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Analysis of Neural Crest Cell Migration in Splotch

George N. Serbedzija*,[1] and Andrew P. McMahon†

**Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th Street, 4th Floor, Charlestown, Massachusetts 02129; and †Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138*

Studies on the mouse *Splotch* (*Sp*) mutation, a deletion in the transcription factor Pax-3, have revealed that Pax-3 is essential for normal development of the neural crest. We have investigated the defect in neural crest development using a Wnt-l::LacZ reporter construct to mark neural crest cells. Staining embryos for β-galactosidase activity at different developmental stages revealed a severe reduction in the number of neural crest cells which emigrated from the neural tube at the vagal and rostral trunk levels. At the caudal thoracic, lumbar, and sacral levels there was a complete loss of neural crest cell emigration. In contrast to previous work in culture, we saw no evidence for any delay in the onset of neural crest cell migration at anterior levels. Pax-3 is expressed in the dorsal neural tube, where the neural crest cells originate, in migrating neural crest cells, and in somitic cells along the migratory pathway. Hence, it is not clear which aspect of the Pax-3 expression accounts for the observed phenotype. We addressed this problem by transplanting neural tissue between mouse and chick embryos. Our studies indicate that the defect in the *Splotch* mutation is not intrinsic to the neural crest cells themselves, but appears to reflect inappropriate cell interactions either within the neural tube or between the neural tube and the somite.

INTRODUCTION

During development of the mouse embryo, neural crest cells arise from the dorsal aspect of the neural tube. From their site of origin, the neural crest cells migrate throughout the embryo in a well-characterized spatial and temporal pattern (Nichols, 1981, 1986; Erickson and Weston, 1983; Serbedzija *et al.*, 1990, 1992) to give rise to a number of neuronal and nonneuronal derivatives (for review see Le Dourain, 1982). Much of our information about neural crest cell development in the mouse has come from vital dye labeling experiments in wild-type embryos (Chan and Tam, 1988; Serbedzija *et al.*, 1990, 1992, 1994; Osumi-Yamashita *et al.*, 1994). While these approaches have shown us a great deal about the pattern of neural crest cell migration and the identity of the neural crest cell derivatives, they have revealed relatively little about the mechanisms by which migration and differentiation of the neural crest cells are initiated and maintained. One way of examining these questions is to analyze mouse embryos carrying mutations which disrupt various aspects of neural crest cell development. Prominent among these is the semidominant mutation *Splotch*.

The original *Splotch* mutation was initially identified because mice which are heterozygous for the mutation have a white patch on their abdomen (Russell, 1947). Subsequently, a variety of other alleles have been identified with variable phenotypes (Dicke, 1964; Beechy and Searle, 1986). Mouse embryos which are homozygous for the *Splotch* mutation die by Embryonic Day 16. Prior to death, mutants are characterized by defects in both neural tube closure and neural crest cell development. The disruption in the neural crest cells results in either a severe reduction or a complete absence of a number of neural crest cell derivatives, including pigment cells, sympathetic ganglia, spinal ganglia, cardiac outflow tract, and enteric nervous system (Auerbach, 1954; Beechy and Searle, 1986; Franz *et al.*, 1989). The severity of these defects increases along the rostrocaudal axis, such that no neural crest derivatives are present in the caudal portion of the *Splotch* mutant embryo (Auerbach, 1954). On the basis of cell culture experiments, it has been suggested that these deficiencies are due to a delay in the onset

[1] To whom correspondence should be addressed. Fax: 617-726-5806. E-mail: serbedzija@cvrc.mgh.harvard.edu.

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of neural crest cell emigration from the neural tube of *Splotch* mutants so that when emigration finally occurs, the environment outside the neural tube is no longer able to support their continued development (Moase and Trasler, 1990). This model has been strengthened by two additional lines of evidence: (1) the gene which is affected by the *Splotch* mutation, the transcription factor Pax-3 (Kessler and Gruss, 1990; Epstein *et al.*, 1991), is expressed in both the dorsal neural tube and somites (Goulding *et al.*, 1991), both of which support neural crest cell development; and (2) recent experiments have shown that the loss of Pax-3 does effect the normal development of the somite (Goulding *et al.*, 1994; Williams and Ordahl, 1994). However, to determine if the defect in neural crest cell derivatives in the *Splotch* mutant is actually caused by a delay in neural crest cell emigration, it is necessary to perform an *in vivo* cell labeling analysis of the neural crest cells in the *Splotch* mutant embryos.

