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Control of dorsoventral pattern in the chick paraxial mesoderm

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SUMMARY

The most profound feature of the mature vertebrate somite is its organisation into dorsal dermomyotome, intermediate myotome and ventral sclerotome. We analysed the role of potential signalling structures in this dorsoventral pattern by ablating them or transplanting them to ectopic locations in chick embryos. Our data suggest that the somite represents a naïve tissue, entirely depending on external cues for its dorsoventral organisation. Dorsalisation by signals from dorsal neural tube and surface ectoderm stimulates the development of the dermomyotome. Likewise, signals from notochord and floor plate ventralise the somite, at high levels overriding any dorsal information and inducing the sclerotome. The dorsalising factors and lower levels of the ventralising factors act in concert to induce the myotome. Finally, the paraxial mesoderm intrinsically controls its competence to respond to the external inducers.

Key words: somite, dorsalisation, ventralisation, dermomyotome, myotome, sclerotome, chick, *MyoD*, *Pax1*, *Pax3*

INTRODUCTION

During vertebrate gastrulation, the paraxial mesoderm is laid down as two strips of mesenchyme laterally flanking the neural tube and the axial mesoderm, the notochord. While the primitive streak continuously adds new material to the posterior ends of these segmental plates, the cells at their anterior ends condense and epithelialise to form discrete somites, enclosing some mesenchymal remnants in the somitocoel (reviewed in Christ and Ordahl, 1995). After somitogenesis, the paraxial mesoderm differentiates dorsoventrally. Ventrally, the somite de-epithelialises and, together with the somitocoel cells, forms the mesenchymal sclerotome, which gives rise to the vertebral column and ribs. Medially, cells leave the edge of the dorsal, still epithelial dermomyotome and spread ventrally beneath it to form the myotome, the source of the epaxial musculature (Denetclaw et al. 1997). Cells at the lateral edge of the dermomyotome give rise to the hypaxial musculature, migrating laterally to form the limb muscles or spreading ventrally to form the body wall. Last, mesenchymal cells leave the dermomyotome dorsally to form the dorsal dermis.

While segmentation appears to be an intrinsic property of the paraxial mesoderm, its dorsoventral pattern is regulated by extrinsic cues. Induction and maintenance of the sclerotome requires either the notochord or the floor plate of the neural tube as shown by ablation and heterotopic grafting experiments in chick embryos (Brand-Saberi et al., 1993; Pourquié et al., 1993; Goulding et al., 1994), by mouse notochord mutants (Dietrich et al., 1993; Koseki et al., 1993) and by coculture experiments (Fan and Tessier-Lavigne, 1994).

The development of the myotome in vivo similarly depends on extrinsic signals (reviewed in Molkentin and Olson, 1996). However, the source of these signals is controversial: myotome formation in the avian embryo may depend on signals from neural tube and notochord (Teillet and Le Douarin, 1983; Rong et al., 1992; Pownall et al., 1996) or from the dorsal neural tube alone (Spence et al., 1996). The whole neural tube has been suggested to be essential (Xue and Xue, 1996), or dispensable for myotome formation (Spence et al., 1996), or to play only a role in its long-term maintenance (Bober et al., 1994). The notochord has been suggested either to have no effect (Teillet and Le Douarin, 1983; Rong et al., 1992), to induce the myotome (Bober et al., 1994; Pownall et al., 1996; Xue and Xue, 1996) or to repress it (Brand-Saberi et al., 1993; Pourquié et al., 1993; Goulding et al., 1994; Xue and Xue, 1996), and an inhibitory function has been ascribed to the lateral plate mesoderm (Pourquié et al., 1996).

In vitro studies on myotome formation are no more conclusive (reviewed in Molkentin and Olson, 1996): autonomous expression of myotomal markers has been reported for high density cultures of segmental plate or somite cells, and for cultured half-somites (George-Weinstein et al., 1994; Gamel et al., 1995). In other studies, the presence of both neural tube and notochord was essential to allow the expression of any myotome-specific gene from segmental plate explants (Münsterberg and Lassar, 1995; Stern et al., 1995). The ventral neural tube/notochord has been implicated as a source of inductive factors (Buffinger and Stockdale, 1995) as has the dorsal neural tube alone (Stern et al., 1995; Stern and Hauschka, 1995; Spence et al., 1996). A myotome-inducing capacity for the surface ectoderm has also been both suggested (Kenny-Mobbs and Thorogood, 1987; Cossu et al., 1996; Spence et al., 1996; Maroto et al., 1997) and denied (Buffinger and Stockdale, 1995; Stern and Hauschka, 1995), whereas the dorsal neural tube (Buffinger and Stockdale, 1995), the lateral plate mesoderm (Cossu et al., 1996) and the intermediate mesoderm (Gamel et al., 1995) have been suggested to delay or inhibit the myogenic response to inducers.

