**Sonic hedgehog is required for survival of both myogenic and chondrogenic somitic lineages**

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**SUMMARY**

In vertebrates, the medial moieties of the somites give rise to the vertebrae and epaxial muscles, which develop in close relationship with the axial organs, neural tube and notochord. The lateral moieties contribute to the ribs and to limb and body wall muscles (hypaxial muscles) after a phase of lateral and ventral migration. Surgical ablation of the neural tube and notochord in the chick embryo during segmentation and early differentiation of the somites (day 2 of incubation) does not affect primary development of the hypaxial muscles, but leads to a complete absence of epaxial muscles, vertebrae and ribs, due to cell death in the somites. Here we demonstrate that cell death, which occurs within 24 hours of excision of the axial organs, affects both myogenic and chondrogenic cell lineages defined, respectively, by the expression of *MyoD* and *Pax-1* genes.

In contrast, *Pax-3* transcripts, normally present in cells giving rise to hypaxial muscles, are preserved in the excised embryos. Backgrafting either the ventral neural tube or the notochord allows survival of *MyoD-* and *Pax-1-*expressing cells. Similarly, Sonic hedgehog-producing cells grafted in place of axial organs also rescue *MyoD-* and *Pax-1-*expressing cells from death and allow epaxial muscles, ribs and vertebrae to undergo organogenesis. These results demonstrate that the ventral neural tube and the notochord promote the survival of both myogenic and chondrogenic cell lineages in the somites and that this action is mediated by Sonic hedgehog.

Key words: Sonic hedgehog, Somite, Chick, Floor plate, Notochord, Cell death, Cartilage, Muscle, *Pax-1*, *Pax-3*, *MyoD"

**INTRODUCTION**

In the vertebrate embryo, trunk paraxial mesoderm is segmented into somites, which give rise to a variety of cell types, including cartilage and bone of vertebrae and ribs, connective tissue forming the dorsal dermis and the connective component of epaxial striated muscles, and endothelial cells. Moreover, all the skeletal muscle cells of the body originate from the somites (see Christ and Ordahl, 1995 for a review), except those of the head, which are derived from the cephalic paraxial and prechordal mesoderm (Wachtler et al., 1984; Couly et al., 1992).

During gastrulation, the cells fated to generate the paraxial mesoderm invaginate through the primitive streak in a region located just caudal to Hensen’s node (Selleck and Stern, 1991; Schoenwolf et al., 1992; Tam and Tan, 1992; Catala et al., 1996). It was recently shown in this laboratory (Catala et al., 1995, 1996) that the group of cells constituting Hensen’s node and the underlying endoderm progresses in a rostral-to-caudal direction laying down the ‘midline cells’, which are the precursors of the notochord, floor plate and dorsal endoderm, while the paraxial mesoderm moves laterally on each side of the newly formed axial organs, thus giving rise to the segmental plates.

Later in development, cells forming the paraxial mesoderm undergo a morphogenetic transition from a mesenchymal to an epithelial state and form spherical structures designated as epithelial somites (see Tam and Trainor, 1994; Brand-Saberi et al., 1996 and references therein). This process of somite segmentation progresses rostrocaudally in a sequential manner (Palmeirim et al., 1997). Somites rapidly become polarized dorsoventrally. Their ventral component turns mesenchymal again forming the sclerotome from which vertebral cartilage, intervertebral discs and ribs develop. Dorsally, the epithelial structure of the original somite is temporarily maintained forming the dermomyotome. This structure is then subjected to movements of cells, yielding both a medial layer of cells, the myotome, which gives rise to the epaxial muscles (Denetclaw et al., 1997 and references therein), and laterally migrating cells, which give rise to the hypaxial muscle cells of the limbs and bodywall. Finally, the superficial epithelial layer gives rise to the dermatome, from which the dorsal dermis develops.

If epithelial somites are subjected to a dorsoventral (DV) rotation (Aoyama and Asamoto, 1988) or to an exchange of the dorsal and ventral moieties (Christ et al., 1992), somite development is normal, indicating that their DV axis is still labile. Similarly, medial and lateral somitic moieties, which
give rise to epaxial and hypaxial muscle cells, respectively, can be exchanged at this stage without any morphogenetic consequences (Orshahl and Le Douarin, 1992). It is therefore clear that the DV as well as the mediolateral (ML) polarities of the somites are progressively acquired in response to morphogenetic signals arising from the environment.

Examination of Pax-3 and Pax-1 transcripts as early dermomyotomal and sclerotomal markers, in various mouse notochord mutants, suggested that the notochord plays a decisive role in the dorsalventral specification of the somites (Dietrich et al., 1993). Moreover, grafting an ectopic notochord or floor plate between the neural tube and the unsegmented paraxial mesoderm resulted in expansion of the sclerotome leading to the differentiation of the entire medial somite into cartilage since neither epaxial muscles nor dorsal dermis develop in these conditions (Pourquié et al., 1993; Brand-Saberi et al., 1993). In cultured quail embryos, Pownall et al. (1996) found that somites, separated by a slit from the neural tube and notochord, did not express QMyoD, a bHLH regulator of myogenesis. In contrast, if the mesoderm remained in contact with the notochord and ventral neural tube, QMyoD was found to be normally expressed suggesting that these axial structures were providing signal(s) involved in the transcriptional cascade of myogenic genes. Similarly, Münsterberg and Lassar (1995), in tissue recombination experiments carried out in culture, showed that the onset of myogenesis in the three last-formed somites required an early signal from the notochord-floor plate complex and a later one from the neural tube. Recently, from multiple types of ablation and grafting experiments, Dietrich et al. (1997) suggested that a balance of dorsalizing factors produced by the dorsal neural tube and ectoderm and ventralizing factors emanating from the ventral neural tube and floor plate are necessary to induce myotomal cells in the intermediate region of the somites.