Direct analysis of neural crest cells in this and other mutant embryos has been difficult for two reasons: (1) the lack of a noninvasive cell marker that labels both migrating neural crest cells and their derivatives and (2) the difficulty in maintaining mutant embryos in culture. Recently, a stable transgenic mouse line was generated, which expresses a LacZ reporter under the control of the *Wnt-1* enhancer. In addition to expressing β-galactosidase in the cell of the dorsal neural tube, embryos from this line show transient β-galactosidase activity in emigrating, migrating, and differentiating neural crest cells (Echelard *et al.*, 1994). We have used this transgenic line to address the neural crest defect in the *Splotch* mutants. Our results suggest that Pax-3 is required for the normal cell interactions either within the dorsal CNS or between the mesoderm and the CNS which lead to the emigration of neural crest cells.

MATERIALS AND METHODS

Animals

Mouse embryos which were both homozygous for the *Splotch* mutation and expressed the *Wnt-1::lacZ* reporter gene were generated as follows: Transgenic male mice which transmitted the *Wnt-1::lacZ* reporter gene (Echelard *et al.*, 1994; identified by β-galactosidase staining in offspring) were mated to female mice which were heterozygous for the *Splotch* mutation (Jax; identified by the characteristic white belly patch) to generate male offspring that were both heterozygous for *Splotch* and transmitted the *Wnt-1::lacZ* reporter gene (*Sp/+; Wnt-1::LacZ*). *Sp/+; Wnt-1::LacZ* males were then crossed to *Sp/+* females to generate *Sp/Sp:Wnt-1::LacZ* embryos. Embryos were removed from the mother between Embryonic Days 9 and 12. Embryos to be used for the direct analysis were placed in a fixation solution (1% formaldehyde, 0.02% glutaraldehyde, 2 m*M* $MgCl_2$, 5 m*M* EGTA, and 0.02% NP-40) for 1–2 hr and stored in phosphate-buffered saline (PBS) before staining. Embryos to be used in transplantation experiments were placed in PBS on ice.

Neural Tube Transplantation

Neural tubes were collected from Embryonic Day 10 *Sp/Sp:Wnt-1::LacZ* and wild-type embryos in the following manner: Embryos were transected at the level of the 19th somite. The caudal portion of the embryos was lightly digested in an enzymatic solution (0.25% trypsin, 0.02% EGTA in calcium-free PBS) for 1 min on ice. The tissue was then transferred to dissecting media (5% fetal calf serum in Dulbecco's modified Eagle's medium) and lightly triturated through a pulled pasture pipet to separate the neural tube from the associated tissue. Fine tungsten needles were used to remove any remaining contaminating tissue. Once the neural tubes were isolated, they were transferred to fresh media and place on ice until they were transplanted into host chick embryos.

Host White Leghorn chicken embryos were incubated at 37°C until stage 12 (Hamburger and Hamilton, 1951). The eggs were then washed in 70% ethanol (EtOH), a window was cut in the shell over the embryo, and India ink (Pelikan, Hanover, FRG) was injected under the blastodisc to aid in visualization of the embryo. The vitelline membrane was removed with a fine tungsten needle to allow access to the embryo.

For neural tube/neural tube grafts, the dorsal portion of the chick neural tube adjacent to the six most recently formed somites was ablated with a fine tungsten needle and an intact section of mouse neural tube was grafted in place (Fig. 3A). For neural tube/lateral mesoderm grafts, an incision was made in the lateral mesoderm adjacent to the segmental plate and the mouse neural tube was placed into the incision (Fig. 4A). After transplantation was complete, the egg was sealed with adhesive tape and returned to the incubator for 24–48 hr before being fixed and stained for β-galactosidase activity.

β-Galactosidase Staining

To detect β-galactosidase activity in mouse embryos or chick embryos with mouse neural tube grafts, the embryos were stained in the following manner: Fixed embryos were incubated 1–3 hr in a staining solution consisting of 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$, 2 m*M* $MgCl_2$, 0.01% Na-deoxycholate, 0.02% NP-40, and 1 mg/ml Xgal at 37°C. The embryos were then washed three times in PBS and postfixed in 4% paraformaldehyde. Whole embryos were then viewed on a dissecting scope equipped with a CCD camera (Sony 3CCD) and an image grabber (Radius).

Embryos were prepared for cryostat sectioning by soaking in a 15% sucrose solution for 8 to 12 hr at 4°C. They were then embedded in 15% sucrose and 7.5% gelatin (Sigma), rapidly frozen in liquid nitrogen, and serially sectioned on a cryostat (HM 500 M, Microm) at 25 μm. Sections were coverslipped in 80% glycerol in PBS and viewed on a photomicroscope (Leitz DMR RD) equipped with Nomarski optics.