Finally, whether extrinsic influences are required for the formation of the dorsal dermomyotome has not been clarified. Some authors have suggested that the paraxial mesoderm is dorsally predisposed (Dietrich et al., 1993; Williams and Ordahl, 1994), while others found that dermomyotomal features develop in response to influences from surface ectoderm or dorsal neural tube (Fan and Tessier-Lavigne, 1994; Kuratani et al., 1994; Brill et al., 1995; Maroto et al., 1997).

The confusion with respect to tissue influences on formation of the myotome may be diagnostic of the complexity of the sytem. In vitro, the myogenic response of mesodermal cells may be stimulated by culture conditions promoting withdrawal from the cell cycle (reviewed in Molkentin and Olson, 1996) or by interruption of intercellular communication (George-Weinstein et al., 1994; Gamel et al., 1995), suggesting that the requirement of inducers may be bypassed by artificial culture conditions. On the contrary, in vivo manipulation of a single signalling structure may cause various responses as signals patterning the paraxial mesoderm may influence the environment, may posses multiple functions or may be redundant. Thus, we undertook a systematic study to identify the structures controlling dorsoventral pattern in the paraxial mesoderm.

We gradually ablated and heterotopically transplanted all the structures suggested to pattern the paraxial mesoderm. The simultaneous detection of dorsoventrally restricted markers for the paraxial mesoderm and the axial structures in triple- and quadruple-labelling in situ hybridisations allowed us to identify complex responses to the microsurgical manipulations. Our observations suggest that the newly formed paraxial mesoderm is a naïve tissue, as only the competence to respond to external signals is regulated intrinsically. Both the dorsal neural tube and the surface ectoderm are sources of dorsalising factors which are essential to induce the dermomyotome. Both the notochord and the floor plate of the neural tube are sources of ventralising factors which, at high concentrations, induce the sclerotome and repress the dermomyotome. Where the ventralising and dorsalising factors are present at appropriate relative levels, as occurs in the intermediate region of the somite, they act together to induce the myotome.

MATERIALS AND METHODS

Chick embryos

Fertilised hens' eggs were obtained from a mixed flock (Poyndon Farm, Enfield) and incubated in a humidified atmosphere at 39°C. The embryos were staged according to Hamburger and Hamilton (1951). For removal of the newly formed and nascent notochord, Hamburger-Hamilton stage 10 (HH10) embryos were used. For all other types of operation, HH10-13 embryos were used.

Somite nomenclature

For the chick somites, we used the nomenclature of Christ and Ordahl (1995). Somites present at the time of operation were assigned positive roman numerals, beginning with the most recently formed somite. Somites that emerged from the segmental plate postoperatively were assigned negative roman numerals in order of their appearance.

Microsurgery and control embryos

After windowing the eggs and ink injection into the yolk beneath the embryos, the vitelline membrane was opened. In the region to be operated, a slit was cut into the ectoderm between neural tube and paraxial mesoderm or lateral to the paraxial mesoderm using flame-sharpened 100 μm tungsten wire. A tiny amount of dispase (1 mg/ml, Boehringer) was applied with a mouth pipette to loosen the contact between the paraxial mesoderm and its environment. The dispase was washed away with Ringer and the operations performed as described in the results section. For grafting operations, donor embryos were transferred into Ringer solution, the ectoderm or endoderm opened and 50 μl dispase applied until the structures to be grafted came loose. The tissues were excised with tungsten needles, washed in Ringer, transferred into the host with a serum-coated pipette and manoeuvred into position with tungsten needles.

To test for induction of mesodermal markers, most operated embryos were reincubated for $6\frac{1}{2}$ or $9\frac{1}{2}$ hours, allowing the formation of 4 and 6 somites, respectively. To assay for marker maintenance, the embryos were harvested after 24 hours, so that 13-17 somites had been formed. Embryos in which the posteriormost notochord had been ablated were re-incubated for 24 hours to test for induction of the markers, and for 36 hours or 48 hours to analyse their maintenance. To check that the procedure did not harm the paraxial mesoderm, the neural tube/notochord complex was ablated between somites XV and –VII, and the embryos harvested immediately (n=7) or after reincubation for 1 hour (n=2). In addition, neural tube/notochord were excised and immediately thereafter re-inserted (Fig. 1A), and the embryos cultivated for 24 hours (Fig. 1B). All control embryos displayed the wild-type expression pattern of the dorsoventrally restricted markers *MyoD*, *Pax1* and *Pax3* (Fig. 1B-D).

