Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10435–10439, October 1994
Developmental Biology

Heterogeneity in the development of the vertebra

(notochord/*Msx* gene/dorsoventral polarity/neural tube/vertebral development)

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ABSTRACT **Vertebrae are derived from the sclerotomal moities of the somites. Sclerotomal cells migrate ventrally to surround the notochord, where they form the vertebral body, and dorsolaterally to form the neural arch, which is dorsally closed by the spinous process. Precursor cells of the spinous process as well as superficial ectoderm and roof plate express homeobox genes of the *Msh* family from embryonic day 2 (E2) to E6. The notochord has been shown to be responsible for the dorsoventral polarization of the somites and for the induction of sclerotomal cells into cartilage. Indeed, supernumerary notochord grafted laterally to the neural tube induces the conversion of the entire somite into cartilage. We report here that a mediodorsal graft of notochord prevents the sclerotomal cells migrating dorsally to the roof plate from differentiating into cartilage. Under these experimental conditions, expression of *Msx* genes is abolished. We thus demonstrate that cartilaginous differentiation is differentially controlled in the dorsal part of the vertebra (spinous process) and in the neural arch and vertebral body.**

The vertebral column is composed of metameric elements, the vertebrae, separated by intervertebral discs ensuring the mobility of the entire structure; both are formed by the ventral sclerotomal moiety of the somites. Each vertebra (except the atlas) comprises a vertebral body arising from sclerotomal cells that have migrated to surround the notochord and a neural arch that surrounds the neural tube. Differentiation of the vertebral cartilage obeys a chronological ventrodorsal gradient beginning around the notochord and extending laterally and dorsally. The last region of the vertebra to be formed is its mediodorsal portion, which closes the arch and develops as the spinous process. The shape and size of the vertebrae vary along the anteroposterior (AP) axis, and their morphogenesis has recently been shown to be controlled by homeobox genes of the *Antennapedia* type (1), whereas that of the intervertebral disc involves the activity of a transcription factor of the Pax family, *Pax1* (2, 3).

The problem that we addressed in the work reported here concerns the mechanisms by which patterning of the vertebrae along the dorsoventral axis is achieved at the genetic level. In previous studies (4, 5), we demonstrated that expression of the homeobox gene *Msx2* is closely related to the development of the vertebral spinous process. The roof plate, the overlying ectoderm, and intermediate mesenchyme, from which the spinous process develops, express *Msx2* from the time of neural tube closure to the beginning of cartilage condensation. Moreover, expression of *Msx2* is induced in the ectoderm and the mesenchyme located dorsolaterally above the somites by ectopic grafts of the dorsal neural tube, provided that the grafted roof plate and the superficial ectoderm are in close proximity, demonstrating that cell–cell interactions between those two tissues are essential. Furthermore, the mesenchyme induced to express *Msx2* differentiates further into ectopic islands of cartilage, subcutaneously located, which can be considered as ectopic spinous processes. In contrast, when the neural tube is rotated 180° dorsoventrally, at the level of the recently segmented mesoderm, *Msx2* no longer is expressed in the roof plate (now in a ventral position), the mediodorsal mesenchyme, or ectoderm. Spinous processes do not develop in this case, and the neural arches remain open at the level of the operation. These results have emphasized the importance of the association between neural tube dorsoventral polarity, *Msx2* expression, and the formation of the dorsal part of the vertebra.

The notochord and floor plate were recently shown to exert a ventralizing effect not only on the neural tube (6–13) but also on the paraxial mesoderm (14, 15). A dorsolateral graft of a supernumerary notochord or floor plate between the neural tube and adjacent somite induces the conversion of the somitic mesenchyme into sclerotome and cartilage while preventing the differentiation of dermomyotomal structures.

This result is at odds with the observation that the spinous process is absent when the neural tube is rotated dorsoventrally—that is, when the floor plate is placed in a position dorsal to the roof plate. To try and resolve the apparent paradox, we decided to further investigate the relationships between *Msx2* gene expression in the dorsal structures, the patterning of the vertebra, and the influence of the notochord on the formation of dorsal cartilage *in vivo*.