RESULTS

Analysis of Neural Crest Cell Formation in Sp Mutants Using the Wnt-1::Lac Z Reporter

The severe reduction of neural crest cell derivatives observed in *Splotch* mutant embryos may be a result of a failure of normal neural crest cell emigration, migration,

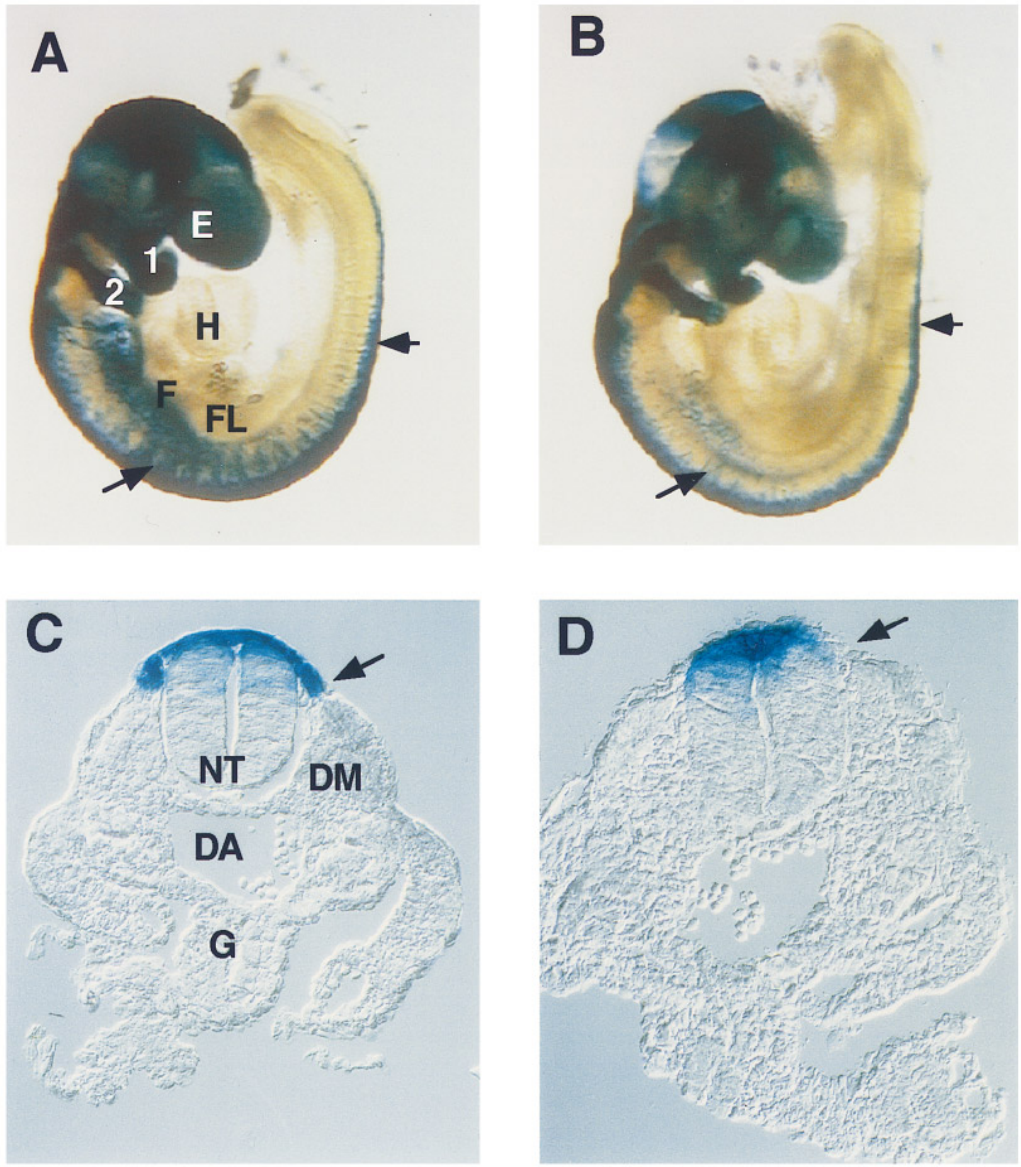
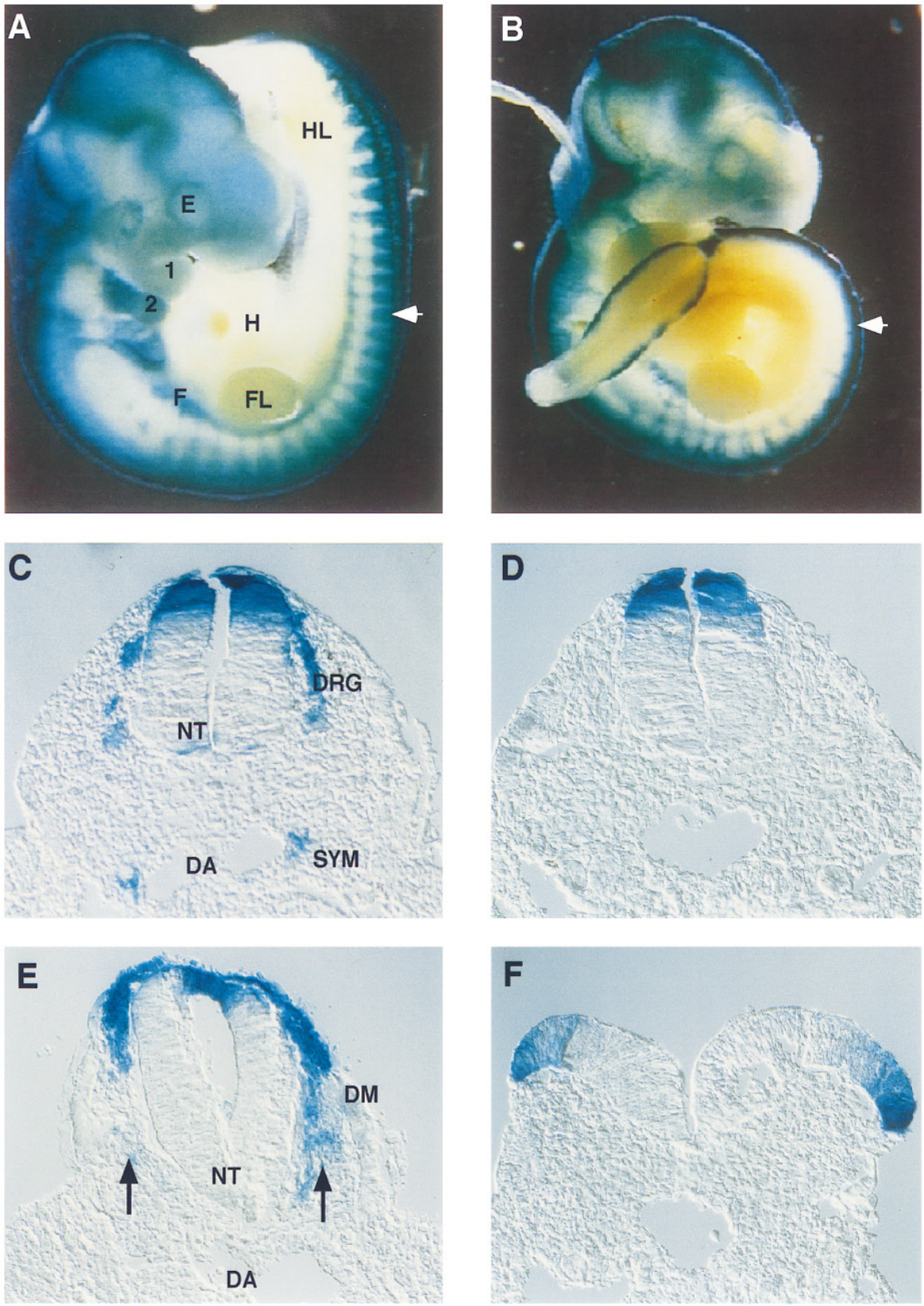