Sonic hedgehog (SHH), a secreted protein produced by both the notochord and the floor plate (Riddle et al., 1993; Echelard et al., 1993; Krauss et al., 1993), may play a role in somite development. SHH, which is known to have an action on cell specification in the neural tube (see Tanabe and Jessell, 1996 for a review), has been shown to activate the expression of Pax-1 in somites in culture (Fan and Tessier-Lavigne, 1994; Fan et al., 1995) and the expression of Pax-1 and MyoD in somites in vivo (Johnson et al., 1994). The processed NH2 terminal active fragment (N-fragment) of the SHH protein can act at a distance from its source on somites (Fan et al., 1995) as well as on the differentiating motoneurons (Roelink et al., 1995). The gene patched (ptc), considered to be a key molecule in the hedgehog signaling system in Drosophila and Vertebrates (Marigo et al., 1996a; Stone et al., 1996), was shown to be expressed in somites and paraxial mesoderm (Marigo and Tabin, 1996; Marigo et al., 1996b).

In previous works (Teillet and Le Douarin, 1983; Rong et al., 1992), we have shown that the neural tube and notochord exert a crucial effect on the paraxial mesoderm. If both axial structures were removed in chick embryos during the second day of incubation, when somites are generated and start to differentiate, a wave of cell death affected all the segmental plate and young somitic cells, as well as the sclerotomal cells of the more mature somites. A tenuous pattern of cell death has been described in normal development of somites (Jeffer and Osmond, 1992; Sanders, 1997). As early as 1 day following neural tube and notochord extirpation, most of the cells of the somites were pycnotic, except for dermomyotomal cells of the more mature somites from which a few myocytes differentiated. The cells giving rise to limb bud and body wall muscles were not affected by this operation. However, hypaxial as well as epaxial muscle cells that formed in the absence of axial organs died after a few days, probably because they failed to become innervated (Rong et al., 1992). Backgrafting either the notochord or the neural tube immediately after the excision rescued a large number of somitic cells from death and allowed them to differentiate further into both cartilage and muscle (Teillet and Le Douarin, 1983; Rong et al., 1992).

The present study aimed to identify the exact source and nature of the factor(s) critical for survival of somitic cells. We found that the ventral but not the dorsal neural tube shares with the notochord the survival effect on sclerotomal and myotomal somitic cells. We hypothesized that this effect could be mediated by SHH, which is secreted by both the ventral neural tube and the notochord. We show that the survival action of axial organs on somites can be mimicked by grafting SHH-expressing quail fibroblasts in the in vivo system designed in this study. We also demonstrate that the survival effect exerted by SHH allows both sclerotomal and dermomyotomal cells to differentiate further.

**MATERIALS AND METHODS**

**Embryos and microsurgery**

Fertilized chick eggs from a commercial source (JA 57 strain, Institut de Sélection Animale (ISA), Lyon, France) were incubated at 38°C under humidified and ventilated atmosphere until the embryos had reached the 15-25-somite stage (2nd day of incubation = E2). We used for the description of our experiments the nomenclature of Christ and Orshahl (1995) in which somite I is the last one formed, somite II the one that is immediately rostral to it and so on.

Microsurgery was performed in ovo (see Fig. 1). The neural tube and notochord were separated from the paraxial mesoderm using a steel microscalpel. Incisions were generally performed from somite X to the posterior end of the neural tube, just ahead of the chordoneural hinge (see Catala et al., 1995). The axial organs were sectioned at the anterior and posterior ends of the segment to be removed and the latter was pulled off from the embryo using a calibrated glass micropipette. In previous experiments (Teillet and Le Douarin, 1983; Rong et al., 1992), the excisions were performed along the whole length of the neural axis.

As a first step, the effects of various components of the axial organs were tested as follows. (i) The neural tube and notochord were excised without subsequent grafting. (ii) The excision was followed by backgraft of a fragment of different components of the axial structures: the neural tube or notochord or both, or the dorsal or ventral moiety of the neural tube (the ventral neural tube was also reimplanted together with the notochord in some cases). Proteolytic enzymes (pancreatin Gibco) were used to separate the notochord from the neural tube when they were grafted separately. In contrast, when the notochord was grafted together with either the total neural tube or with its ventral part, the dissection was carried out microsurgically without enzymatic treatment. (iii) In the last experiments, the excised notochord and neural tube were replaced by aggregates of cells of the QT6 line of quail fibroblasts programmed (or not) to produce SHH.

In all cases, the graft was made at a length of approximately 10 somites including the three to five last-formed somites and an equivalent length in the non-segmented region, leaving the rostral and caudal regions of the excision without graft (Fig. 1).

The operated embryos were reincubated for various times depending on the type of analysis. Embryos were treated with Nile
Blue Sulphate (18-24 hours after the operations at E3). Others were fixed for whole-mount in situ hybridization (6-24 hours after the operations at E2, E3). 5 days after the operations (at E7), they were prepared for immunostaining. 9 days after the operations (at E11), they were treated for whole-mount staining of the skeleton.

**Nile Blue Sulphate staining**

We used the protocol of Jeffs and Osmond (1992). Operated or control embryos (30- to 35-somite stage) were removed from the egg and transferred to Pannett and Compton saline (PCS). Extraembryonic embryos (30- to 35-somite stage) were removed from the egg and we used the protocol of Jeffs and Osmond (1992). Operated or control embryos were fixed for whole-mount in situ hybridization (6-24 hours after the operations at E3). 5 days after the operations (at E7), they were prepared for whole-mount in situ hybridization (6-24 hours after the operations at E3). 9 days after the operations (at E11), they were treated for whole-mount staining of the skeleton.