We show here that the early ablation of the notochord results in the complete absence of vertebra; the somite differentiates exclusively into muscle and dermis, despite the maintenance of *Msx2* expression in the dorsal part of the neural tube. This implies that the notochord is required for the determination of the somite into sclerotome. We also have found that if the notochord is grafted dorsally to the neural tube in the as yet unsegmented region of the paraxial mesoderm, *Msx2* expression in the dorsal ectoderm, mesenchyme, and roof plate is inhibited; thereafter, superficial cartilage differentiation and formation of a spinous process are totally suppressed. Therefore, development of the vertebra requires the influence of the notochord for early determination of the sclerotome and for its subsequent differentiation into the cartilage of the vertebral body and lateral parts of the neural arches; the formation of the spinous process involves tissue interactions between the roof plate and the superficial ectoderm. This process is apparently linked to the activation of genes of the *Msh* family.

MATERIAL AND METHODS

Heterospecific grafts between chicken and quail embryos were performed at embryonic day 2 (E2).

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Abbreviations: DRG, dorsal root ganglia; E2–E10, embryonic days 2–10; AP, anteroposterios.

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Notochord Grafting. The notochord, enzymatically dissected from the level of the 10 last somites formed of quail embryos (9–24 somite stage), was grafted into chicken embryos (12–21 somite stage) either laterally or mediodorsally to the neural tube. The levels of implantation are indicated in Fig. 1. In control embryos, similar fragments of fixed notochord (4% paraformaldehyde), hair, or various types of membranes (Millipore, silastic) were similarly implanted.

Notochord Ablation. The notochord and the neural tube were surgically excised from 13–17 somite-stage embryos for the length of the still unsegmented paraxial mesoderm *in ovo*. The neural tube was separated from the notochord by mild enzymatic digestion (4) and reimplanted; the notochord was then discarded.

***In Situ* Hybridization.** The operated embryos were fixed at E4 and treated as described (4). A 320-bp fragment located 3′ to the homeobox of quail Msx2 cDNA was used to synthesize antisense and sense RNA probes.

Histology and *in Toto* Skeletal Staining. Operated embryos were fixed from E3 to E10 for standard histological techniques and at E8–E10 for *in toto* staining according to the classical alcian blue/alizarin red procedure.

Immunocytochemistry. Sections were treated with a monoclonal antibody raised against the BEN glycoprotein (14), a marker for floor plate and motoneurons identical to SC1 (10).

RESULTS

Effect of Implants of Notochord on *Msx2* Expression. When the notochord was inserted laterally at E2 between the neural tube and the somites (Fig. 1 *A* and *B*), the implant was found within the somitic mesenchyme at E4. When the graft was placed at a level where the paraxial mesoderm was still unsegmented, a characteristic wedging of the lateral neural tube and the formation of a supernumerary floor plate were induced as described (6). The roof plate was then shifted slightly to the contralateral side, where it continued to express *Msx2* as did the ectoderm and the mesenchyme located dorsally to it (Fig. 2 *A–D*). When the notochord was implanted mediodorsally, it was found at E4 to be dorsal to the neural tube, covered by superficial ectoderm, and surrounded by mesenchymal cells. The influence of the graft on *Msx2* expression varied greatly with respect to its site of implantation along the AP axis. When it was implanted within the segmented area (n = 10), virtually no effect on *Msx2* expression was seen in 7 of 10 embryos. In three cases,

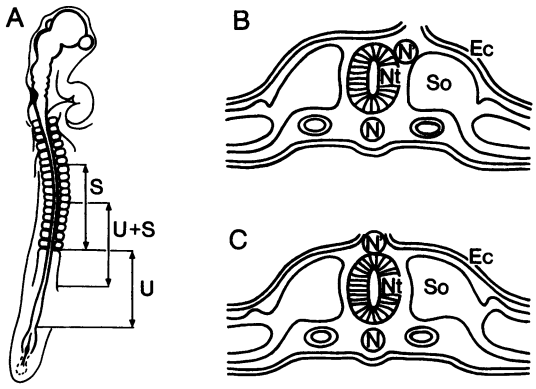

FIG. 1. Lateral and mediodorsal grafts of the notochord. The AP level of implantation varied from the segmented area (S) to the unsegmented area (U) (*A*). After incision of the ectoderm, the quail notochord was inserted between the neural tube and the somites and/or the unsegmented plate for the lateral implantation (*B*) or above the dorsal aspect of the neural tube (*C*). Ec, ectoderm; N, endogenous notochord; N′, grafted notochord; Nt, neural tube; So, somitic mesoderm.