Whole-mount in situ hybridisation

Entire chick embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight, and subsequently hybridised simultaneously with two digoxygenin (DIG, Boehringer)-labelled and one or two fluorescein isothiocyanate (FITC, Boehringer)-labelled antisense RNA probes according to Henrique et al. (1995). After hybridisation, the DIG-labelled molecules were detected using NBT/BCIP (Boehringer) as substrate for the anti-DIG-antibody-coupled alkaline phosphatase. After overnight treatment with 4% PFA and extensive washes with PBS, the alkaline phosphatase-coupled anti-FITC-antibody was applied in the same way, and the staining reaction performed with Fast Red (Sigma). The staining reaction was optimised for the detection of the mesodermal markers, leading to some variation in the signal intensity of the notochord or neural tube markers.

In situ probes and wild-type expression

The *Bmp4* probe described by Francis et al. (1994) labels the roof plate of the neural tube and the overlying ectoderm, the lateral mesoderm and the endoderm (Liem et al., 1995; Pourquié et al., 1996; Fig. 1D).

The *Wnt4* probe represents a PCR product encompassing the 3′ end of the coding region (L. Tumiotto, A. Graham, A. Lumsden, unpublished results). Expression is found in dorsal neural tube and the intermediate mesoderm, in line with Hollyday et al. (1995).

The *Pax3* probe has been described by Goulding et al. (1993). The mRNA is found in the dorsal neural tube, the anterior segmental plate and the dermomyotome, and at high levels in progenitors of hypaxial musculature at the lateral edge of the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994; Fig. 1B,C).

The *Pax6* probe of Goulding et al. (1993) detects messages in the intermediate neural tube, dorsally overlapping with *Pax3*.

The *Shh* probe and the expression pattern of the gene in notochord, floor plate, endoderm, branchial arches and limbs has been described by Johnson et al. (1994) (Fig. 1B-D).

MyoD (Fig. 1B,C) represents the earliest expressed myotomal marker in the avian somite. Expression starts in the medial halves of somites I or II along the ventral border of the *Pax3* expression domain