In order to assess the massive cell death observed in excised embryos, we fixed two samples in paraformaldehyde (4%) for the TUNEL technique on tissue sections (Gavrieli et al., 1992; Wijsman et al., 1993) and used the kit supplied by Boehringer-Mannheim according to the protocol described by Sanders (1997).

**In situ hybridization of whole mounts and sections**

Embryos at 20- to 35-somite stage (E2 to E3) were dissected from extraembryonic membranes and heart were removed and the embryos were placed in a dissecting dish containing 100 μl of 1 mg/ml H2O of Nile Blue Sulphate (NBS) (Sigma), in 10 ml of PCS. Embryos were incubated at room temperature for 30-40 minutes and washed in PCS for approximately 1 hour, then photographed using a Wild Leica M10 stereomicroscope equipped with a diascopic Wild Heerbrugg apparatus. After observation, some of NBS-stained embryos were fixed and treated for whole-mount in situ hybridization.

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**Immunohistological analysis**

Control and grafted embryos were fixed at E7 in Carnoy’s fluid, dehydrated through ethanol, cleared in toluene and embedded in paraffin. Serial sections at 5 μm were immunostained with the 13F4 monoclonal antibody (mAb), which recognizes early myogenic and differentiated muscle cells (Rong et al., 1987), and the QCPN mAb (Hybridoma Bank), which recognizes all quail cells including QT6 cells.

**SHH-producing cell preparation**

RCASBP(A)-chick Shh encoding chick SHH was donated by C. Tabin (Riddle et al., 1993). Chicken embryonic fibroblasts (CEF) from O-line chick embryos were transiently transfected with the recombinant construct Shh-RCAS, as described previously (Watanabe and Le Douarin, 1996). QT6 cells were cultured in the presence of supernatant of transfected CEF for 1 week. Once confluent, the culture of RCAS(A)-chick Shh-infected QT6 cells was trypsinized, seeded in 90 mm bacteriological Petri dishes where they formed cellular aggregates used for grafting. These cells are further designated as Shh-RCAS-QT6 cells. We have observed that the RCASBP (A) virus is not propagated into the cells of the chick host of the chick strain (ISA JA57) used in our experiments.

Another Shh construct carrying the chick SHH-coding region under the influence of the CMV promoter in the pBK plasmid (Stratagen) was constructed by Hermann Rohrer (Germany). This construct was stably transfected to the QT6 cell line and selected in G418 (Duprez et al., 1998). These cells were used in some of our experiments.

**Skeletal staining**

Embryos at E11 were fixed overnight in acetic alcohol containing 15% Alcian blue. Bones were stained using alizarin red in alcohol and non-skeletal tissues were cleared with 1% KOH in 20% glycerol (Ojeda et al., 1970).

**RESULTS**

Somatic cells destined to die after surgical ablation of axial organs are rescued by factor(s) arising from the ventral neural tube and notochord

In all our experimental series, axial organs were excised from chick embryos in ovo at the 15- to 25-somite stage from the ventral neural tube and notochord.
level of somite X to the posterior part of the already formed neural tube including or not the chordoneural hinge (Fig. 1). At this stage, the non-segmented paraxial mesoderm corresponds to the presumptive territory of the next approximately 10 somites to be formed. As previously observed (Christ et al., 1972; Packard and Jacobson, 1976; Teillet and Le Douarin, 1983; Bober et al., 1994), elimination of the axial organs did not preclude further segmentation of the segmental plate but caused profound morphological changes in the development of the paraxial mesoderm facing the site of ablation. The two rows of already formed somites became closely apposed on the midline, remaining individualized. In contrast, somites that became segmented after the excision fused on the midline forming a single medial row of segments (Fig. 2A). 18 hours after the excision, massive cell death was already occurring in the somites all along the rostrocaudal length of the ablation (Fig. 2A; n=8). In embryos incubated for 24 hours following ablation, cell death was at its peak even in the most recently formed somites, indicating that axial organs are necessary for survival of cells of newly formed as well as of older somites. On transverse sections stained with the TUNEL technique, somites that were segmented before the ablation, as well as the newly formed somites that became fused by pair on the midline, exhibited many labeled cells principally in the sclerotome but also in the dermomyotome (Fig. 2B).

In order to determine which part of the neural tube-notochord complex is important for somitic cell survival, we reimplemented different portions of these structures into the groove created by the ablation (Fig. 1). Backgrafted fragments included (i) the neural tube and notochord, (ii) the neural tube alone, (iii) the ventral or the dorsal half of the neural tube and (iv) the notochord alone or with the ventral neural tube. Grafts were inserted along a length of no more than 10 somites including at most the five last-segmented somites and an equivalent length caudal to it, thus leaving two regions, caudal and rostral to the graft, where no axial organ was present. These regions served as controls to evaluate the effect of the graft.

At E3, the embryos in which fragments of neural tube and notochord were backgrafted together (n=9) showed, in the region of the graft, NBS staining equivalent to that which occurs in normal development (Fig. 2C). In fact, at this stage, a restricted zone of cell death, visible in each somite, has been attributed to the death either of some neural crest cells aggregating segmentally (Jeffs and Osmond, 1992) or of sclerotomal cells at the site of the intersclerotomal tissue (Sanders, 1997). Thus extensive somitic cell death due to the

