

Dorsoventral axis determination in the somite: a re-examination

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SUMMARY

We have repeated classic dorsoventral somite rotation experiments (Aoyama and Asamoto, 1988, *Development* 104, 15-28) and included dorsal and ventral gene expression markers for the somitogenic tissue types, myotome and sclerotome, respectively. While the histological results are consistent with those previously published, gene expression analysis indicates that cells previously thought to be 'sclerotome' no longer express Pax1 mRNA, a sclerotome marker. These results, together with recent quail-chick transplantation experiments indicating that even very late sclerotome tissue fragments are multipotential (Dockter and Ordahl, 1998, *Development* 125, 2113-2124), lead to the conclusion that sclerotome tissue remains phenotypically and morphogenetically plastic during early embryonic somitogenesis. Myotome precursor cells, by contrast, appear to be determined

within hours after somite epithelialization; a finding consistent with recent reports (Williams and Ordahl, 1997, *Development* 124, 4983-4997). Therefore, while these findings support a central conclusion of Aoyama and Asamoto, that axis determination begins to occur within hours after somite epithelialization, the identity of the responding tissues, myotome versus sclerotome, differs. A simple model proposed to reconcile these observations supports the general hypothesis that determinative aspects of early paraxial mesoderm growth and morphogenesis occur in early and late phases that are governed principally by the myotome and sclerotome, respectively.

Key words: Experimental embryology, Chick-quail chimera, Dorsoventral polarity, Somite, Sclerotome, Myotome, Dermomyotome, Paraxial mesoderm, Epaxial domain

INTRODUCTION

The pre-cartilage models of the vertebrae, ribs and a portion of the scapula are derived from the embryonic sclerotome in vertebrate embryos. The sclerotome arises from cells within the ventral region of the newly formed, epithelial somite that undergo an epithelial-mesenchymal transition and begin to migrate around the notochord and neural tube (Fig. 1A). Although classic experimental embryology had shown that rotation could alter somite development (see for example, Gallera, 1966), Aoyama and Asamoto raised this type of experimentation to new heights by systematically rotating all three axes of somites at specific, well-defined stages in their development (Aoyama and Asamoto, 1988). Their results regarding somite dorsoventral axis determination led them to conclude that the somite ventral tissue, the sclerotome, is determined rapidly after somite formation. Thus within 24 hours after rotation, a stage III somite forms an ectopic mesenchyme, dorsal to the dermomyotome and myotome, that was scored as 'sclerotome' (Aoyama and Asamoto, 1988). A similar dorsal mesenchyme that appeared 24 hours post-surgery in sclerotome challenge experiments (Dockter and Ordahl, 1998) was not expressing *Pax-1*, an established sclerotome marker (Deutsch et al., 1988). Furthermore, longitudinal analysis indicated that this mesenchyme was destined to form either muscle or dermis, but not cartilage, leading to the overall conclusion that determination of

sclerotome is a late event in somitogenesis, occurring about embryonic day (ED)4 (Dockter and Ordahl, 1998).

Such disparities in interpretation are not uncommon in experimental embryology and are often attributable to differences in experimental approach. For example, Aoyama and Asamoto rotated whole, intact somites while, in the challenge experiments noted above, only sclerotome fragments were analyzed. It is not difficult to imagine differences in behavior of whole somites and somite fragments. In order to minimize such differences we repeated the somite rotation experiments of Aoyama and Asamoto and analyzed the results by both histology and in situ hybridization, using somite marker probes that have become recently available. The results support the conclusion that sclerotome determination occurs late during development, but also suggest that determination of other tissues, such as muscle, begins early in somite development, consistent with previous reports from this laboratory (Williams and Ordahl, 1997). A simple model is proposed to reconcile these observations and the implications of that model for understanding axis determination during early paraxial mesoderm growth and morphogenesis are discussed.

MATERIALS AND METHODS

Embryos

White leghorn chicken (*Gallus gallus domesticus*) (Petaluma Farms,

Petaluma, CA, USA) and Japanese quail (*Coturnix coturnix japonica*) (Strickland Quail Farm, Pooler, GA, USA) eggs were incubated at 37.6°C in a forced-draft incubator. Embryos were staged according to Hamburger and Hamilton (HH) (1951). Somites were developmentally staged (Ordahl, 1993) whereby the most newly formed caudal somite is designated stage I and older, more cranial somites are progressively designated by increasing Roman numeral values. The relative ages of donor and host embryos were closely matched, usually within the same or next HH stage. For uniformity and ease of surgery, all experiments were restricted to somites of the upper thoracic and brachial levels.