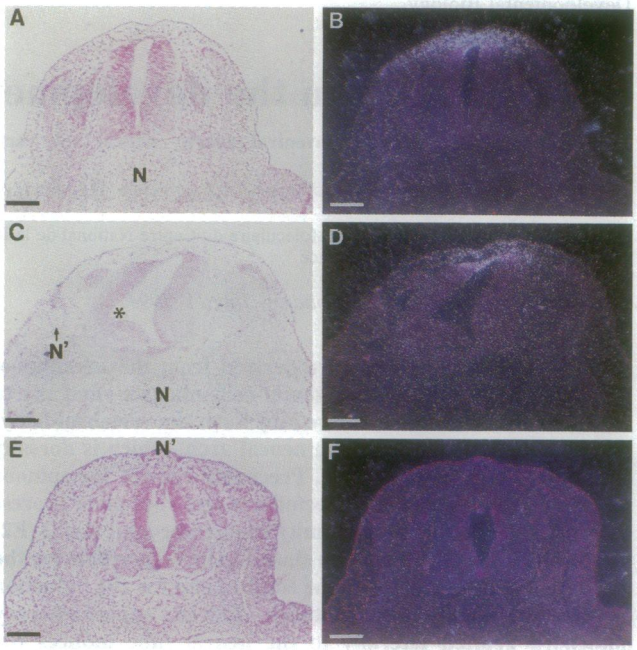

FIG. 2. *Msx2* expression after notochord grafts. *Msx2* expression was analyzed by *in situ* hybridization followed by bright-field (*A*, *C*, and *E*) and dark-field (*B*, *D*, and *F*) photomicroscopy. In normal embryos, the gene is expressed in the roof plate, the dorsal mesenchyme, and the ectoderm. The dorsal root ganglia (DRG) and the other somitic derivatives are negative for *Msx2* expression (*A* and *B*). In embryos that received a lateral notochord graft (N′) (*C*), the neural tube shows a lateral wedging facing the graft (asterisk), revealing the induction of a floor plate laterally. In these embryos, the roof plate is slightly displaced to the contralateral side. The DRG on the operated side is smaller than in controls and develops abnormally in a dorsal position. *Msx2* expression is restricted to the roof plate in its new location, the overlying mesenchyme and the ectoderm (*D*). In contrast, mediodorsal grafts of the notochord (*E*) result in direct contact between the roof plate and the implant. DRG form on each side of the neural tube. When the graft was implanted within the unsegmented area, the complete inhibition of *Msx2* expression in the roof plate, the dorsal mesenchyme, and the ectoderm is observed (*F*). (Bar = 100 μm.)

however, inhibition of *Msx2* labeling was evident only at the caudal level of the graft insertion. When the notochord was grafted in the unsegmented area, *Msx2* expression was completely inhibited in all cases (n = 9) (Fig. 2 *E* and *F*). Neither the roof plate nor the overlying mesenchyme or superficial ectoderm expressed *Msx2*. In two cases, the notochord was inserted within the neural groove. The graft was then found at E4 to be localized between the two neural folds, and *Msx2* expression was abolished just as it had been when the notochord was placed mediodorsally to the roof plate. In grafts encompassing the five last somites formed and an equivalent length in the unsegmented area (n = 5), a transition was seen between the responding and the nonresponding territories. Implantation of a neutral obstacle of about the size of a notochord was used to assess possible alterations of ectoderm–neurectoderm interactions (n = 8). In these cases no perturbation of *Msx2* was seen in the neural tube, the mesenchyme, or the ectoderm. In conclusion, mediodorsal grafts of notochord specifically inhibit the expression of *Msx2* in the dorsal axial structures provided that the graft is implanted at the level of the unsegmented paraxial mesoderm.