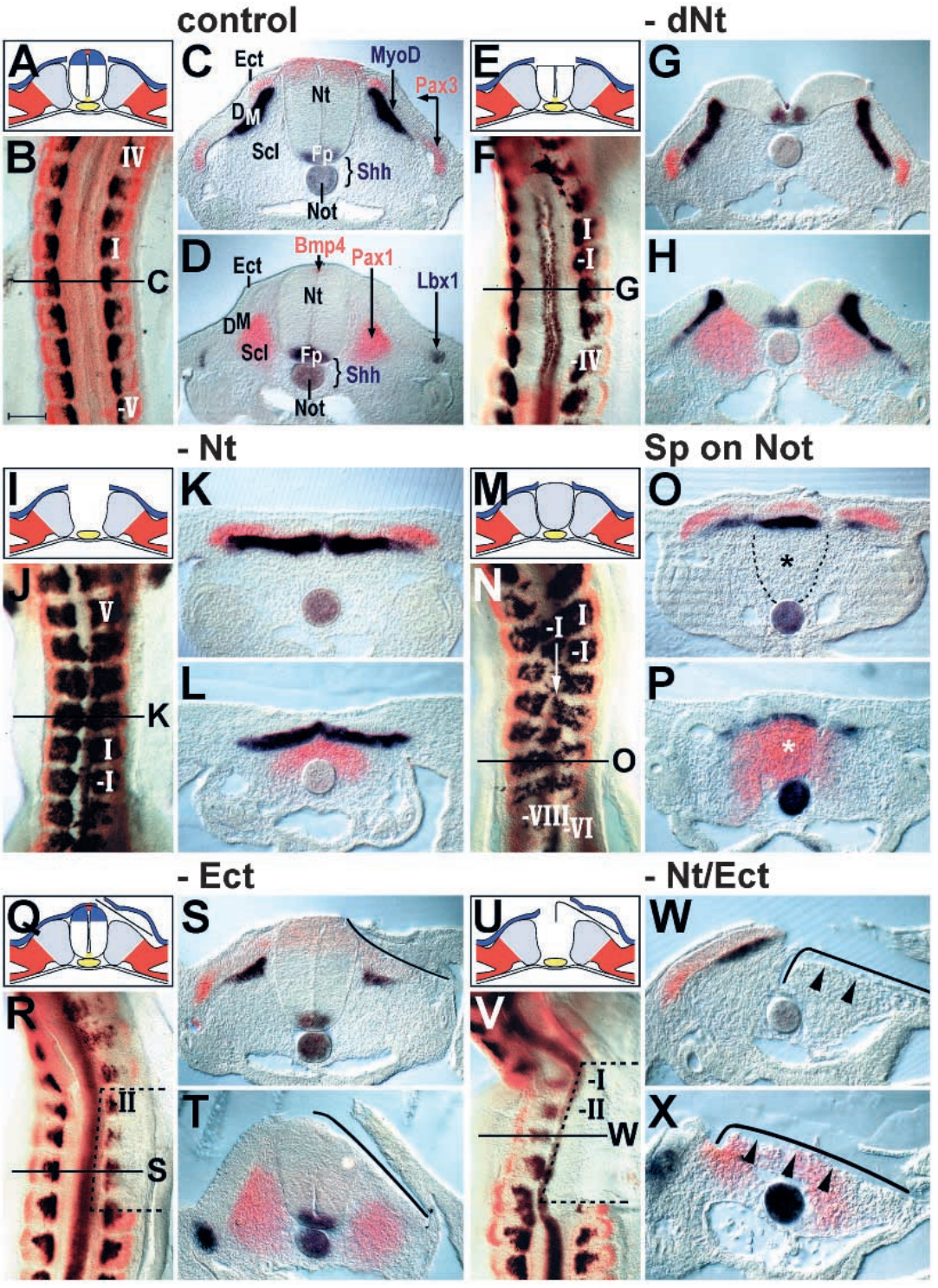

Fig. 1. Consecutive loss of dorsalising structures. (A,E,I,M,Q,U) Schemes of operations performed at the level of the anterior segmental plate and youngest somites. Surface ectoderm and dorsal neural tube are depicted in blue, notochord and floor plate in yellow, intermediate and lateral mesoderm and roof plate in red, and paraxial mesoderm in grey. (B,F,J,N,R,V) Dorsal views of the operated areas, 24 hours after surgery. The developmental age of the paraxial mesoderm at the time of operation is indicated with roman numerals. All whole-mounted embryos are labelled with *Shh* and *MyoD* probes in blue, and *Pax3* probe in red. (C,G,K,O,S,W) Cross sections corresponding to the levels indicated on the whole-mounted embryos, showing expression of *Shh/MyoD* in blue and *Pax3* in red. (D,H,L,P,T,X) Similar sections, stained for *Shh*/*Lbx1* expression in blue and *Pax1*/*Bmp4* expression in red (D), for *Shh*/*MyoD* expression in blue and *Pax1*/*Pax6* expression in red (H), for *Shh*/*MyoD* in blue and *Pax1* in red (L,P), and for *Shh*/*Lbx1* in blue and *Pax1* in red (T,X). Variations in the intensity of the *Shh* labelling are due to variations of the probe cocktail. The scale bar in B represents 67 μm in B,F,J,N,R,V and 50 μm in C,D,G,H,K,L,O,P,S,T,W,X. Abbreviations: d, dorsal; D, dermomyotome; Ect, ectoderm; Fp, floor plate; M, myotome; Not, notochord; Nt, neural tube; Scl, slerotome; Sp, segmental plate. (A-D) Controls. (A,B) Ablation and reinsertion of neural tube and notochord allows normal marker expression as shown for (C) *Shh*, *MyoD* and *Pax3* and (D) *Shh*, *Lbx1*, *Pax1* and *Bmp4*. Normal expression pattern are also found in R-T on the left sides of these unilaterally operated embryos. (E-H) Ablation of dorsal neural tube (–dNt) or (I-L) ablation of entire neural tube (–Nt) does not alter marker expression. (M-P) Segmental plate replacing the neural tube (Sp on Not). The anteroposterior orientation of the graft is indicated by an arrow, its position on the sections is marked by a star. Donor and host somites show the correct dorsoventral distribution of the markers. (Q-T) Insertion of tantalum foil separating ectoderm from paraxial mesoderm and neural tube (–Ect). For photography and sectioning, the foil had been removed. Its former position is indicated by a dotted line on the whole-mounted embryo and by a black line on the sections. In the medial somite territories, the markers are expressed normally. (U-X) Removal of the neural tube and tantalum foil insertion (–Nt/Ect); the position of the foil is indicated as before. *Pax1* labels the entire dorsoventral perimeter of the fully de-epithelialised paraxial mesoderm, at the expense of *MyoD* and *Pax3* (arrowheads).

(Williams and Ordahl, 1994; reviewed in Molkentin and Olson, 1996). Details of the probe are described in Bober et al. (1994).

The *Pax1* probe is described in Ebensperger et al. (1995). Expression starts simultaneously with or one somite behind *MyoD* in the ventromedial region of the epithelial somite. After somite de-epithelialisation, the transcription of *Pax1* is confined to the medial sclerotome (Johnson et al., 1994; Ebensperger et al., 1995; Fig. 1D).

The homeobox of the chick *Lbx1* gene was isolated by RT-PCR. The antisense probe derived from this clone labels the lateral edge of the dermomyotome (Fig. 1D) and the migrating progenitors of the limb musculature, coincident with the lateral expression domains of *Pax3* (S. Dietrich, F. Schubert, S. C. Chapman and A. Lumsden, unpublished observations), similar to its murine homologue (Jagla et al., 1995).