Embryo surgery

ED2 quail donor embryos were cut from the yolk in a bowl containing Tyrode's salts (Sigma) with iridectomy scissors (Fine Science Tools) and pinned dorsal side up in a glass dish coated with black Sylgard (Fig. 2A). Under a dissecting microscope, incisions in the ectoderm were made with tungsten microscalpels lateral to the stage I-III somites on the left side of the embryo, followed by subsequent ectoderm incisions cranial to the stage III somite and caudal to the stage I somite (Fig. 2A,B). After 2-3 minutes treatment with pancreatin (4× Gibco-BRL), the ectoderm was removed with tungsten microscalpels and the stage I-III somites were teased away from the neural tube and notochord with the pancreatin still in place to allow digestion of the extracellular matrix contacts between the somites and these tissues. The endoderm was then incised longitudinally, between the somites and the axial tissues, followed by transverse incisions between the stage I somite and the segmental plate and between the stage III and stage IV somites. Finally, incisions were made along the lateral edges of the intermediate mesoderm immediately adjacent to the stage I-III somites allowing the 3-somite-containing fragment to be removed from the donor embryo. Animal carbon was placed on the dorsal face of the fragment for orientation purposes (arrow, Fig. 2B). The graft tissue fragment, consisting of the stage I-III somites, the underlying endoderm and adjacent intermediate mesoderm, was then placed in a drop of Tyrode's salts containing 2% fetal calf serum and held at room temperature until grafting (15 minutes-1 hour).

ED2 chick host eggshells were opened by cutting out a small circle of eggshell. Ink (Pelikan black no. 17) was mouth pipetted under the blastoderm for visualization of the embryo (Fig. 2C) using a finely drawn micropipet. After opening the vitelline membrane, incisions were made in the ectoderm on the right side of the embryo, immediately laterally, cranially and caudally to the stage I-III somites with a tungsten microscalpel. After 2-3 minutes treatment with pancreatin solution, the ectoderm was teased back or removed with microscalpels. Additional pancreatin solution was gently pipetted onto the exposed somite region of the embryo and the stage I-III somites teased away from the neural tube and notochord and removed (Fig. 2C,D). The excised area was then liberally rinsed with 10% fetal calf serum in Tyrode's salts. The quail somite fragment was then pipetted onto the embryo and implanted into the space left by the removal of the host somites. The graft was oriented such that only the dorsoventral axis of the somites was inverted (Figs 1, 2E,F). Eggs were sealed and re-incubated until harvested for histological analysis.

Histology

Chimeric embryos were harvested at 24 hours post-surgery, preserved in Carnoy's fix and embedded in paraffin. 7 µm sections were cut on a rotary microtome and stained with the Feulgen reaction to visualize quail nucleoli (Le Douarin, 1973). Chimeric embryos were harvested at 24 hours post-grafting and alternate adjacent sections stained by the Feulgen reaction to visualize the grafted quail cells or processed for in situ hybridization as previously described (Dockter and Ordahl, 1998). Feulgen-stained sections were observed using light microscopy. In situ hybridization patterns were analyzed using dark-field microscopy. All microscopy was performed on a Zeiss Axiophot microscope. Images were acquired using a DEI 470 Optronics CCD

camera system and a RasterOps frame capture board and were imported by a plug-in module directly into Adobe Photoshop 3.5.