Effect of Early Ablation of the Notochord on *Msx2* Expression. Ablation of the notochord was carried out at E2; two embryos were observed at E3, and the sections were treated with anti-BEN monoclonal antibody; three other embryos

were fixed at E4 and treated for *in situ* hybridization with the *Msx2* probe. In four of the five cases, the neural tube was devoid of floor plate structures as revealed by morphological criteria and by the absence of BEN immunoreactivity; this nonpolarized morphology was conspicuous in the medial part of the operated region, whereas, in its rostral and sometimes caudal parts, the neural tube progressively resumed a normal morphology. In the nonpolarized region, the DRG and the myotomes fused underneath the neural tube (Fig. 3*A*). *Msx2* expression in the dorsal part of the depolarized neural tube, the ectoderm, and the intervening mesenchyme was not perturbed even in the most modified segment of the spinal cord (Fig. 3 *A* and *B*). In particular, no lateral or ventral extension of the labeling was seen in the neuroepithelium. Thus, the roof plate-restricted expression of *Msx2* is independent of the polarizing influence of the ventral notochord. Interestingly, when, as a consequence of the operation, the round-shaped neural tube was distanced from the ectodermal surface (Fig. 3*C*), *Msx2* expression disappeared (Fig. 3*D*). This demonstrates once more that ectoderm–roof plate interactions are critical in the regulation of *Msx2* expression.

Influence of the Notochord on the Development of the Vertebral Cartilage. Vertebral morphogenesis was first studied at E8–E10 in embryos previously subjected to laterodorsal grafts. These grafts, which do not modify *Msx2* expression, did not prevent the formation of the spinous process (n = 8) (Fig. 4*C*). However, formation of the neural arch was profoundly perturbed. As already described (14), a large mass of cartilage developed on the side of the graft where dermis, feather buds, and muscle did not differentiate (Fig. 4*C*).

Mediodorsal grafts of notochord in the unsegmented area abolished the differentiation of the dorsal cartilage that normally forms the spinous process (n = 27). As a result, the neural arch remained open dorsally, along the length of several vertebrae, at the level of the graft (as seen in skeletal preparations; Fig. 5). Transverse sections show that the lateral and ventral parts of the vertebra are well developed

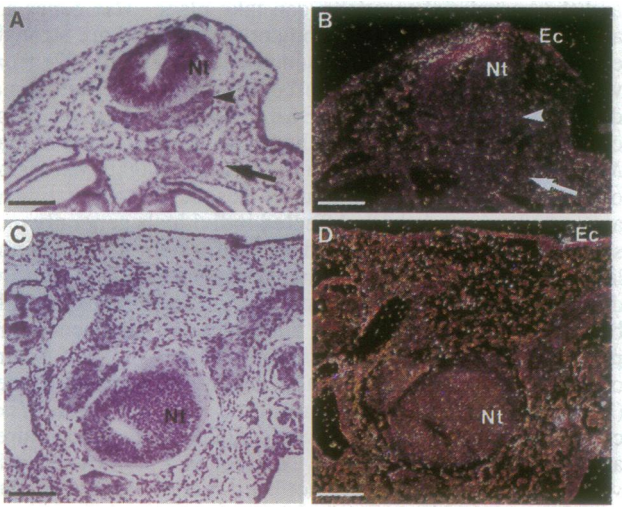

FIG. 3. Effect of ablation of the notochord on *Msx2* expression. The neural tube (Nt) has no floor plate, assumes a rounded shape, and is thinner at the level of the roof plate (*A*). The DRG (arrowhead) and the myotomes (arrow) have fused under the ventral aspect of the neural tube, in the midportion of the excised territory. Some mesenchymal cells have migrated to the dorsal aspect of the neural tube. *Msx2* expression is detected in the roof plate, the loose mesenchyme, and weakly in the ectoderm. There is no extension of the labeled domain in the lateral neuroepithelium (*B*). When the neural tube is deeply implanted and separated from the ectoderm (Ec) by a thick mass of mesenchymal tissue (*C*), *Msx2* is no longer expressed (*D*). Thus, the ectoderm–neurectoderm proximity is important for *Msx2* expression or maintenance or both. (Bar = 100 μm.)