Fig. 2. Cell death staining with (A-C,G) the Nile Blue Sulfate dye (NBS) and (B) the TUNEL technique. (A) 18 hours after excision of the neural tube-notochord complex, most somitic cells have absorbed the dye indicating massive cell death. In the rostral region (r), somites are closely apposed but remain individualized in two rows. In contrast, newly formed somites of the caudal region (c) are fused on the midline in a single row. (B) Transverse section 24 hours after the excision. The TUNEL technique shows that many sclerotomal cells(scl) and dermomyotomal cells (dm) are dying in the somites deprived of axial organs. (C) Backgraft of a fragment of the neural tube-notochord complex (NT/NC) restores completely the development of the somites, strictly in the region of the graft (gr). This lateral view shows that, in the rostral (r)- and caudal (c)-ablated non-grafted regions, somites are considerably flattened and have absorbed the dye (NBS). Arrows indicate the normal pattern of cell death described by Jeffs and Osmond (1992) and Sanders (1997) in the somites of E3 embryos. (D) Backgraft of the dorsal moiety of the neural tube (dNT). Somites in contact with the grafted fragment of dorsal neural tube and separated by it, exhibit cell death as in somites situated in the rostral and caudal regions where neural tube and notochord are absent. (E) Backgraft of a fragment of notochord (NC). At the level of the graft (gr) somites are fused on the midline dorsally to the notochord. Cell death is reduced and shows a segmented pattern where blue colored bands (cell death) alternate with non-stained bands (healthy tissue). (F) Graft of QT6-control cells (QT6C). Non-infected QT6 aggregates were grafted between somites 16 and 21 in an embryo excised from somite 7, at 17-somite stage. 24 hours after the graft, all the somites including those situated on each side of the grafted cells (gr) show a heavy blue staining indicating massive cell death. (G) Graft of Shh-RCAS-QT6 aggregates (SHH) at the level of somites 12-22 in a 17-somite-stage embryo excised from somite 6. Somites in the grafted region (gr) have a nearly normal size and somitic cell death is considerably reduced compared to rostral and caudal control regions and to QT6C graft (F). (A,C,D) Bar, 250 μm; (E-G) bar, 350 μm, (B) bar, 150 μm.
ablation of axial organs was completely inhibited in the precise region of the backgraft showing that the surgery was not responsible of it. In contrast, massive cell death was evident in the regions rostral and caudal to the graft where no axial organ was present (Fig. 2C).

When a fragment of neural tube alone was backgrafted (n=7), considerable rescue was also observed in the medial somitic component, but some spots of cell death were, however, visible laterally (not shown). The same effect was observed when only the ventral neural tube was backgrafted (n=8) (not shown). In contrast, after backgrafts of the dorsal neural tube (n=10), cell death was nearly as important on each side of the grafted tissue as in the control rostral and caudal regions (Fig. 2D).

When a fragment of notochord alone was backgrafted (n=12), both already formed and newly formed somites covered the graft and fused by pairs dorsally to the notochord. They showed a strongly reduced segmented pattern of cell death compared to the rostral and caudal regions where no axial organ was present (Fig. 2E).

In each experimental series, in toto NBS staining showed the same general pattern of survival whatever the rostrocaudal level considered, thus indicating that requirement of somitic cells for factors derived from axial organs is the same at all somitic developmental stages.

After excision of the axial organs, the backgraft of the ventral neural tube and/or the notochord restores somitic expression of Pax1 and MyoD

In order to evaluate more precisely the effect of putative morphogenetic substances released by ventral neural tube and notochord on somitic development, we have analyzed in the various experimental conditions recorded above the expression patterns of three genes, Pax-1, Pax-3 and MyoD, which are known to be expressed by cells belonging to different somitic compartments and giving rise to different derivatives. In situ hybridizations of the operated embryos were performed 6, 12, 18 or 24 hours after the surgery.

Pax-1 expression characterizes the sclerotomal part of the somites (Ballinger et al., 1988; Deutsch et al., 1988; Dietrich and Gruss, 1995). In the avian embryo, Pax-1 transcripts appear first in the ventromedial angle of somite IV, then becoming restricted to the sclerotome as dorsoventral segregation of the dermomyotomal and sclerotomal somitic components takes place (Barnes et al., 1996; Borycki et al., 1997 and see Figs 3A, 6A). Pax-3 is first weakly expressed in the segmental plate and in the whole epithelial somites. Later on, it becomes restricted to the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994 and see Figs 4A, 6B). As myotomal differentiation occurs in the dorsomedial part of the somites, MyoD, the first myogenic bHLH regulator to be expressed in the avian species (Charles de la Brousse and Emerson, 1990; Pownall and Emerson, 1992), becomes detectable mediadorsally in the myotome (Figs 5A, 6C). Pax-3 remains highly expressed in the medial and lateral areas of the dermomyotomes as previously described by Williams and Ordahl (1994) and Pourquié et al. (1996).

Chick embryos, in which both the neural tube and the notochord had been ablated as described above, were analyzed for these genes 6 to 24 hours after the surgery. 6 hours after the excision, the embryos (n=3) had 4 to 5 more somites. All along the excision site, the somites were smaller than in the area where the axial organs were left in situ. However, Pax-1 transcripts were present, as normally, in all the somites down to somite IV inclusively (Fig. 3B). This observation shows that three already segmented somites plus 1 or 2 newly segmented...
somites acquired Pax-1 transcripts in the absence of neural tube and notochord. 12 hours after the excision (n=3), the same number of somites showed Pax-1 transcripts albeit in smaller amounts due to cell death (Fig. 3C). Embryos analyzed 18 hours (n=3) and 24 hours (n=3) after excision, when cell death was at its peak, showed no Pax-1 transcripts over the whole length of the excision while normal patterns of expression were seen in the cervical and caudal regions where the neural tube and notochord had been left in situ (not shown). Backgraft of a fragment of the dorsal moiety of the neural tube (n=4) did not restore Pax-1 expression (Fig. 6D). In contrast, backgrafts of either neural tube-notochord complex or of neural tube alone or of ventral neural tube and notochord or of notochord alone resulted in all cases (n=9) in strong expression of Pax-1 strictly restricted to the site of the graft (Figs 3D,E, 6G).

