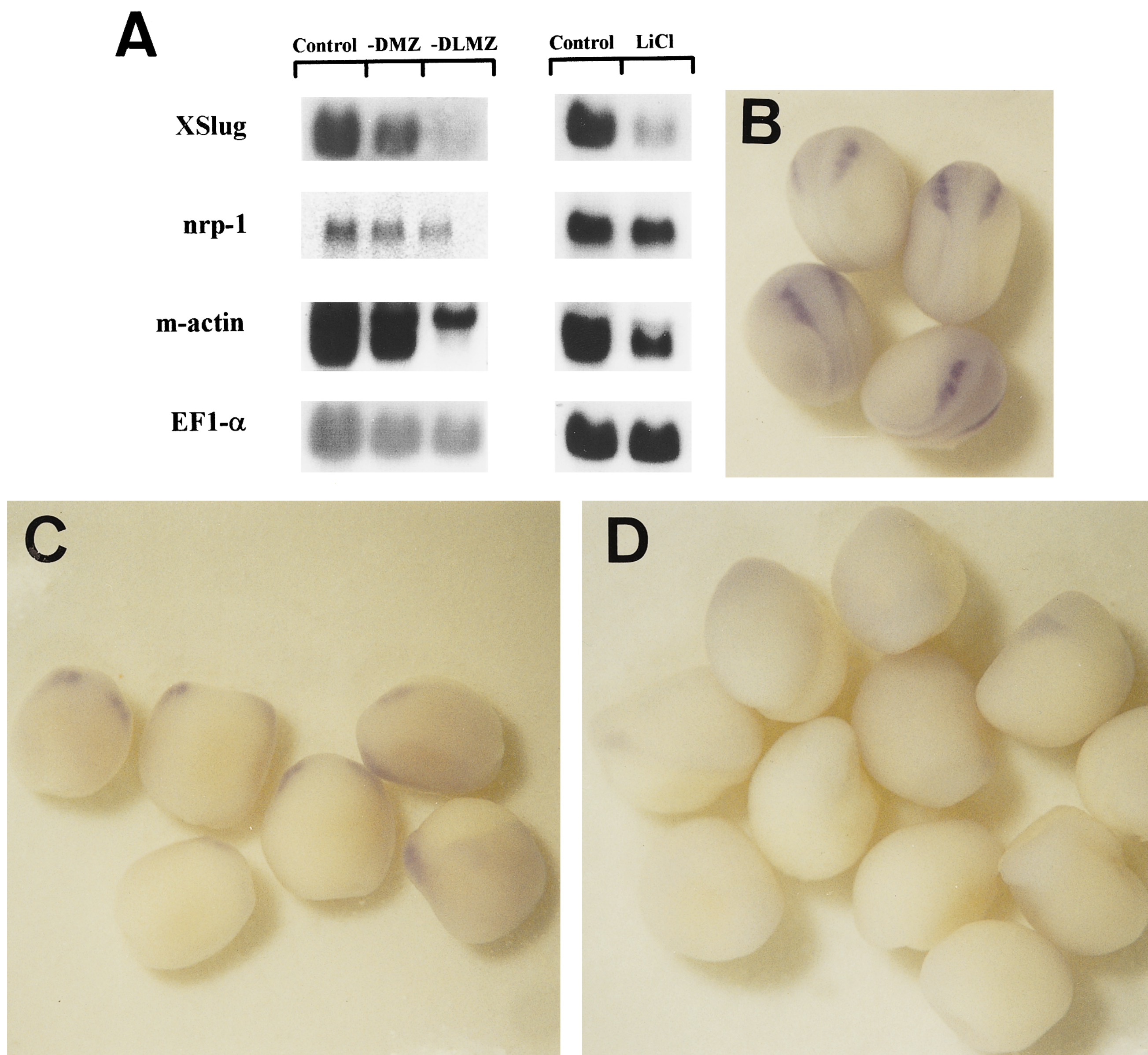

FIG. 5. Expression of XSlug in embryos lacking paraxial or axial mesoderm. (A) Northern analysis of RNA isolated from stage 17 embryos. Total RNA was isolated from 4 control, -DMZ, or -DLMZ embryos and from 10 control or LiCl-hyperdorsalized embryos. Two embryo equivalents of RNA were loaded per well for Northern analysis. Filters were sequentially hybridized with *Xenopus* cDNA probes for XSlug, nrp-1, muscle-specific actin (m-actin), and EF1α. (B) Whole-mount *in situ* hybridization with XSlug was performed on mid-neurula-stage embryos (see Materials and Methods), dorsal view. (C) XSlug expression in mid-neurula-stage embryos (-DMZ), dorsal view. (D) XSlug expression in mid-neurula-stage embryos (-DLMZ), dorsal view.

al., 1995). XSlug mRNA levels were induced at least 10-fold higher in DLMZ–AC explants in comparison to DMZ–AC explants (Fig. 3C). In addition, the Xtwi (Hopwood *et al.,* 1989) cranial neural crest marker was also induced in DLMZ–AC recombinant explants (not shown). We have also shown that DLMZ induces panneural markers such as nrp-1 and NCAM in the ACs like the DMZ, but unlike the DMZ, the DLMZ does not induce markers characteristic of anterior neural induction, such as the XAG-1 cement gland marker (Fig. 3C).