RESULTS

The somite rotation experiment involves transplanting a block of three somites (stages I, II and III) from the left side of a quail embryo into the contralateral side of a chick embryo in which the right-hand somites I, II, III had been removed (Aoyama and Asamoto, 1988). In this manner the dorsoventral axis of the somite could be changed without disrupting the mediolateral or craniocaudal axes. Of the 31 chimeric embryos that survived surgery, only six (approximately 20%) produced results comparable to those previously reported. In those specimens, the somites that were stage I and stage II at the time of rotation and transplantation matured relatively normally with regard to the dorsoventral arrangement of the tissues (i.e. an epithelial dermomyotome as the most dorsal structure, then myotome, and finally a ventral mesenchymal sclerotome; see

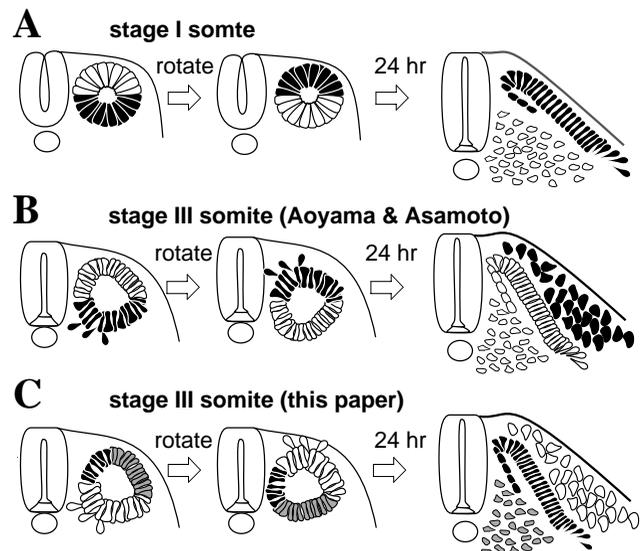


Fig. 1. Diagram of the somite rotation experiment and alternative interpretations of the results. (A) After inversion of a stage I somite, the presumptive sclerotome (black) and dermomyotome (white) become re-specified to form dermomyotome/myotome and sclerotome, respectively. (B) Aoyama and Asamoto (1988) interpretation of the consequences of inversion of a stage III somite; the presumptive sclerotome (black) forms a dorsolateral mesenchyme superimposed over a relatively normal-appearing dermomyotome, myotome and sclerotome that arose from the presumptive dermomyotome (white). (C) Alternative interpretation of somite rotation experiment, based upon the results presented here. By stage III of somite development only the early myotome precursor cells are determined (black) while the dorsal and ventral somite epithelium (white and grey, respectively) are undetermined. After rotation, the determined myotome precursor stem cells grow in the dorso-medial direction, generating myotome cells and new dermomyotome cells in a manner described elsewhere (Denetclaw et al., 1997), while the dorsal epithelial cells become re-specified as sclerotome (Pourquie et al., 1993). The ventral epithelium (presumptive sclerotome) when placed dorsally, on the other hand, remains as an undifferentiated mesenchyme that retains potency for multiple morphogenetic and phenotypic endpoints (Dockter and Ordahl, 1998).