FIG. 1. Embryonic Day 9.5 wild-type and *Sp*/*Sp:Wnt-1::LacZ* embryos stained for β-galactosidase activity. (A and B) Whole mounts of E9.5 wild-type and mutant embryos, respectively. Labeled cells are present in the cranial mesenchyme, including the nasotemporal region around the eye and the branchial arches. In the anterior portion of the trunk, labeled cells are present in the neural tube, somites (long arrows), and the region of the foregut. Notice that there are fewer labeled cells in the mutant when compared to the wild type. (C and D) Transverse sections through the level of the 19th somite (short arrows) of embryos shown in A and B. Labeled cells are present in the dorsal portion of the neural tube (NT) and in migrating neural crest in the wild-type embryo (arrows) but restricted to the dorsal neural tube in *Splotch* mutant embryos. For orientation, the eye (E), first (1) and second (2) branchial arches, foregut (F), heart (H), forelimb (FL) in the wild-type whole mount, and dorsal aorta (DA), dermamyotome (DM), and gut (G) are labeled.

differentiation, or survival. To determine which, if any, of these explanations accounts for the phenotype, we crossed the *Splotch* mutant line with the *Wnt-1::LacZ* line and analyzed the distribution of β-galactosidase expressing cells in the resulting mutant embryos. The *Wnt-1::LacZ* reporter gene, like the *Wnt-1* gene, is expressed in much of the dorsal CNS ,including the spinal cord. β-Galactosidase activity in the neural crest cells results from perdurance of either the mRNA or the protein inherited from the neural crest progenitors in the dorsal neural tube (Echelard *et al.*, 1994).