A recent work concludes that all dorsal mesoderm, including Spemann's organizer, is a strong inducer of XSlug (Mayor *et al.,* 1995). These organizer explants may have contained dorsal–lateral mesoderm, since muscle markers were not examined in this study. In Urodeles, medial regions of the archenteron roof were excellent inducers of

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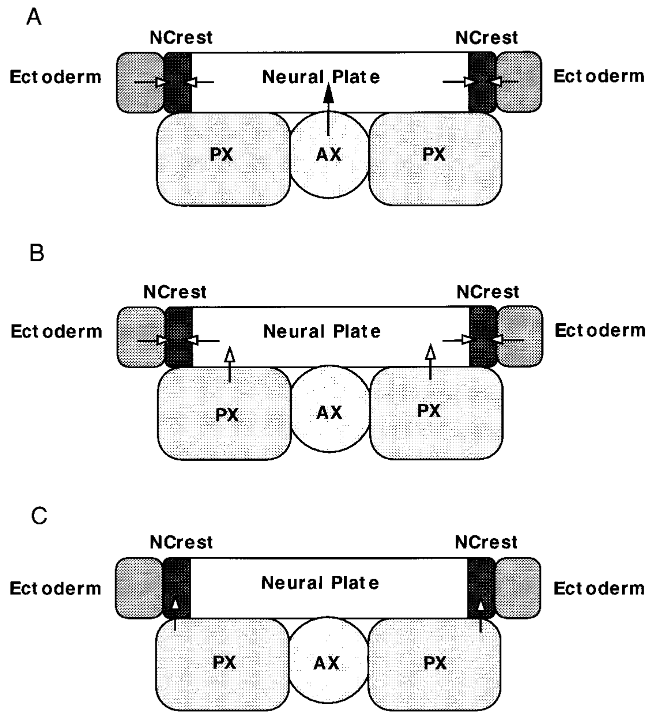

FIG. 6. Model of *Xenopus* neural crest induction. (A) The underlying axial mesoderm (AX) induces neural plate in ectoderm. Lateral neural plate interacts with the nonneural ectoderm to form neural crest cells in the neural folds region. (B) The underlying paraxial mesoderm (PX) induces/modifies lateral neural plate. Modified lateral neural plate interacts with the nonneural ectoderm to form neural crest cells in the neural folds region. (C) The underlying PX directly induces neural crest in the overlying cells of the neural folds region.

neural crest (Raven and Kloos, 1945). In these experiments early neurulae explants were inserted into early gastrulae in an Einstecken assay and tissue differentiation was assayed. However, no distinction was made between paraxial and axial mesoderm, since the grafted medial explant pieces differentiated into both notochord and muscle. Thus the inductive differences observed between paraxial and axial mesoderm in our studies were not distinguished in this work.

A study in chick suggests a role for paraxial mesoderm in melanocyte induction (Selleck and Bronner-Fraser, 1995). In these experiments, paraxial mesoderm from stage 10 embryos induces melanocytes in stage 4–6 neural plate explants. Presumptive neural plate and not naive animal cap ectoderm was used as the source of induced tissue. At the time of surgical removal from the embryo, these neural plate explants were already specified to express the HNK-1 neural/neural crest marker, suggesting that the neural plate had already been modified by an earlier mesoderm–neural plate interaction such as we have described in our results. These neural plates are also competent to make other neural crest cell types when juxtaposed to epidermal ectoderm. By stage 10 in the chick (the time of paraxial mesoderm explant removal), Slug is already expressed in the neural ridges, so the mesoderm–neural plate interactions addressed in this study are of a later nature. Thus, the question of early mesoderm–ectoderm interactions is not ascertained in this work.

Embryos Lacking Paraxial Mesoderm Have Fewer Neural Crest Cells

Embryos lacking a DMZ, while having perturbed head structures, made large numbers of trunk melanocytes; in contrast, embryos lacking DLMZ made heads but lacked melanocytes (Fig. 4; Table 1). A sharp reduction of XSlug expression was also seen in the neural folds of embryos lacking a DLMZ in comparison to embryos lacking a DMZ (Fig. 5). In addition, we examined XSlug and muscle actin expression in neurula-stage embryos that were hyperdorsalized by LiCl at the 64-cell stage. These embryos have an expanded DMZ and a reduced DLMZ region (Kao and Elinson, 1988). Similar to the embryos in which the DLMZ was removed, LiCl-treated embryos also expressed about threefold less XSlug and muscle actin mRNA in comparison to control embryos (Fig. 5A). A correlation between muscle actin (paraxial mesoderm) and XSlug expression was always observed; when muscle actin expression was low, XSlug expression was also reduced. These results suggest that the paraxial-fated mesoderm is essential for proper neural crest induction in the whole embryo and that axial-fated mesoderm alone is a weak inducer of neural crest cells.