The somitic expression pattern of Pax-3 was not fundamentally modified by excision of the neural tube and notochord whatever the stage of analysis (n=10). As shown on whole-mount preparations (Fig. 4B) and on sections (Fig. 6E) 24 hours after the excision, expression was fully maintained in the lateral moieties of the dermomyotomes, which escaped cell death. This was expected since, in our previous neural tube and notochord ablation experiments, the development of the hypaxial muscle component was not affected by this operation (Rong et al., 1992). The only difference between the site of excision and the normal control regions was that the lateral halves of the somites became close to each other on the midline since the cells from the medial somitic halves had died. Backgrafting a fragment of either the neural tube-notochord complex, or of the neural tube alone resulted in restoration of a more normal pattern of Pax-3 expression (not shown). After grafting the notochord alone (n=2), both lateral zones of high expression were separated by a medial zone where Pax-3 expression was weak or absent depending on the anteroposterior level considered (Figs 4C, 6H).

Like Pax-1, MyoD transcripts were totally absent in the operated embryos at the site of neural tube and notochord excision 18 and 24 hours after the surgery (n=5; see Fig. 5D,E). However, 6 and 12 hours after the excision, some of the more rostral somites at the level of the excision were lightly positive (Fig. 5B,C), showing that MyoD-expressing cells disappear progressively. Bober et al. (1994) also observed gradual disappearance of MyoD in chick embryos after extirpation of axial organs. In our backgraft experiments, MyoD expression was restored strictly at the sites where at least a fragment of the ventral neural tube (n=6) or the notochord (n=2) was backgrafted (Figs 5E, 6I). Backgrafting of the dorsal moiety of the neural tube (n=3) rescued only very few MyoD-expressing cells localized in the rostral region of the graft where the paraxial mesoderm was already segmented at the operation time but not at more caudal levels (Figs 5D, 6F). As shown on Fig. 6D, Pax-1 transcripts were totally absent in these embryos at the same level.

From these findings, ventral neural tube and notochord backgrafts can be assumed to rescue both sclerotomal cells and myotomal cells of the epaxial domain from death and to allow them to continue to express cell-type-specific gene activities.

**SHH rescues somitic cells from death and allows the expression of genetic markers of dorsoventral somitic specification**

Since the ventral neural tube and notochord were shown to be highly efficient in ensuring survival and differentiation of paraxial mesodermal cells, we thought that the protein SHH produced by both the floor plate and the notochord (Marti et al., 1995) was a possible candidate in mediating this effect. To test this hypothesis, we substituted quail fibroblasts (QT6 cells) infected with Shh-RCAS retrovirus (Shh-RCAS-QT6) to neural tube-notochord complex, according to the experimental paradigm used before for neural tube or notochord backgraft experiments (see Materials and methods and Fig. 1). Adhesion of QT6 cells to embryonic tissues was very efficient and ensured correct positioning of the graft. 1 day after the
operation, Shh transcripts were seen to be strictly localized to the midline of chick embryos in which Shh-RCAS-QT6 were grafted (not shown).

The graft of non-infected control QT6 cells after neural tube and notochord excision (n=4) did not change the pattern of cell death in the paraxial mesoderm, whether it was segmented or not at the time of operation (Fig. 2F). The only difference between these embryos and those that were subjected to excision alone was that fusion of the somites over the dorsal midline did not occur at the level of the graft, due to the presence of grafted cells. 6 and 12 hours after the graft, Pax-1 (n=6) and MyoD (n=6) expression progressively decreased due to cell death. 24 hours after the graft, these transcripts were completely absent in the somites at the level of the operation (n=3 and 2). QT6 cells could be visualized because they themselves produce low levels of MyoD transcripts. Whatever the time after the operation, Pax-3 expression (n=6) was still maintained in the lateral somitic half as in excised, non-grafted embryos (not shown).

In contrast, implantation of Shh-RCAS-QT6 at the level of the last-formed somites and the non-segmented paraxial mesoderm, markedly prevented somitic cell death (n=8; Fig. 2G). 6 and 12 hours after the operation, Pax-1 (n=8) and MyoD (n=8) expression were present at the level of the graft while decreasing or absent in rostral and caudal control regions (not shown). 24 hours after the graft, Pax-1 (n=4) was expressed all along the graft (Fig. 3F) in the ventral somitic half (Fig. 6J). Pax-3 (n=5) exhibited a normal pattern of mediolateral expression (Figs 4D, 6K) and MyoD (n=5) showed a normally segmented pattern in the mediadorsal part of the somites of the entire grafted region (Fig. 5F and 6L).

It thus appears that SHH produces by itself the same effect as the notochord and the floor plate on somitic cell survival and on their further development to dorsal and ventral somitic cell types at least up to 24 hours after the operation.

**SHH supports organogenesis of vertebral column, ribs and epaxial muscles**

Our previous experiments (Teillet and Le Douarin, 1983 and Rong et al., 1992) showed that the Shh-RCAS-QT6-grafted cells (n=5) remained localized mostly in the site of the graft, while the stable SHH-expressing cell line (n=3) became more dispersed in the grafted embryos. In both cases, vertebrae, ribs and muscles developed. Vertebral cartilage was found to surround the Shh-RCAS-QT6 aggregates, whereas muscle masses were disposed dorsolaterally on each side of the vertebra-like structures as in normal embryos (Fig. 7A,B). As seen on skeletal preparations at E11, while excised non-grafted embryos were completely devoid of vertebrae and showed scarce remnants of ribs (Fig. 7D), implantation of Shh-RCAS-QT6 (n=3) allowed a large portion of vertebral column and ribs to develop (Fig. 7E). Interestingly, after grafting SHH-producing cells, the vertebral arches were always open dorsally and the spinous process of the vertebrae was absent (see Fig. 7F).