Therefore, if the LacZ reporter is expressed in the dorsal neural tube in the *Splotch* mutants, any neural crest cells emigrating from the neural tube should contain β-galactosidase activity. This is supported by the neural tube grafting experiments described below.

Mutant and wild-type embryos were stained at a variety of developmental stages ranging from E8 to E12. In all of the embryos which expressed the *Wnt-1::LacZ* reporter gene, labeled cells were present in the dorsal portion of the neural tube (Fig. 1). Both mutant and wild-type embryos contained labeled cells in a pattern that was consistent with the normal distribution of neural crest cells in the cranial region anterior to the otic vesicle. Specifically, blue cells were observed throughout the cranial mesenchyme in the first and second branchial arches and cranial ganglia V, VII, and VIII (Figs. 1A, 1B, 2A, and 2B). In contrast to the cranial region, a significant difference in the distribution of labeled neural crest cells was observed caudal to the otic vesicle. These differences were quite obvious by E10.5, when the spinal neural tube defects were apparent in the mutant; however, these differences also were evident at younger stages, prior to any morphological defects in the trunk (Fig. 1B).

In both mutant and wild-type embryos examined at E9.5 and E10.5, the rostral portion of the trunk (somites 1–18) contained labeled cells in the regions of the dorsal root ganglia and the sympathetic ganglia (Figs. 1A, 1B, 2A, and 2B). However, far fewer labeled cells were observed in mutant embryos when compared with wild-type embryos (compare Fig. 1A with 1B and Fig. 2A with 2B). While fewer labeled cells were present in the mutants, the distribution of labeled cells in both mutant and wild-type embryos was appropriate for neural crest cells, i.e., labeled cells in the rostral portion of the somites (Figs. 1B and 2B).

The difference in the distribution of labeled neural crest cells was even more marked in the caudal portion of the trunk (somites 19–39). Embryos stained at E9.5 show that at the level of the 19th somite, wild-type embryos contained labeled neural crest cells in the space bordered by the neural tube, somite, and ectoderm, while mutant embryos do not (compare Fig. 1C with 1D, arrows). In E10.5 wild-type embryos, labeled cells were present either in the neural crest cell derivatives or along neural crest cell migration pathways through the rostral portion of the somites (Figs. 2A, 2C, and 2E) depending on the axial level. In contrast, at E10.5 and later stages mutant embryos contained no labeled cells outside the neural tube in this region (Figs. 2B, 2D, and 2F and data not shown). The absence of labeled neural crest cells between the 19th somite and the rostral region of the hindlimb was not due to any detectable difference in the level of *Wnt-1* expression in the *Sp* mutants (data not shown). Rather, it would appear that there is a failure of neural crest cell migration.

Neural Tube to Neural Tube Grafts

To determine whether the failure of the neural crest cells to migrate in the *Splotch* mutant was due to an inherent defect in the neural crest cells themselves, we performed a series of grafts into chick embryos. In the first series we grafted portions of neural tube from regions just caudal to the 19th somite of either wild-type or *Sp* embryos into stage 15 chick embryos adjacent to the six most recently formed somites (Fig. 3A). The chimeric embryos were then allowed to develop for an additional 24 to 48 hr before sectioning. In most grafts, the donor mouse neural tissue fused with the host chick neural tube.

In all cases in which fusion was successful, irrespective of whether the graft was from wild-type (17/23) or *Splotch* mutant (23/30) embryos, β-galactosidase expressing cells were observed outside the neural tube grafts in locations appropriate for neural crest cell migration (Figs. 3B and 3C). In chimeric embryos which were allowed to develop for an additional 24–48 hr, blue cells were observed in the rostral sclerotome adjacent to the graft and in the dorsal root ganglia and sympathetic ganglia adjacent to the dorsal aorta, all of which are sites of normal neural crest cell migration and colonization. Because of variations in the intensity of the LacZ staining, it was not possible to determine whether there were any absolute differences in the number of blue cells outside the neural tube between the wild-type and mutant grafts. However, there did not appear to be any obvious differences in either the distribution or the number of blue cells outside the grafts. Thus, grafting of *Sp* neural tubes to wild-type hosts rescues a neural crest cell defect within the mutant neural tissue.