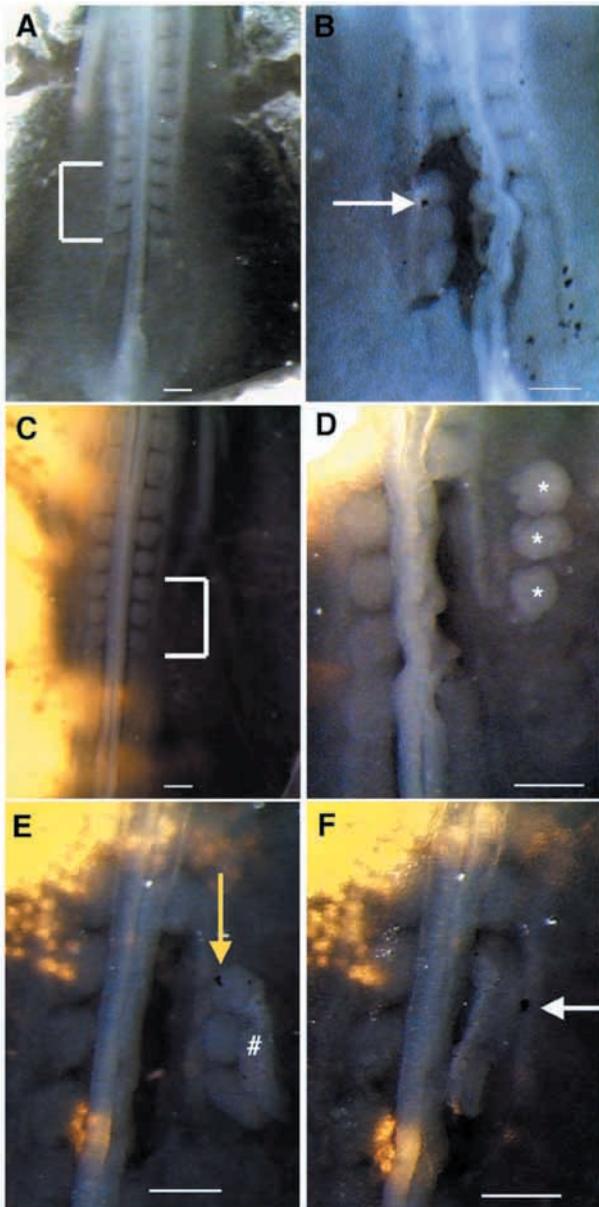


Fig. 2. Surgical implantation and rotation of quail somites in chick embryos. (A,B) Preparation of quail donor somites. (A) Brackets show the targeted stage I, II and III somites on the left-hand side of a quail embryo. (B) After surgical incisions the quail somites are liberated from attachment to the neural tube and the adjacent intermediate mesoderm. A carbon particle is implanted on the dorsocranial surface for orientation purposes (white arrow). (C,D) Preparation of chick host embryo. (C) Stage I, II and III somites on the right hand side of chick embryo are bracketed. Black ink has been injected under the blastoderm to provide contrast. (D) After surgical incisions, the host stage I, II and III somites (asterisks) are removed leaving the intermediate mesoderm in place. (E,F) Implantation and rotation of quail donor somites into chick host embryo. (E) Chick host embryo from D with quail somite fragment from B. Note that the position of a second carbon particle (yellow arrow) indicates that the fragment is lying dorsal side up (in same orientation as B). This particle is out of view in B due to medial rotation of the somite fragment. The '#' symbol marks a fragment of donor endoderm that has curled up from the ventral surface of the donor fragment. (F) Insertion of fragment into host somite field. Note the position of the carbon particle (white arrow) indicates that the fragment is now lying dorsal side up, with intermediate mesoderm lying in a lateral position adjacent and parallel to the host intermediate mesoderm. This is the same carbon particle seen in B. Bars, 200 μ m.

adjacent sections examined by Feulgen stain for the presence of quail cells and by in situ hybridization for the expression of the following markers: *Pax-3*, which marks the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994), *MyoD*, which marks the myotome (Pownall and Emerson, 1992), and *Pax-1*, which marks the ventral sclerotome (Deutsch et al., 1988; Borycki et al., 1997). In all three cases, the morphologically identifiable dermomyotome and myotome tissues formed by the inverted stage I-III somites were found to express the expected markers for these two tissues (Fig. 5B-D and data not shown). *Pax-1* mRNA expression by grafted somites, however, was limited to sclerotome that was ventral to the myotome and this mRNA was not detected in the dorsolateral mesenchyme derived from stage III somites (Fig. 5D).

DISCUSSION

In the experiments reported here we have repeated a classic somite rotation experiment of Aoyama and Asamoto and analyzed the resulting tissue by morphogenesis and in situ hybridization. Six chimeric embryos gave somite development patterns corresponding to the predicted pattern: (1) somites that were at stage I or II at the time of inversion developed normally, while (2) somites that were stage III at the time of inversion developed essentially normally with respect to the dorsal-to-ventral placement of dermomyotome, myotome and ventral mesenchyme (sclerotome), except for the presence of an ectopic mesenchyme dorsal to the dermomyotome epithelium (see Fig. 1C). In situ hybridization showed that the dermomyotome, myotome and (presumptive) sclerotome, respectively, express the marker genes, *Pax-3*, *MyoD* and *Pax-1*, consistent with their morphological identity. The dorsal mesenchyme, by contrast, expresses none of these markers. The ventralmost cells of a stage III somite express *Pax-1*, so it

Fig. 1A) and the morphology of the tissues themselves (Fig. 3A-D). The somite that was stage III at the time of transplant, on the other hand, developed dermomyotome, myotome and sclerotome that appeared normal with regard to morphology and relative position in the embryo, but formed in addition a large mesenchyme dorsolateral to the dermomyotome (Fig. 3E,F). This ectopic, dorsal mesenchyme corresponded to that observed and designated 'sclerotome' by Aoyama and Asamoto (1988).