**Fig. 5. In toto MyoD in situ hybridization.** (A) 38-somite-stage control chick embryo. MyoD transcripts are present dorsomedially in every somite. Bar, 800 μm. (B,C) 6 and 12 hours after excision of the neural tube and notochord MyoD transcripts are disappearing at the level of the surgery, due to cell death. (D) 35-somite-stage operated embryo. Backgraft of the dorsal moiety of a fragment of neural tube (dNT) from somites 18-23 in a 20-somite chick embryo excised from somite 10. Several somites in the rostral region of the graft show a weak MyoD expression, while the caudal ones do not, like in the excised non-grafted regions. (E) 37-somite-stage operated embryo. Backgraft of a fragment of notochord (NC) between somites 13 and 19 in a 17-somite-stage embryo. MyoD expression is completely restored at the level of the graft, while rostrally and caudally to it no 24 transcripts appear in the excised region. (F) 35-somite-stage operated embryo. Graft of Shh-RCAS-QT6 (SHH) at the level of somites 10-18 in a 16-somite-stage embryo excised from the level of somite 5. MyoD is strongly expressed in somites 10-18 (brachial level) where SHH-producing cells have been grafted and not at all rostrally and caudally to it. (B-F) Bar, 400 μm; Ex, level of excision; gr, level of the graft.
Substitution of the notochord and neural tube development of the vertebral column

7A,E). In contrast, embryos grafted with QT6-control cells (n=2) like the ones that were not grafted (n=3; Fig. 7D) showed neither vertebrae nor ribs (not shown). The skeleton of the girdles and limbs were always present. Body wall muscles that develop from the lateral somitic half were also observed as already described by Rong et al. (1992) in embryos totally deprived of axial organs (n=5). However, in the absence of derivatives of the medial somitic half, scapulae were closed together dorsally (Fig. 7D) and body wall muscles were located laterodorsally.

DISCUSSION

SHH protein rescues paraxial mesoderm from cell death

Removal of both neural tube and notochord in E2 chick embryos in ovo results in massive cell death in the medial somitic compartment. In contrast, the cells belonging to the lateral part of the dermomyotomes, the fate of which is to give rise to the hypaxial musculature, survive the ablation and migrate to their target sites in the embryo (i.e. the limbs and body wall) where they further differentiate. Backgrafting of the neural tube or the notochord restores normal somitic development (Teillet and Le Douarin, 1983; Rong et al., 1992).

We demonstrate here that the source of the signal(s) responsible for the survival of the medial moiety of the somites is located in the ventral neural tube and the notochord. Since the ventral neural tube includes the floor plate, which shares several gene activities with the notochord, we suspected that SHH, a secreted factor produced by both these structures, could be involved in the survival of paraxial mesodermal cells. SHH-producing cells (Shh-RCAS-QT6), introduced into the groove resulting from the neural tube and notochord ablation, prevented cell death in paraxial mesoderm apparently to the same extent as either one of the two axial organs, ventral neural tube or notochord. Gene activities specific for each of the dorsoventral somitic components, i.e. Pax-1 for the sclerotome and MyoD for the myotome, were observed in the vicinity of Shh-RCAS-QT6 graft in a pattern very similar to that in normal somites. In contrast, the parental QT6 cell line had no effect on somitic cell survival. Since the retroviral construct that we use cannot infect host cells (see Materials and methods section), one can conclude that SHH protein acts as a diffusible signal.

SHH is required for sclerotomal cell survival, Pax-1 maintenance and development of the vertebral column

Substitution of the notochord and neural tube with a source of SHH protein rescues sclerotomal cells from death and allows Pax-1 expression to be maintained. SHH also promotes the formation of vertebra-like structures, which have most of the normal morphological characteristics of vertebrae, in particular ‘neural arches’ embracing the source of the signal as they do for the neural tube.

It has been reported in un and Sd mutant mice that de-epithelialisation of the ventral somites can occur in the absence of Pax-1 expression (Koseki et al., 1993). However, in these mutants, differentiation of the sclerotome into cartilage forming the axial skeleton is severely impaired (Grüneberg, 1954; Balling et al., 1988; Wallin et al., 1994), showing that Pax-1 is required for the formation of the vertebrae. In the chick, Pax-1 starts to be expressed in the ventromedial part of somite IV (Deutsch et al., 1988; Johnson et al., 1994; Borycki et al., 1997) so that, at the stage when our experiments are performed, Pax-1 transcripts are present at least in the rostral
third of the ablation site. We observed that ablation of the source of SHH did not prevent appearance of Pax-1 expression in the somites already segmented at the operation time and in one or two somites which segmented after the excision (see the results obtained 6 hours postoperation). Pax-1 expression, however, is not maintained much more than 12 hours due to cell death. Therefore, the last-formed somites, in the experiments where the embryos were fixed 18 and 24 hours after excision, could not express Pax-1 before being the site of massive cell death. In fact, whether or not somitic cells expressed Pax-1, they died if they were deprived of the influence of the ventral neural tube and notochord. Cells producing SHH grafted in the place of the excised axial organs promoted the survival of sclerotomal cells and their further differentiation and vertebral organogenesis. This finding is in agreement with the phenotype of mice carrying a null mutation of the Shh gene, in which the vertebral column and most of the ribs are absent (Chiang et al., 1996). In these mutants, Pax-1 expression is initiated and rapidly lost. In the light of the results presented here, one important role of SHH on the maintenance of Pax-1 expression is due to its effect on somitic cell survival. This effect may be accompanied by a direct effect on cell proliferation as previously suggested by Johnson et al. (1994) and Fan et al. (1995).