Neural Tubes to Lateral Plate Grafts

To determine if contact between the host neural tube and/or host somites is essential for neural crest cell emigration from the donor tissue, portions of caudal neural tubes

FIG. 2. Embryonic Day 10.5 wild-type and *Sp*/*Sp:Wnt-1::LacZ* embryos stained for β-galactosidase activity. (A and B) Whole mounts of E10.5 wild-type and mutant embryos, respectively. Notice the absence of labeled cells outside the neural tube in the caudal portion of the trunk. (C and D) Transverse sections through the level of the 19th somite of embryos shown in E and F. In the wild type (G), labeled cells are present in the dorsal portion of the neural tube (NT), the dorsal root (DRG), and the sympathetic (SYM) ganglia. In contrast, mutant embryos (H) contains no labeled cells outside the neural tube. (E and F) Transverse sections through the level of the hindlimb (HL in A and B) of embryos shown in E and F. Labeled cells are present in the sclerotome (arrows) of the wild type, but not the mutant embryo. For an explanation of the labels, see Fig. 1.

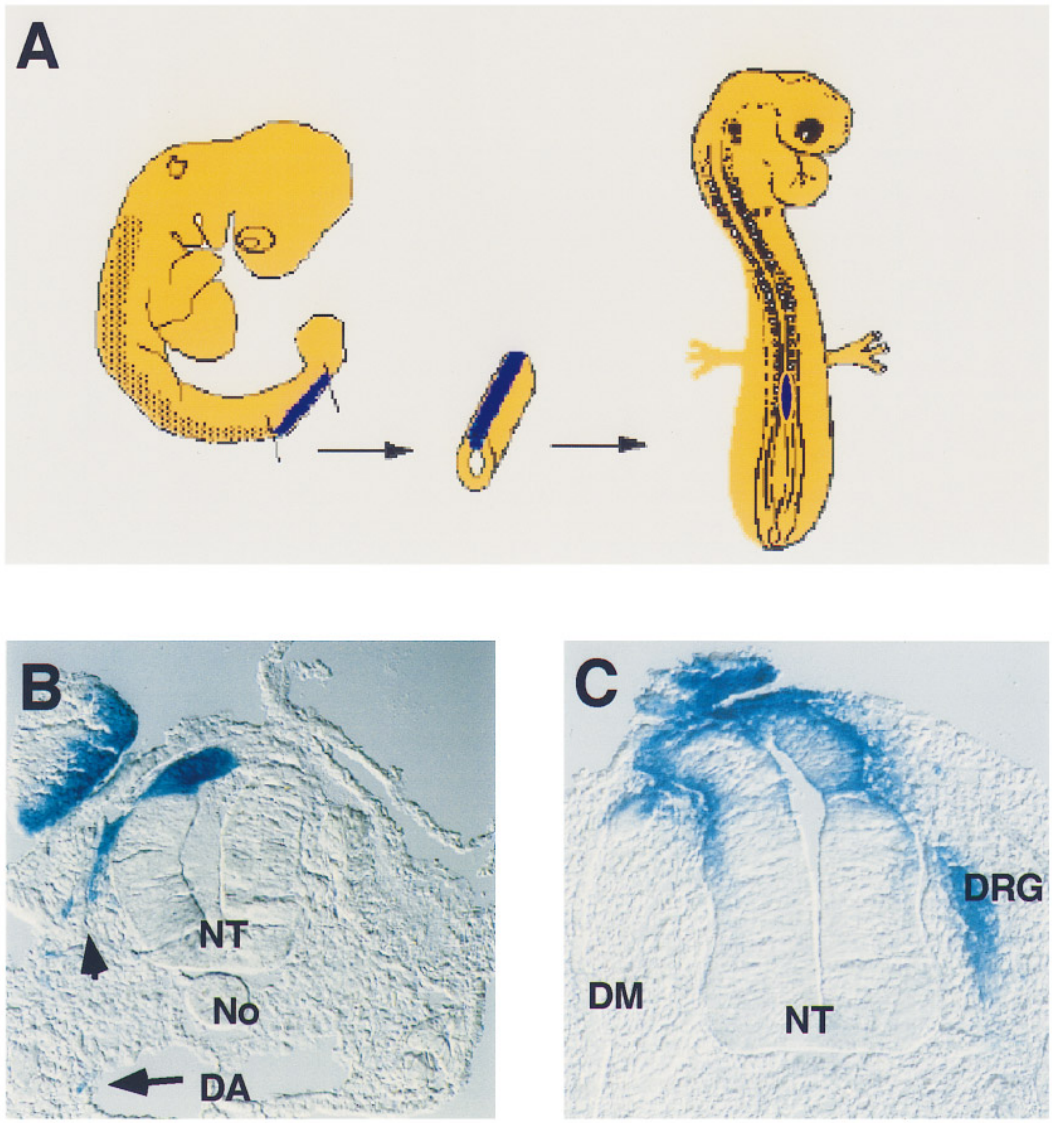


FIG. 3. Neural tube to neural tube transplantation. (A) Schematic representation of the mouse neural tube to chick neural tube transplantation. (B and C) Transverse sections through the center of the *Sp* mutant neural tube grafts in chimeric embryos allowed to develop 24 or 48 hr after the transplantation, respectively. After 24 hr, labeled cells are present in the chimeric neural tube (NT), in the sclerotome (arrowhead), and in the region of the sympathetic ganglia (arrow). After 48 hr, labeled cells are present in the dorsal root ganglia (DRG) and in the chimeric neural tube (NT). In many of the experiments, small pieces of donor tissue remained attached to the graft site outside the host (B). The dermamyotome (DM) and dorsal aorta (DA) are labeled for orientation.

from mutant and wild-type embryos were grafted into the lateral plate mesoderm of host chick embryo (Fig. 4A).

Grafts of neural tubes from wild-type embryos (9) extended streams of labeled cells from the graft to the sclerotome and dorsal aorta (Fig. 4B). In some cases, cells were also observed at the apex of the gut (arrowhead in Fig. 4B). In contrast, when mutant mouse embryos were used as donors (12), no significant migration of labeled cells was observed (Fig. 4C).

DISCUSSION

In these studies, we have analyzed the effect of the *Splotch* mutation on development of the mouse neural crest. Analysis of transgenic reporter gene expression in the dorsal CNS and neural crest of *Splotch* mutants demonstrated (1) that neural crest cell emigration and differentiation appeared normal in the regions anterior to the otic vesicle; (2) a severe reduction