Models of Neural Crest Induction

Models of neural crest induction generally suggest a two-step induction pathway. In the first step, the axial mesoderm initially induces neural plate; in the second step, cells in the neural folds region are redirected to a neural crest fate by the adjacent nonneural epidermal ectoderm (Fig. 6A). Our results suggest at least two variations to this model. In one model, paraxial-fated mesoderm borders/underlies the presumptive neural plate at the proper time to potentially act as an inducing tissue; a factor in the DLMZ may be secreted to the overlying ectoderm (Fig. 6B). This factor could then make the neural plate competent to neural crest induction signals (Liem *et al.,* 1995) coming from the adjacent nonneural ectoderm (Fig. 6B). Thus, the paraxial mesoderm may be inducing a novel type of lateral neural plate or modifying a preexisting neural plate that was induced by the organizer (Fig. 6B). This model is compatible with results suggesting that epidermal/neural plate interactions induce neural crest formation. In both chick and frog, transplantation of neural plate to the lateral ectoderm region leads to induction of Slug expression in the transplanted neural plate (Selleck and Bronner-Fraser, 1995, 1996; Dickinson *et al.,* 1995; Mancilla and Mayor, 1996). At the relatively late stages at which these neural plates were removed, it is likely that they were already modified by paraxial mesoderm signals. In preliminary studies, we have

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found that melanocytes are efficiently induced in recombinant explants made between noggin-treated ACs (anterior neural plate) and DLMZs. In addition, we have never detected significant levels of melanocyte formation or XSlug expression in recombinant explants made between noggin-treated ACs (anterior neural plate) and control ACs (nonneural epidermal ectoderm), thus suggesting that an additional signaling event is necessary for induction of neural crest (unpublished). A thorough investigation of these cell–cell interactions is presently being addressed.

An alternative model suggests that neural crest may be induced by a combination of two independent pathways (Figs. 6A and 6C). Paraxial mesoderm underlies the presumptive neural crest region at the proper time to potentially act as a direct inducer. A factor could be secreted from the DLMZ which directly recruits overlying ectoderm cells to neural/neural crest fates (Fig. 6C) and this induction could act independently of the neural plate/ectoderm interaction which also induces neural crest (Fig. 6A). Paradoxically, only cells underlying paraxial mesoderm in the neural folds region will make neural crest. Neural crest formation does not occur in medial–ventral neural plate regions underlying paraxial mesoderm. Possibly, the proximity of axial mesoderm or neuroepithelial cell contacts may inhibit neural crest formation in these medial regions. In preliminary studies, we have observed that the addition of DMZ to DLMZ–AC explants inhibits neural crest induction, thus suggesting that a signal emanating from the organizer could inhibit neural crest formation in neural plate regions.

Other studies also support the idea that neural plate cells are under negative regulation. Ablation studies in chick have shown that when neural crest is removed, medial–ventral neural plate cells are redirected to neural crest fates (Scherson *et al.,* 1993). Quite possibly, these cells have received a neural crest inducing/modifying signal from paraxial mesoderm. It is possible that in their normal embryonic position, these cells have the potential to make neural crest but are under local inhibition. Therefore, these cells can only make neural crest when juxtaposed to ectoderm.

Paraxial mesoderm can act as a neural inducer (Jones and Woodland, 1989; Hemmati-Brivanlou *et al.,* 1990b), but its specific role in neural induction is unclear. Most models of early neural/neural crest induction generally ignore signals originating from the paraxial mesoderm (Fig. 6A). With regard to early induction of cell fates in the neural plate, the consensus seems to be that whatever dorsal–lateral mesoderm can do, the organizer can do better. In this study, we demonstrate that paraxial-fated mesoderm is required for neural crest induction and that the organizer participates minimally in this process. We show that signaling molecules secreted from paraxial mesoderm induce or redirect neural plate cells to unique fates. Paraxial mesoderm induces panneural markers (similar to the organizer) in addition to inducing neural crest. Unlike the DMZ, anterior neural markers are not induced by the DLMZ. This observation suggests that the neural inducing/modifying molecules present in the DLMZ are likely different from those found in the DMZ and are required for neural crest induction. Future experiments will be designed to examine the nature of these neural inducing/modifying molecules.

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