That the other 25 embryos (80%) did not reproduce the original result was probably due to altered orientation of the graft, either at the time of grafting or during subsequent incubation. The somitic pattern observed in these embryo was often disrupted and graft-derived intermediate mesoderm (a lateral surgical marker) was frequently found in an incorrect position in the embryo (Fig. 4A,B). These non-corresponding chimeras were therefore not analyzed further.

Three of the six positive embryos were sectioned serially and

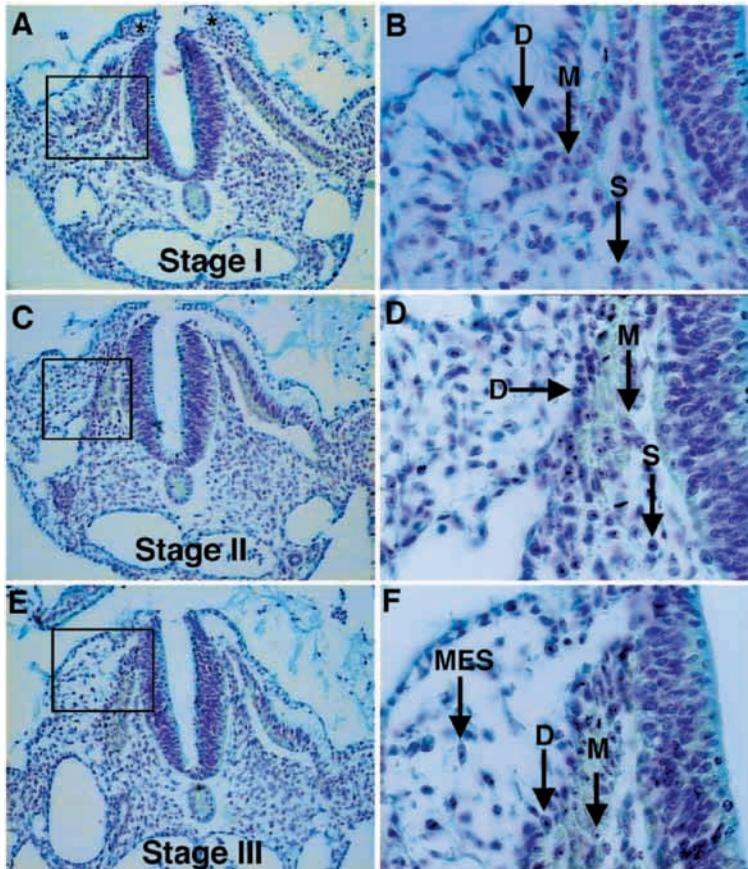


Fig. 3. Somite rotation experiments; histological results. Sections of a chimeric embryo harvested 24 hours after the right stage I-III somites of the chick host were replaced by the left stage I-III somites of a quail embryo; the grafted somites were inverted on the dorsal-ventral axis. The operated side is to the left in all panels. (A,C,E) 20 \times objective views of the somites which were at stage I (A), stage II (C) and stage III (E) at the time of inversion. The dorsal cells (*) in A are of chick origin on both operated and unoperated sides. Note the relatively normal dermomyotome, myotome and sclerotome formed by the graft in A and C and the presence of a mass of dorso-lateral mesenchyme in E. (B,D,F) 63 \times oil objective views of the areas boxed in A, C and E, respectively. The black arrows point to quail cells present in dermomyotome (D), myotome (M), sclerotome (S) and dorso-lateral mesenchyme (MES).

can be concluded that the dorsal mesenchyme (which we assume arose from previously ventral cells) downregulated *Pax-1* gene transcription in the 24 hours between inversion and harvest and, therefore, has lost sclerotome identity. By contrast, previously dorsal cells gain sclerotome identity and begin expressing *Pax-1* (Fig. 5E). This conclusion is consistent with previous experiments that indicated that sclerotome determination occurs relatively late in development (Dockter and Ordahl, 1998) because sclerotome fragments from somites much older than stage III consistently gave rise to muscle and dermis after being translocated to a dorsal position. Interestingly, such implants also went through a mesenchyme phase, which, at 24 hours post-transplant, expressed none of the somite markers used in this study. The present findings strengthen the conclusion that sclerotome tissue in the early embryo is plastic with respect to gene expression, ultimate final cell type and morphogenesis until relatively late in development,