Sections

Embryos were embedded in 20% gelatine in PBS and stored at 4°C overnight. Blocks were trimmed, fixed in 4% PFA overnight, washed in PBS and sectioned to 40 μm on a Pelco 1000 Vibratome. The sections were collected on gelatinised slides and covered with Moviol (Hoechst).

Photomicroscopy

After in situ hybridisation, the embryos were cleared in 80% glycerol in PBS. Whole embryos and sections were photographed on a Zeiss Axiophot, using Nomarski optics.

RESULTS

We first studied the requirement of possible signalling tissues for the dorsoventral pattern of the somite by systematically ablating dorsal and ventral structures from the chick embryo. We then confirmed our results by transplanting these structures to ectopic locations.

Ablation of dorsal structures

Dorsal structures in proximity to the paraxial mesoderm are the dorsal neural tube and the surface ectoderm. To determine their influences on dorsoventral somite pattern, we ablated the dorsal neural tube, the entire neural tube and the surface ectoderm, either alone or in combination, and analysed the operated embryos for the expression patterns of dorsoventrally restricted genes.

(1) Ablation of dorsal neural tube (*n*=6)

Following a vertical incision between the axial structures and the paraxial mesoderm at the level of somites VII to –V, the dorsal half to two thirds of the neural tube was ablated with a horizontal cut (Fig. 1E). As a consequence, the expression domains of *Bmp4*, *Wnt4*, *Pax3* and occasionally *Pax6* were lost, but *Shh* persisted in floor plate and notochord. After $6\frac{1}{2}$-$9\frac{1}{2}$ hours, the ectoderm had re-established contact to the cut edge of the neural tube rudiment, which failed to re-close dorsally and to reinstate expression of the dorsal markers (Fig. 1F-H). Possibly as a result of the dorsally flared neural tube, the medial lips of the dermomyotomes appeared less strongly curved than in unoperated embryos (Fig. 1G,H). However, the time course and the dorsoventral distribution of *Pax3*, *MyoD* and *Pax1* in the paraxial mesoderm remained unaltered (Fig. 1F-H; compare to controls in Fig. 1B-D).

(2) Ablation of complete neural tube (*n*=22)

After separating neural tube and paraxial mesoderm with a vertical incision, the neural tube was removed (Fig. 1I), leaving the notochord as the only source of *Shh* signalling (Fig. 1J-L). In the operated area, extending from somites XIII to –IX, the left and right segmental plates and somites approached each other, fusing above the notochord. Similarly, the edges of the cut ectoderm fused, so that the gap was closed after 24 hours. In all cases, *Pax3*, *MyoD* and *Pax1* were expressed normally (Fig. 1J-L).

(3) Replacement of the neural tube by segmental plate (*n*=7)

To verify that the paraxial mesoderm can be fully dorsoventrally patterned in the absence of the neural tube, we removed the neural tube between somites XIII and –VIII and inserted one or two segmental plates of a stage-matched donor embryo into the slit on top of the notochord, maintaining anteroposterior orientation (Fig. 1M,N). After $6\frac{1}{2}$ hours, the grafts had segmented in the same way as the host paraxial mesoderm and the newly formed somites had activated *Pax1* in a normal fashion in ventral regions adjacent to the notochord. Dorsally, the segmental plates and somites expressed *Pax3* as soon as they were re-covered by ectoderm. In a similar, ectoderm-dependent fashion *MyoD* was induced in the newly formed somites just below the *Pax3* expression domain (data not shown). After 24 hours, the operated region was fully covered by ectoderm, and the normal dorsoventral expression pattern of *Pax3*, *MyoD* and *Pax1* established (Fig. 1N-P). However, while the host paraxial mesoderm showed high levels of *Pax3* expression in the lateral edge of the dermomyotome and no *Pax1* expression in the lateral aspect of the sclerotome, this high level expression of *Pax3* never appeared in the graft (Fig. 1O), and the ventral mesenchyme expressed *Pax1* throughout (Fig. 1P), possibly because the graft is distanced from lateral structures.

(4) Isolation from surface ectoderm (*n*=17)

After cutting the ectoderm between neural tube and paraxial mesoderm, a drop of dispase was applied to loosen the contact between the ectoderm and the tissues underneath. The embryo was washed with Ringer solution, and a piece of 10 μm thick tantalum foil inserted between ectoderm and paraxial mesoderm (*n*=7) or between ectoderm and both neural tube and paraxial mesoderm (*n*=17) at the level of somites V to –III and V to –VII, respectively (Fig. 1Q). In both types of operation, the dorsoventrally restricted expression pattern of *Pax6*, *Bmp4* (not shown), *Shh* (Fig. 1R-T) and *Pax3* (Fig. 1R,S) in the neural tube was unaltered. Similarly, in the medial half of the somite *Pax3*, *MyoD* and *Pax1* were induced normally (Fig. 1R-T).