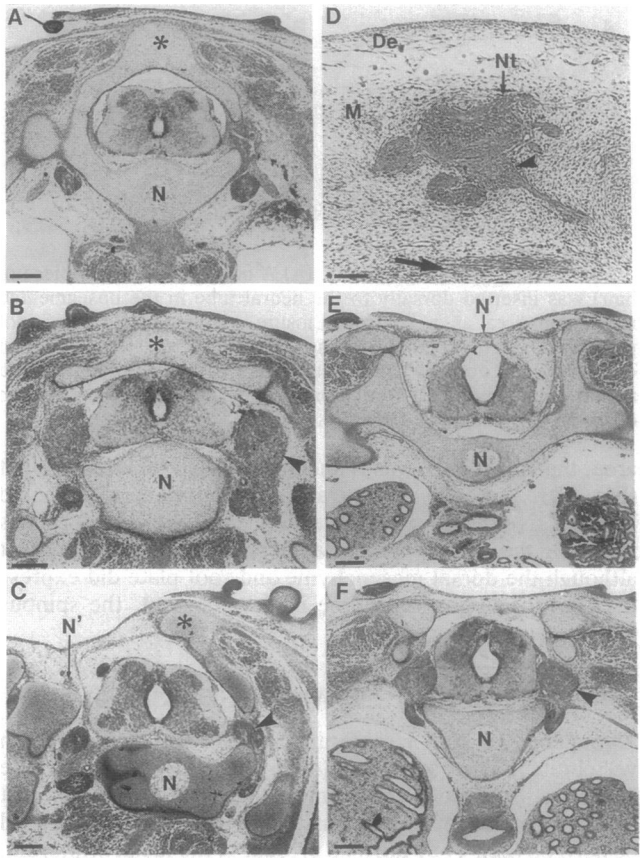

FIG. 4. Effect of notochord grafts on vertebral formation. Formation of vertebral cartilage was observed from E7 to E10. At the truncal level, the spinous process (asterisk) is well developed in controls (*A* and *B*). The vertebral body surrounds the notochord (N). Dorsal muscles, dermis, and feather buds are formed. In the case of a lateral graft of notochord (N′) (*C*), the spinous process, although present (asterisk), is smaller than it is in controls and is displaced to the side contralateral to the graft. On the grafted site, a large mass of cartilage has developed laterally beneath the graft (N′), but no muscle, dermis, or cartilage is seen dorsal to the graft. At E7, early ablation of the notochord (*D*) has resulted in the complete absence of cartilage around the neural tube (Nt). As noticed at E4, the DRG (arrowhead) and the myotomes (arrow) are fused ventrally, and loose mesenchymal tissue (M) surrounds the neural tube. Dermis (De) has developed under the ectoderm. In the case of mediodorsal grafts (*E* and *F*), the notochord totally has inhibited the formation of the spinous process, along the length of several vertebrae. In contrast, the vertebral body and the lateral arches are formed (*E* and *F*). In some sections, the lateral arches curve laterally (*E*). When the notochord graft (N′) is positioned above the dorsal aspect of the neural tube (*E*), mesenchymal cells surround the graft, but neither cartilage nor dermis is seen. At the level immediately caudal to the graft (*F*), mesenchymal cells have migrated to the neural tube but do not form a spinous process. (Bar in *A–C*, *E*, and *F* = 200 μm and in *D* = 100 μm.)

(Fig. 4*E*), although the spinous processes are totally missing. Mesenchymal cells located above the roof plate, close to the rostral and the caudal ends of the graft, have not differentiated into cartilage (Fig. 4*F*). This implies that the dorsally grafted notochord exerts a negative effect on dorsal chondrogenesis, which extends over a certain distance along the AP axis. Neutral grafts such as a piece of hair (Fig. 5*A*) or a fixed notochord inserted above the roof plate did not perturb the normal development of the vertebra.

The effect of notochordectomy was further examined at E5 (n = 3) and E7 (n = 4). Serial histological sections were alternatively treated with anti-BEN monoclonal antibody and the Feulgen–Rossenbeck's technique. In the most affected

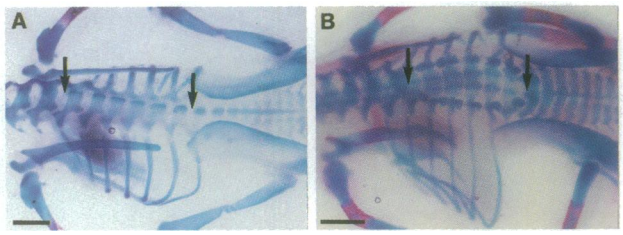

FIG. 5. Skeletal preparations. (*A*) A neutral obstacle (a piece of hair) was inserted dorsally to the neural tube in the unsegmented mesoderm area as a control. Closure of the vertebra was not prevented, and the spinous processes formed normally. (*B*) Insertion of a notochord dorsally to the neural tube in the unsegmented area inhibits spinous process formation, hence several vertebrae remain open dorsally (arrows). (Bar = 0.1 cm.)

area, the spinal cord was reduced to a small tubular remnant (Fig. 4*D*). At this level, no trace of vertebral cartilage could be seen. Neither the vertebral body nor the neural arches including the spinal process developed. It is noteworthy that although the dorsal mesenchyme and roof plate did express *Msx2* at the appropriate time in development, the spinous process did not develop.