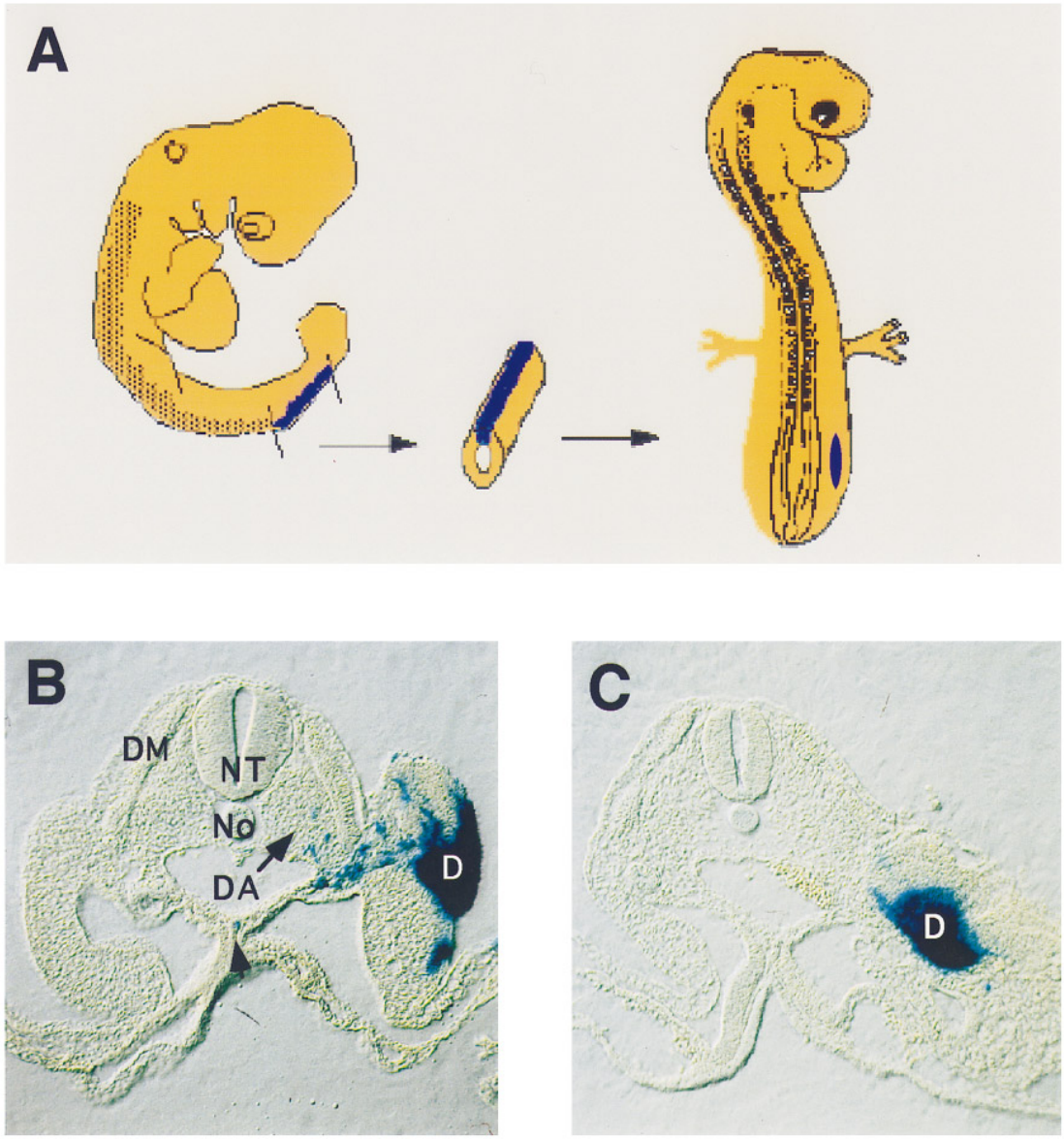


FIG. 4. Neural tube to lateral plate transplantation. (A) Schematic representation of the mouse neural tube to chick lateral plate transplantation. (B and C) Transverse sections through chimeric embryos containing either a wild-type (B) or mutant (C) mouse neural tube 24 hr postgrafting. Labeled cells are present in the donor mouse neural tube (D) in both chimeras. However, the chimera with the wild-type neural tube also contains labeled cells in the lateral plate mesenchyme, in the sclerotome (arrow), and in the gut (arrowhead). The chick neural tube (NT), notochord (No), dermamyotome (DM), and the dorsal aorta (DA) are labeled for orientation.

in the number of neural crest cells emigrating from both the vagal and rostral trunk regions; and (3) a complete loss of neural crest cell emigration from caudal trunk. Thus, the loss of Pax-3 in the *Splotch* mutant leads to a decrease in the number of neural crest cells which emigrate from the neural tube, and this effect increases in severity along the rostrocaudal axis. The phenotype presumably reflects either a decrease in the number of neural crest cells which form or failure in emigration from the neural tube. Redundancy between Pax family members may explain the difference in phenotype between cranial versus trunk regions of the neural tube (Masouri *et al.*, 1996).

Where neural crest cells do emerge in the *Splotch* mutant, they do so at the same time as wild-type littermates, in

contrast to *in vitro* studies(Moarse and Trasler, 1990). No evidence for delayed emigration was ever observed, even though we analyzed mutant embryos up to E12.5, approximately 72 hr after neural crest cell emigration begins at the level of the hindlimb (Serbedzija *et al.*, 1991).

By transplanting neural tubes from mutants, we were able to show that the defect caused by the *Splotch* mutation was not intrinsic to the neural crest cells. Transplanted neural tubes, when fused with the chick host neural tubes, gave rise to labeled neural crest cells migrating along the appropriate ventral neural crest cell migration pathway and colonizing both sympathetic ganglia and dorsal root ganglia. In contrast, when neural tubes from *Sp* mutants were transplanted into the lateral plate of chick, no labeled cells were observed outside the grafts. Since the neural tubes were isolated and handled in an identical manner, the only difference between the neural tube grafts and the lateral plate grafts was their location. Control neural tubes grafted to the chick lateral plate mesoderm were able to generate migrating neural crest cells which migrated toward the somite and dorsal aorta. From these experiments, it is clear that (1) the ability to generate neural crest cells is not lost in homozygous *Splotch* mutant embryos, even in caudal regions of the neural tube where no cells emigrate from the neural tube; and (2) some interaction either within the neural tube or between the neural tube and adjacent regions (most likely the somites) is required for neural crest cell development and this interaction is defective in *Splotch* mutants. Our results would be consistent with a model in which the loss of Pax-3, a transcriptional regulator, disrupts cell interactions which are required to either initiate neural crest cell formation or initiate emigration from the neural tube.

In addition to providing important controls, the wild-type mouse neural tube transplantations have led to several interesting observations. The most obvious of these is that mouse neural crest cells appear to be capable of following the cues that are present in the chick embryos, as has been observed for neural crest cells which populate the gut (Rothman *et al.*, 1993). Another less obvious observation comes from the transplantations into the lateral plate. In these experiments, neural crest cells from wild-type neural tubes not only emigrated from the graft, but also ended up in locations appropriate for neural crest cells, for example adjacent to the dorsal aorta and in the rostral portion of the somitic sclerotome. In many of the chimeric embryos, thin streams of labeled cells extended directly through the lateral plate mesoderm from the graft to the somitic sclerotome and dorsal aorta. As neural crest cells do not normally migrate through the lateral mesoderm, these results suggest that the mouse neural crest cells may be following some target-derived signal rather than a pathway of extracellular matrix molecules (for review see Bronner-Fraser, 1993, 1994). Thus, mouse and chick embryos appear to use conserved mechanisms to guide different aspects of neural crest cell development.

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