One part of the vertebra, the spinous process, was always lacking after SHH-producing cells were grafted in place of neural tube and notochord. It has been shown that the spinous process forms from somitic cells that migrate dorsomedially, between the neural tube and the superficial ectoderm, from E4 onward (Takahashi et al., 1992). These precartilaginous cells do not express the Pax-1 gene but instead Msx-1 and Msx-2 genes and their development is controlled by the TGF-β family member BMP4 whose transcripts are expressed by both the superficial ectoderm and the roof plate of the neural tube (Monsoro-Burq et al., 1996). The graft of a notochord between the dorsal neural tube and the mediodorsal ectoderm inhibits expression of Msx genes and prevents the formation of a spinous process (Monsoro-Burq et al., 1994). In contrast, grafts of BMP-2- or BMP-4-producing cells increase the size of the spinous process (Monsoro-Burq et al., 1996). Interestingly, however, in the absence of the axial structures, i.e. the normal sources of SHH, neither the ventrolateral nor the dorsal parts (spinosus process) of the vertebrae develop (Monsoro-Burq et al., 1994). The fact that most somitic cells (including sclerotomal cells) die could account for the absence of spinous process development under these conditions. Thus, development of the spinous process requires first SHH provided by the notochord and floor plate for ensuring somitic cell survival and a second signal involving BMP-4, originating from both mediodorsal ectoderm and roof plate, essential for chondrogenesis in this subectodermal environment. Formation of the vertebral body and neural arches necessitates the continuous action of a single signal, SHH for both survival and chondrogenesis.

**SHH is required for myotomal cell survival, MyoD expression and further muscle differentiation**

In the absence of neural tube and notochord, MyoD expression was rapidly abolished while Pax-3 transcripts remained, although they were distributed differently due to the absence of axial organs and the death of medial somitic component. Thus, muscles that differentiated in these embryos were hypaxial muscles, which did not require the notochord-floor plate SHH signal to survive (Rong et al., 1992 and these experiments). It is important to note that, in the absence of epaxial muscles and vertebrae, hypaxial muscles come to occupy a mediadorsal position, whereas normally they are within the ventrolateral body wall. It is interesting that in the Shh<sup>−/−</sup> mutant mice, myf5 transcripts, which are normally expressed in the myotomes (bwm) are normally located. Bar, 250 μm. (B) The grafted cells have been stained with the QCPN Mab. They are surrounded by vertebral cartilage (v) stained with Alcian Blue. Bar, 60 μm. (C-E) Skeleton preparations of E11 chick embryos at the trunk level. (C) Normal embryo. The scapulae (sc) are well separated on each side of the vertebral column. The spinous processes are visible on the midline of the vertebral column. The spinous processes are visible on the midline of the vertebrae at the thoracic level where the ribs develop. (D) Embryo deprived of neural tube and notochord from somite 8 at 18-somite-stage (Exc). This embryo lacks vertebral column and ribs at the level of excision. Scapulae (sc) are apposed in the thoracic level while skeleton of anterior and posterior limbs (al, pl) are normally developed. md, mandible; cv, cervical vertebrae. (E) Aggregates of Shh-RCAS-QT6 cells were grafted at the brachial level of a 20-somite-stage chick embryo excised from somite 10 (SHH). Vertebral body and neural arches are present at the level of the graft. Scapulae (sc) are separated as in normal embryos. (C-E) Bars, 1500 μm.

**Fig. 7.** (A) Transverse section of a E7 chick embryo deprived of axial organs at 19-somite stage and grafted with Shh-RCAS-QT6 aggregates (arrow) in the site of the excision. Organogenesis of vertebrae (v), ribs (r) and epaxial muscles (em) has occurred in the vicinity of the grafted SHH-producing cells. Body wall muscles (bwm) are normally located. Bar, 250 μm. (B) The grafted cells have been stained with the QCPN Mab. They are surrounded by vertebral cartilage (v) stained with Alcian Blue. Bar, 60 μm. (C-E) Skeleton preparations of E11 chick embryos at the trunk level. (C) Normal embryo. The scapulae (sc) are well separated on each side of the vertebral column. The spinous processes are visible on the midline of the vertebral column. The spinous processes are visible on the midline of the vertebrae at the thoracic level where the ribs develop. (D) Embryo deprived of neural tube and notochord from somite 8 at 18-somite-stage (Exc). This embryo lacks vertebral column and ribs at the level of excision. Scapulae (sc) are apposed in the thoracic level while skeleton of anterior and posterior limbs (al, pl) are normally developed. md, mandible; cv, cervical vertebrae. (E) Aggregates of Shh-RCAS-QT6 cells were grafted at the brachial level of a 20-somite-stage chick embryo excised from somite 10 (SHH). Vertebral body and neural arches are present at the level of the graft. Scapulae (sc) are separated as in normal embryos. (C-E) Bars, 1500 μm.
present in the medial part of the mouse somites, are only weakly expressed, while MyoD transcripts, which are localized laterally in the dermomyotomes, are expressed (Chiang et al., 1996). We therefore suggest that the striated muscles observed in the Shh<sup>−/−</sup> mutants are mostly hypaxial muscles that have reached a dorsal position in the absence of the epaxial muscles and vertebrae that failed to develop due to the absence of SHH signaling.