¹It is predicted that the dorsolateral mesenchyme derived from a stage III somite in a dorsoventral axis inversion experiment would exhibit multipotentiality and integration into host anatomical structures when examined at a later time point. To test this hypothesis, chimeric embryos with inverted stage I-III somites would be harvested at a later time point to allow the examination of the ultimate differentiated cell types formed. Unfortunately, the low success rate (6/31 chimeric embryos) in obtaining correct dorsoventral inversion of the grafted stage I-III somites renders meaningful interpretation of such experiments difficult, due to the inability to determine whether the results came from correctly or incorrectly oriented somite grafts.

a period corresponding to the onset of chondrogenic differentiation (Dockter and Ordahl, 1998)¹.

We therefore propose an alternative interpretation of this result (Fig. 1C), which involves the early and rapid determination of myogenic progenitor cells in one region of the somite and the plasticity of cells in all other regions of the somite (Fig. 1C). Once induced to grow, myogenic progenitor cells in stage IV somites form dozens to hundreds of myogenic daughter cells (Williams and Ordahl, 1997) that in a native environment form the epaxial myotome, which grows in a lateral-to-medial direction (Denetclaw et al., 1997). We suggest that in the inverted stage III somite, such determined myogenic progenitor cells initiate myotome formation while the remaining cells of the somite come under the influence of their new environment.

Previously dorsal cells that are not yet determined come under the influence of the notochord and floor plate when placed in a ventral position (Pourquie et al., 1993) and

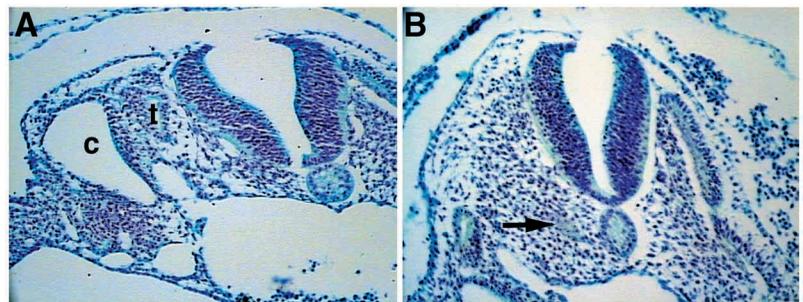
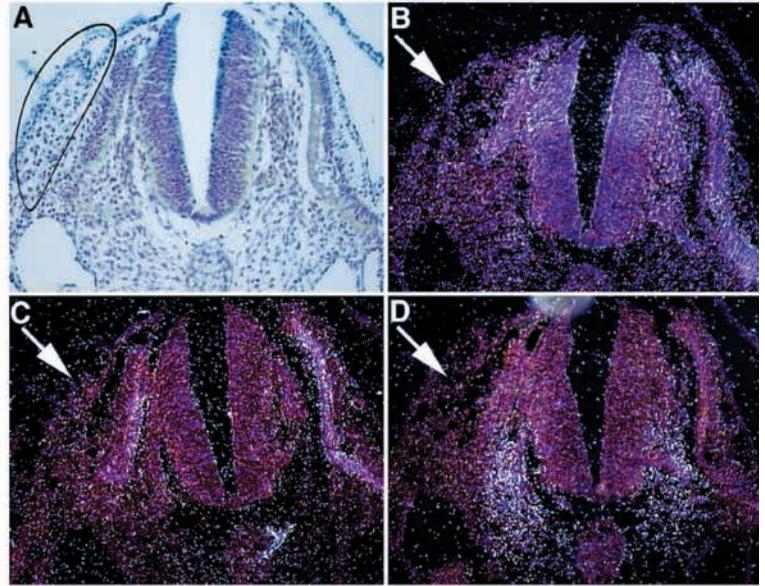