(5) Ablation of neural tube and isolation from surface ectoderm (*n*=16)

When contact between surface ectoderm and paraxial mesoderm was prevented with tantalum foil and the neural tube removed (Fig. 1U), between somites IV and –V the somite fully de-epithelialised (Fig. 1W,X, arrowheads). Of our mesodermal markers, only *Pax1* was activated, occupying all dorsoventral positions (Fig. 1X). Both *MyoD* and *Pax3* failed to be induced or maintained (Fig. 1V,W). The same type of operation performed more anteriorly, between somites XIV and V, allowed a weak expression of *MyoD* and *Pax3*, with signals fading from anterior to posterior (data not shown). These findings suggest that the neural tube and the surface

ectoderm are the only two structures in the embryo that provide dorsalising signals. Their redundant signals are necessary for the development of both dermomyotome and myotome.

Ablation of ventral structures

Ventral structures in proximity to the paraxial mesoderm are the notochord, floor plate and endoderm. The endoderm expresses the signalling molecule *Sonic hedgehog*, as do the notochord and floor plate, and interacts extensively with the splanchnic mesoderm during the formation of the caudal intestinal port (Roberts et al., 1995). However, the endoderm is insufficient for ventralisation of the paraxial mesoderm, due to the absence of required signals or due to limited apposition during development: after ablation of the neural tube/notochord complex no ventral somitic markers were expressed, either in the endogenous paraxial mesoderm or in paraxial mesoderm grafted into the slit (S. Dietrich, F. Schubert, S. C. Chapman and A. Lumsden, unpublished observations). Therefore, we focussed our attention on notochord and floor plate. We ablated either the notochord or both notochord and floor plate at two axial levels, and analysed the expression pattern of the markers as before.

(1) Ablation of notochord from mid-segmental plate to somitic levels (*n*=40)

The axial structures were mechanically and enzymatically separated from the paraxial mesoderm in the region of somite XIII to –IV as described above, and the neural tube of the same or a stage-matched donor embryo was grafted back (Fig. 2A). This neural tube, although deprived of notochord, showed the normal dorsoventrally restricted expression of *Bmp4*, *Wnt4*, *Pax6* (not shown), *Pax3* (Fig. 2B,C) and *Shh* (Fig. 2D). The paraxial mesoderm expressed *Pax3*, *MyoD* and *Pax1* in a normal fashion (Fig. 2B-D), suggesting that the loss of the notochord was compensated for by the fully established floor plate. In one embryo, the caudalmost area of the neural fold was included in the graft. Here, the *Pax6* expression domain was shifted into the ventral midline. In the adjacent paraxial mesoderm, the *Pax3* and *MyoD* signals were shifted ventrally too, with *MyoD* residing ventrally beneath the neural tube (data not shown).

(2) Ablation of newly formed and nascent notochord at the level of the posterior segmental plate (*n*=25)

To ablate the newly formed and nascent notochord, an incision was made between the posterior end of the segmental plate and the neural plate of a HH 10 embryo. After brief enzymatic treatment, the notochord was excised, cutting as far posterior into the node region as possible. The neural plate was flipped back into position (Fig. 2E,M), where it re-established contact to the surface ectoderm and closed dorsally. After 24 hours, the embryos had added 13 to 17 somites. They fell into two categories: in half of the animals, in the operated area a repeatedly interrupted *Shh* pattern was observed below the neural tube, indicating that the notochord had partially regenerated. Both paraxial mesoderm and neural tube expressed the markers normally (not shown). In the other half of the embryos, as judged by morphological inspection (Fig. 2G,H,O,P) and *Shh* staining (Fig. 2F-H), the notochord was absent between somites –V and approximately somite –XX. Because the length of this notochord-less area at the time of inspection reached further posterior than the segmental plate at the time of operation, notochord precursors residing in the node must also have been removed. Between somites –VI and –VII, the neural tube appeared dorsoventrally compressed, but the floor plate was still *Shh*-positive, and *Pax6*, *Wnt4* and *Pax3* displayed their normal dorsoventral distribution (not shown). More posteriorly, the neural tube acquired a more radially symmetrical shape. Its diameter remained small, and no morphologically defined floor plate developed. In line with Goulding et al. (1994), in the region of somites –VII to –XIV, the *Wnt4* (not shown) and *Pax3* signals (Fig. 2F-H, arrows) spread through the neural tube, leaving out the ventral midline where *Pax6* (Fig. 2N-P, arrows) and occasionally weakly *Shh* (not shown) was expressed. In the posteriormost region where the notochord precursors had been removed from the node, neither *Shh* (Fig. 2F,H) nor *Pax6* (Fig. 2N) activity was found, and the neural tube expressed *Wnt4* (not shown) and *Pax3* (Fig. 2F,H) throughout.