CONCLUSIONS AND DISCUSSION

Two genes of the *Msh* family have been discovered in vertebrates, *Msx1* and *Msx2* (4, 16–21). Transcripts of these genes are localized in the premigratory cephalic neural crest from which most of the skull including the facial skeleton will be derived (22). They are also present in the facial primordia, developing teeth, apical ectodermal ridge, and underlying mesenchyme of the limb bud (4, 16, 17, 23). Tissue interactions between ectoderm and mesenchyme have been shown to be critical for the development of some of these structures. Recombination experiments have revealed that, in most of these sites, *Msx* gene expression and further skeletal development are dependent upon these interactions (19, 24, 25).

In our previous work, the following two facts were evident: first, *Msx2* expression can be induced in the nonexpressing somitic mesenchyme and ectoderm by the roof plate, provided that the two latter tissues develop in close proximity. This results in the differentiation of extrapieces of superficial cartilage. Second, rotation of the neural tube 180° abolishes *Msx2* expression, both in the roof plate and in the mediodorsal mesenchyme and ectoderm, resulting in failure of the spinous processes to develop. It thus appeared that when the dorsoventral polarization of the neural tube was altered (e.g., by the rotation of the neural tube), the differentiation of cartilage within the dorsal mesenchyme was severely compromised as was the morphogenesis of the vertebral neural arch. Therefore, we decided to try to answer the following two questions. When the neural tube is rotated 180°, what causes extinction of *Msx2* expression in the roof plate, dorsal ectoderm, and mesenchyme? Particularly, is the interaction with the notochord responsible for extinction of *Msx2* expression by the roof plate when this structure assumes a ventral position? By grafting a fragment of notochord mediodorsally above the neural tube at E2, we found that when mediodorsal notochord grafts were carried out in the region of the still unsegmented paraxial mesoderm, the complete inhibition of *Msx2* expression in the roof plate, the dorsal mesenchyme, and the ectoderm resulted; moreover, cartilage differentiation in the dorsal part of the neural arch failed to occur. This inhibitory effect can occur only within a well-defined temporal window. It was less pronounced when the graft was done more rostrally, at the level of the last somites formed where the neural tube was more mature. It should be noted that preliminary experiments on *Msx1* expression in these circumstances yielded similar results (not shown).

One can assume, therefore, that in the experiments where the neural tube is rotated 180°, the absence of *Msx* gene expression and subsequent cartilage differentiation from the dorsal mesenchyme is due to two additional causes: the absence of the roof plate, which in conjunction with the superficial ectoderm is necessary to maintain *Msx2* expression in the dorsal mesenchyme, and/or the presence of the floor plate, which in other experiments has been shown to mimic the effect of the notochord [e.g., on polarization of the neural tube (10) and of the somite (14)]. The extinction of *Msx2* expression in the roof plate, now in contact with the notochord, is evidently triggered by the notochord itself.

The fact that the mediodorsal graft of notochord inhibits cartilage differentiation in the dorsal mesenchyme is very striking when we consider that placement of the notochord a few micrometers more laterally induces the somite to differentiate virtually exclusively into cartilage (14). This demonstrates the extreme subtlety of local regulatory cues responsible for the patterning of axial structures in the vertebrate embryo. Moreover, this result shows that the dorsal part of the vertebral arch obeys different developmental controls from the rest of the vertebra. In our experiments, the corpus and the lateral parts of the neural arches differentiate normally, provided that the notochord is located in a ventral position with respect to the neural tube, and this differentiation appears not to involve *Msx* genes. In contrast differentiation of the dorsally located mesenchyme into the spinous process depends for its development on an induction arising from complex cellular interactions between the dorsal part of the neural tube and the superficial ectoderm, this induction is positively correlated with the expression of *Msx* genes.