Fig. 4. Representative examples of unsuccessful somite rotation grafts. Feulgen-stained sections of two chimeric embryos harvested at 24 hours post-replacement of chick right side somites stage I-III with quail left side somites stage I-III with inversion of the dorsal-ventral axis are shown. The operated side is to the left; 20 \times objective views. (A) Section through an area of quail origin; determination of the original stage of the somite from which these cells derived was not possible. Notice the large cyst (c) that has formed. The presence of tubular structures (t) next to the neural tube, presumably derived from the grafted intermediate mesoderm (Ordahl and Le Douarin, 1992), indicate that this result is probably due to incorrect reorientation of the donor somites after grafting. (B) Section through an area of quail cell origin; determination of the original stage of the grafted somite from which these cells derived was not possible. The cells have formed a large field of mesenchyme, which fills the operated side; no somitic structures can be distinguished. The arrow marks a ventral patch of quail cells that resembles a clump of muscle tissue.

Fig. 5. Gene expression in inverted somites. Adjacent sections of a chimeric embryo harvested 24 hours after the right stage I-III somites of the chick host were replaced by the left stage I-III somites of a quail embryo; the grafted somites were inverted on the dorsal-ventral axis. These sections contain the somite which was at stage III at the time of grafting and the operated side is to the left in all panels. (A) Feulgen-stained section. Note the relatively normal-appearing, quail-derived dermomyotome, myotome and sclerotome superimposed by a large field of mesenchyme dorsolateral to the dermomyotome (outlined). (B) In situ hybridization of *Pax-3* probe. *Pax-3* mRNA is detected in the dermomyotome epithelium and the dorsal neural tube on both the operated and unoperated sides but is not detected in the dorsolateral mesenchyme on the operated side (white arrow). (C) In situ hybridization of *MyoD* probe. *MyoD* mRNA is detected in the myotome, which is located immediately subjacent to the dermomyotome, on both the operated and unoperated sides. It is not being expressed in the dorsolateral mesenchyme (white arrow). 20× objective view. (D) Expression of *Pax-1*. *Pax-1* is detected in the ventral sclerotome, which lies subjacent to the myotome, on both the operated and unoperated sides. *Pax-1* is not detected in the dorsolateral mesenchyme (white arrow). All photographs were taken with a 20× objective.



express *Pax-1* (Fan and Tessier-Lavigne, 1994). Previously ventral (presumptive sclerotome) cells placed dorsally form a quiescent mesenchyme that consists of undetermined cells. As later developmental events take place these dorsal mesenchyme cells are swept into several differentiation pathways and contribute to different tissues. Thus, the growing myotome divides the inverted somite and supports epaxial growth that is remarkably comparable to that on the contralateral (unoperated) side of the embryo (compare operated and unoperated sides, Fig. 3).

A most interesting aspect of the ectopic sclerotome is its morphogenetic malleability. Dorsalized sclerotome, when it finally enters into the formation of organized tissue around ED4, does so in concert with host tissues, and transplanted and contralateral sides are always perfectly symmetrical and proportioned (Dockter and Ordahl, 1998). Even when ectopic sclerotome cells were found in cartilage, they conformed to the endogenous cartilage model, regardless of whether the structure was dorsal spine or ventral lamina. This property of morphogenetic malleability abruptly ends coincident with the onset of determination, as indicated by the formation of ectopic, morphologically inappropriate cartilage nodules. Sclerotome, therefore, appears to be plastic both in terms of cell type fate as well as morphogenetically, classically defined as material and dynamic determination (Spemann, 1938). Myotome growth seen in the somite rotation experiments indicates that both aspects are also determined essentially simultaneously for myotome progenitor stem cells because the myotome/dermomyotome structures are consistent with the dorso-medial growth vector of the early epaxial myotome (Denetclaw et al., 1997). These observations support the hypothesis that dermomyotome-myotome development plays an active role in the overall growth and morphogenesis of the epaxial domain during early embryonic development and that the sclerotome plays a relatively passive role during these early stages. During subsequent development, however, and concomitant with the onset of chondrogenic differentiation,

sclerotome morphogenesis and cell type determination becomes fixed and resistant to change. If this hypothesis is correct, early defects in epaxial morphogenesis, including vertebral morphogenesis, may result from alterations in the early growth and morphogenesis of the myotome and dermomyotome.

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Dedication: The authors dedicate this paper to the memory of James (Jay) Lash, somite pioneer and friend and mentor.

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