The expression of the mesodermal markers followed the same principle: after 24 hours, in embryos retaining notochord or floor plate, the mesodermal markers were expressed in a normal fashion (not shown). In notochord-less animals, in the region of somites –VI to –IX, the somitic *Pax3* staining extended ventrally (Fig. 2F,G, arrows). However, some weak expression of *Pax1* (Fig. 2N) and *MyoD* (Fig. 2F,G,N,O, arrowheads) was still present in ventral locations. In the middle of the operated area, first the *Pax1* and subsequently the *MyoD* staining disappeared (Fig. 2N). Finally, in the region where the neural tube failed to express *Pax6*, a strong *Pax3* signal occupied the entire paraxial mesoderm (compare Fig. 2F,H,N). The same observations were made after reincubation for 36 hours and 48 hours (not shown), suggesting that the removal of the nascent notochord is incompatible with the development of the floor plate in the neural tube (Yamada et al., 1991), and of sclerotome and myotome in the paraxial mesoderm.

(3) Ablation of notochord and floor plate from mid-segmental plate to somitic levels (*n*=40)

To test whether *MyoD* expression depends on ventralising structures in a region where the floor plate is established, the neural tube was opened in the dorsal midline between somites XIV and –XII, and cut horizontally dorsal to the floor plate. After dispase treatment, notochord and floor plate were excised, while the dorsal neural tube was left in place (Fig. 2I). This neural tube rudiment re-closed and expressed *Bmp4*, *Wnt4* (not shown), *Pax3* (Fig. 2K) and *Pax6* (Fig. 2J,L). Occasionally, some *Shh* staining was found at its ventral edges (Fig. 2L, left side). The adjacent paraxial mesoderm showed weak to normal *Pax1* and *MyoD* staining, with the *MyoD* signals being more robust than the *Pax1* signals (Fig. 2J,L, left side). In regions devoid of any *Shh* staining both *Pax1* (Fig. 2J,L, right side) and *MyoD* (Fig. 2K; Fig.2 J,L, right side) failed to be activated and maintained. These findings suggest that redundant ventralising signals provided by notochord and floor plate are required for both sclerotome and myotome development. The dose sufficient for myotome induction seems to be lower than the dose essential for the formation of the sclerotome.

Ectopic ventralising and dorsalising structures

The ablation experiments in chick suggested that dorsalising signals are required for dermomyotome formation, and in concert with ventralising factors, for the formation of the myotome. The response of the paraxial mesoderm to ventral-