The positive correlation between *Msx2* expression and skeletal morphogenesis was demonstrated in other systems. Thus, formation of membrane bones in the mandibular arch (26) and *Msx2* expression (24) occur only if interactions between the neural crest-derived branchial arch mesenchyme and the superficial ectoderm can take place. *Msx* genes are also involved in teeth development (23, 25) and are strongly expressed in the cranial mesenchyme (4, 16, 17, 27) from which the skull develops. Moreover, the genetic autosomal dominant craniosynostosis (Boston type) was found to be correlated with a mutation in the homeodomain of the human *Msx2* gene. In this disease, the sutures ensuring the extension of the skull bones are prematurely closed, thus generating an abnormally shaped skull. Affected individuals generally present additional defects including malformations of ear and limb (28), other structures where *Msx2* is expressed during development. The null mutation of *Msx1*, which has recently been produced in the mouse, has generated cleft secondary palate, deficiencies in alveolar mandible and maxilla, failure of tooth development, and abnormalities in cranial bones (29). Therefore, it can be considered that *Msx1* and *Msx2* control the development of superficial skeletal structures arising from the neural crest. The fact that abnormalities in vertebral development have not been recorded in the human mutant may be accounted for by the incomplete penetrance of the mutation detected in craniosynostosis (Boston type). In the case of the *Msx1* murine knock out, possible vertebral abnormalities may not have been looked for by the authors. The involvement of *Msx* genes in the development of the vertebra is of particular interest because it develops from paraxial mesoderm and not from mesectoderm. The common feature between the development of the neural crest-derived bones and the spinous process is that they all have a superficial localization and involve the activation of *Msx* genes. It is worth noting in this respect that *Msx* genes are also involved in the development of the limb: cells proliferating in the progress zone at the apex of the limb bud express *Msx*

genes. In the "limbless" mutant, the apical ectodermal ridge is not able to induce proliferation and survival of the mesenchymal cells, and *Msx* genes expression thus becomes extinguished (19).

Removal of the notochord at the level of the unsegmented mesoderm prevents the formation of the whole vertebra including the spinous process. This result demonstrates that tissue interactions triggering vertebral morphogenesis appear to proceed in two steps. First, the notochord is necessary for the ventralization of the somitic mesenchyme and for its differentiation along the sclerotomal pathway (14). In the absence of notochord, the somitic mesenchyme expresses only dermomyotomal potentialities. Hence, any differentiation into cartilage is prevented under these conditions, and the dorsal mesenchyme, although expressing *Msx2*, is not competent to differentiate into cartilage. This is in agreement with the fact that the cephalic paraxial mesoderm does not differentiate into cartilage rostral to the anterior limit of the notochord; the more anterior regions of the paraxial mesoderm yield muscles but no bones (30). The second step requires distinct influences according to the localization of the precartilage cells within the embryo. If they are in a superficial position (i.e., in contact with ectoderm), they require signals arising from the roof plate and superficial ectoderm and their further evolution involves the expression of *Msx2*. If, in contrast, they are not in close proximity to the superficial ectoderm, they can complete their terminal differentiation into cartilage under the continuous influence of the notochord.

One can conclude from these studies that development of the vertebra is highly dependent on influences arising from the axial structures, notochord, and neural tube. Particularly, the dorsoventral patterning of the somitic mesenchyme and later on of the sclerotome itself depends on genes whose expression is at least in part regulated by tissue interactions occurring sequentially with the notochord, the neural tube, and the superficial ectoderm. The cells migrating from the sclerotome to surround the neural tube and the notochord are thus subjected to different microenvironments. Those migrating dorsally to a superficial position appear to need signals involving the expression of *Msx2* gene to complete their developmental program, as do the cells of the neural crest-derived dermal skeleton and limb skeletal primordia, which develop under the control of epithelio–mesenchymal interactions.

We thank C. Ordahl, T. Rothman, and O. Pourquié for critical reading of the manuscript; Y. Rantier, F. Viala, T. Guérot, and S. Gournet for the illustrations; and M. Scaglia and E. Bourson for typing the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Ligue Nationale contre le Cancer, and the Association Française Contre les Myopathies.

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