In the experiments reported here, grafting SHH-producing cells in place of the neural tube and notochord restored the normal pattern of expression of MyoD by rescuing somitic cells from death. Thus, SHH maintains the activity of this gene by promoting myotomal cell survival as is the case for Pax-1 in the sclerotome. Since in the absence of SHH signaling the somitic cells die, we conclude that the effect of notochord and floor plate on muscle differentiation already observed by other authors (Buffinger and Stockdale, 1994; Cossu et al., 1996b; Münsterberg and Lassar, 1995; Stern and Hauschka, 1995; Pownall et al., 1996) is primarily due to the essential requirement of SHH for somitic cell survival. This survival effect is probably associated with an effect on the proliferation of muscle cells. Overexpression of Shh indeed results in an expansion of the MyoD expression domain in the paraxial mesoderm in zebrafish (Weinberg et al., 1996; Hammerschmidt et al., 1996; Concordet et al., 1996) and in chick (Johnson et al., 1994). In addition, SHH was shown to induce the proliferation of already committed skeletal muscle cells in the chick limb (Duprez et al., 1998). The SHH effect on myotomal cell survival and proliferation does not exclude a specific role for this molecule on muscle differentiation: in zebrafish embryos, overexpression of a combination of Echinoderm Hedgehog and SHH induces supernumerary muscle pioneers (Currie and Ingham, 1996) and Sonic hedgehog mediates induction of slow muscle (Blagden et al., 1997).

It has been proposed that the initiation of the myogenic programme in the dorsal somite is mediated by factors originating from the dorsal neural tube (Fan and Tessier-Lavigne, 1994; Stern and Hauschka, 1995; Cossu et al., 1996b; Spence et al., 1996; Dietrich et al., 1997) and/or from the superficial ectoderm overlying the somites (Fan and Tessier-Lavigne, 1994; Cossu et al., 1996b; Dietrich et al., 1997). The WNT proteins constitute good candidates for this role, since it has been shown that WNTs are able to induce myogenesis in explants of paraxial mesoderm (Münsterberg et al., 1995; Stern et al., 1995; Maroto et al., 1997; Fan et al., 1997). It has been demonstrated that WNTs and SHH can act in combination (Münsterberg et al., 1995; Maroto et al., 1997). Recently, several models of control of dorsoventral somite patterning, implicating antagonistic activities of WNT and SHH pathways through various mechanisms, have been proposed (Hirsinger et al., 1997; Marcelle et al., 1997; Capdevila et al., 1998; Reshef et al., 1998).

In the experimental design where the dorsal neural tube was grafted back into the groove left by the ablation of the axial organs, we observed the survival of some MyoD-expressing cells but only to a very small extent (Figs 5D, 6F). This suggests that the dorsal neural tube has the capacity to initiate and sustain myogenesis from the dorsal somite in the absence of a SHH source, but is unable to increase the committed muscle cell population to a normal level, a function that is fulfilled by SHH. This view is supported by another type of experiments in which Hensen’s node is removed (see Catala et al., 1996). Caudally to the ablation, the dorsal part of the neural tube alone develops while, in the absence of notochord and floor plate, its ventral part degenerates (our unpublished observations). At this level, some muscle cells differentiate ventrally to the remaining neural tube in the total absence of a source of SHH (Catala et al., 1996). This indicates that, in normal development, signals from the dorsal neural tube and ectoderm (probably WNT factors) act in close cooperation with SHH expressed in the ventral structures to respectively initiate, and maintain and increase myogenesis.

In the experiments reported here, the axial structures were removed before muscle specification has appeared in the nonsegmented paraxial mesoderm. One has therefore to assume that the influence emanating from the superficial ectoderm is sufficient for specifying myogenesis. However, SHH is necessary to enable these presumptive myogenic cells to survive, proliferate and further differentiate even in the absence of a dorsal neural tube. One hypothesis is that, in the absence of the WNT source normally provided by the dorsal neural tube, the WNT proteins released by the ectoderm (Cossu et al., 1996b) are able to initiate myogenesis in dermomyotome in the presence of SHH. This is in concordance with the findings and Dietrich et al. (1997). However, the level of WNT proteins expressed in the ectoderm might not be sufficient to allow further development of myotomal cells in the experiments where the survival factor SHH was lacking.

**Does SHH have a ventral polarizing effect?**

It has been shown that SHH induces sclerotomal markers in somitic explants (Fan and Tessier-Lavigne, 1994; Fan et al., 1995) and that ectopic expression of Shh gene in the dorsal cells of the segmental plate induces dorsal expansion of the Pax-1 expression in the somites, at the expense of myotomal derivatives (Johnson et al., 1994). These results, together with the conversion of the whole somite into cartilage following a dorsolateral graft of the notochord or floor plate (Pourquié et al., 1993; Brand-Saberi et al., 1993; Goulding et al., 1994), were interpreted as meaning that SHH secreted by the notochord and the floor plate acts as a somitic ventralizing factor. The results reported here were thus surprising, as replacing axial organs (neural tube and notochord) by cells producing SHH could be thought to result in the sole (or highly dominant) differentiation of sclerotomal derivatives. The fact that MyoD expression was initiated and maintained at the level of insertion of SHH-producing cells was an indication that the entire pattern of somitic differentiation was actually maintained in this situation. It was striking to see, at E3, that in the presence of a large and uniform source of SHH occupying the whole space left by the ablation of the axial structures, the relative disposition of Pax-3- (dermomyotome), MyoD- (myotome) and Pax-1- (sclerotome) expressing cells was the same as in normal embryos (see Fig. 6J-L). This was confirmed when the embryos were examined at later stages of development. At E7, the arrangement of muscles and vertebral cartilage along the mediolateral axis was found to be normal. Cartilage was found surrounding the SHH-secreting cells and muscle masses were located dorsolaterally to the pieces of cartilage (Fig. 7). The fact that SHH-producing cells can replace the neural tube and notochord in permitting the entire somites to differentiate can be explained by a complementary
role played by the superficial ectoderm, in the determination of the dermomyotome. Thus, patterning of the somites can result from a balance between dorsalizing and ventralizing factors in agreement with results obtained from various experimental paradigms (Dietrich et al., 1997; Fan et al., 1997).

As a conclusion, SHH appears to be an essential factor for somitic cell survival and is also likely to have a positive effect on the proliferation of the somitic cells, be they sclerotomal or dermomyotomal in nature.

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