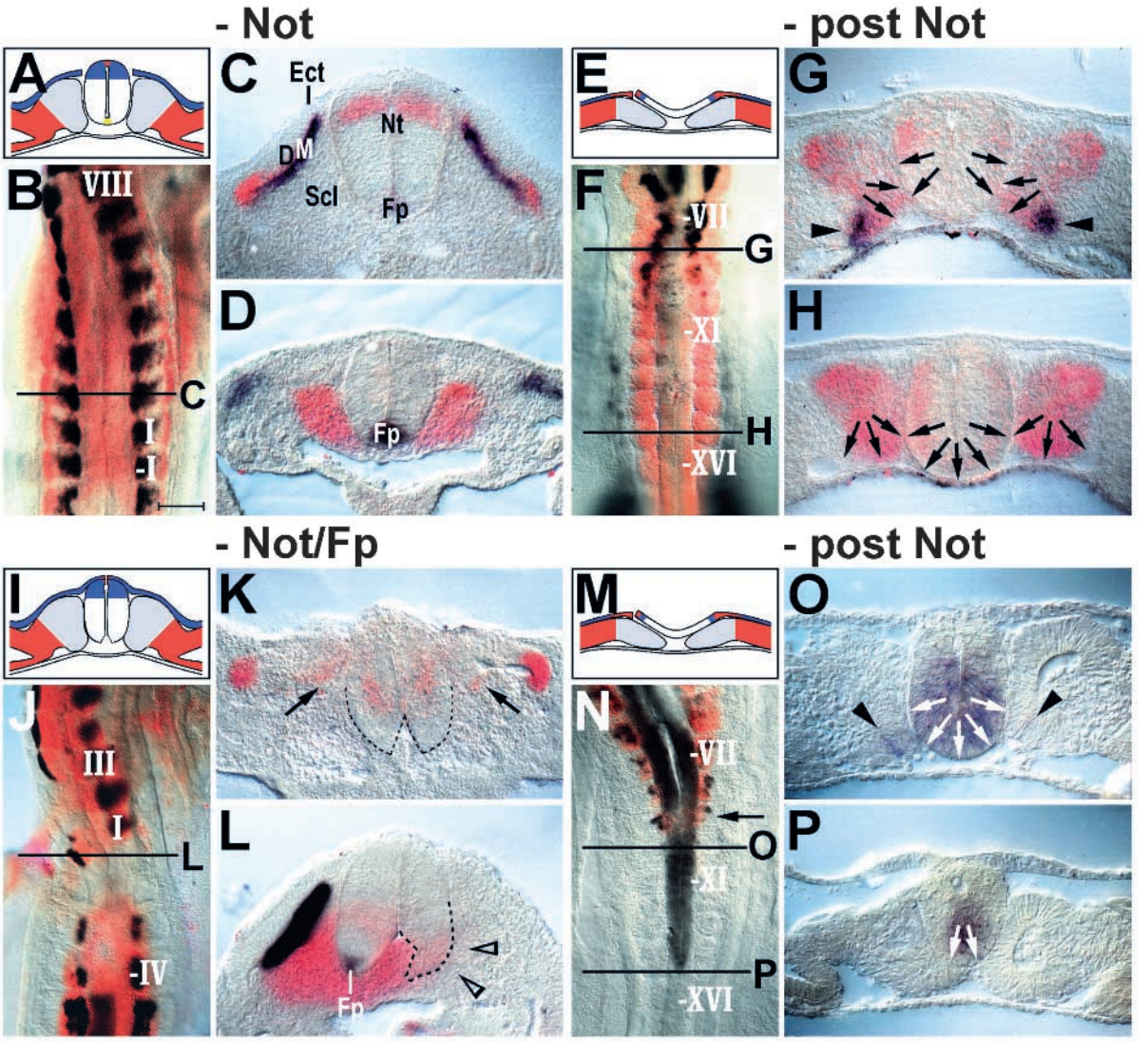

Fig. 2. Consecutive loss of ventralising structures. (A,E,I,M) Schemes of operations performed at the level of the anterior segmental plate and youngest somites (A-D,I-L) or at the posterior end of the segmental plate (E-H,M-P); colour code as before. (B,F,J,N) Dorsal views of the operated areas 24 hours post-surgery. The developmental age of the paraxial mesoderm and the level of the cross sections (C,G,H,L,O,P) are indicated as before. (C,D,G,H,K,L,O,P) Cross sections. The scale bar in B represents 67 μm in B,F,J,N, 50 μm in C,D and 25 μm in G,H,K,L,O,P. Hl, hindlimb; post, posterior; other abbreviations as before. (A-D) Notochord-ectomy (–Not). (B) Chick embryo labelled with *Shh*/*MyoD* probes in blue and *Pax3* probe in red. (C) Section of B. (D) Similar section stained for *Shh*/*Lbx1* expression in blue and *Pax1* expression in red. Note the presence of the floor plate in the neural tube and the normal expression pattern of the mesodermal markers. (E-H,M-P) Removal of the newly formed and nascent notochord (–post Not). (F-H) Entire and sectioned embryo stained for *Shh*/*MyoD* in blue and *Pax3* in red. In somites –VIII to –X (F), the last, ventrally located *MyoD* signals are found (G, arrowheads), and the *Pax3* domains are expanded ventrally (G, arrows). Posteriorly, neural tube and paraxial mesoderm express *Pax3* throughout the dorsoventral perimeter (H, arrows), at the expense of *MyoD*. (M-P) Entire and sectioned embryo hybridised with *MyoD/Pax6* probes in blue and *Pax1* probe in red. Note the ventral shift of the *Pax6* domain (O,P, arrows), the last *Pax1* signal in somite –IX (N, arrow), the last *MyoD* signal in somite –X (O, arrowheads), and the loss of *Pax6* signals posterior to somite –XV (N). (I-L) Ablation of notochord and floor plate (–Not/Fp). (J,L) Embryo/section stained for *Shh/MyoD* in blue and *Pax1/Pax6* in red. (K) Section showing the pattern of *Shh/MyoD* in blue and *Pax3* in red. Absence of notochord and floor plate prevents the expression of *MyoD* and *Pax1* (L, arrowheads) and the dorsal restriction of *Pax3* (K, arrows).

ising signals seems to be dose dependent, as a low dose appears sufficient for myotome, but not for sclerotome induction. To test these hypotheses, we grafted the signalling structures to ectopic sites and analysed the spatiotemporal expression of *MyoD*, *Pax1*, *Pax3* in the paraxial mesoderm, and *Shh*, *Pax6*, *Pax3*, *Wnt4* and *Bmp4* in notochord, neural tube and lateral mesoderm.

(1) Transplantation of notochord fragments to ectopic sites

(A) Notochord grafts dorsomedially between neural tube and paraxial mesoderm (*n*=17)

Donor notochords were obtained from the posterior region of dispase-treated HH 10-12 chick embryos and inserted dorsally between neural tube and paraxial mesoderm of stage-matched

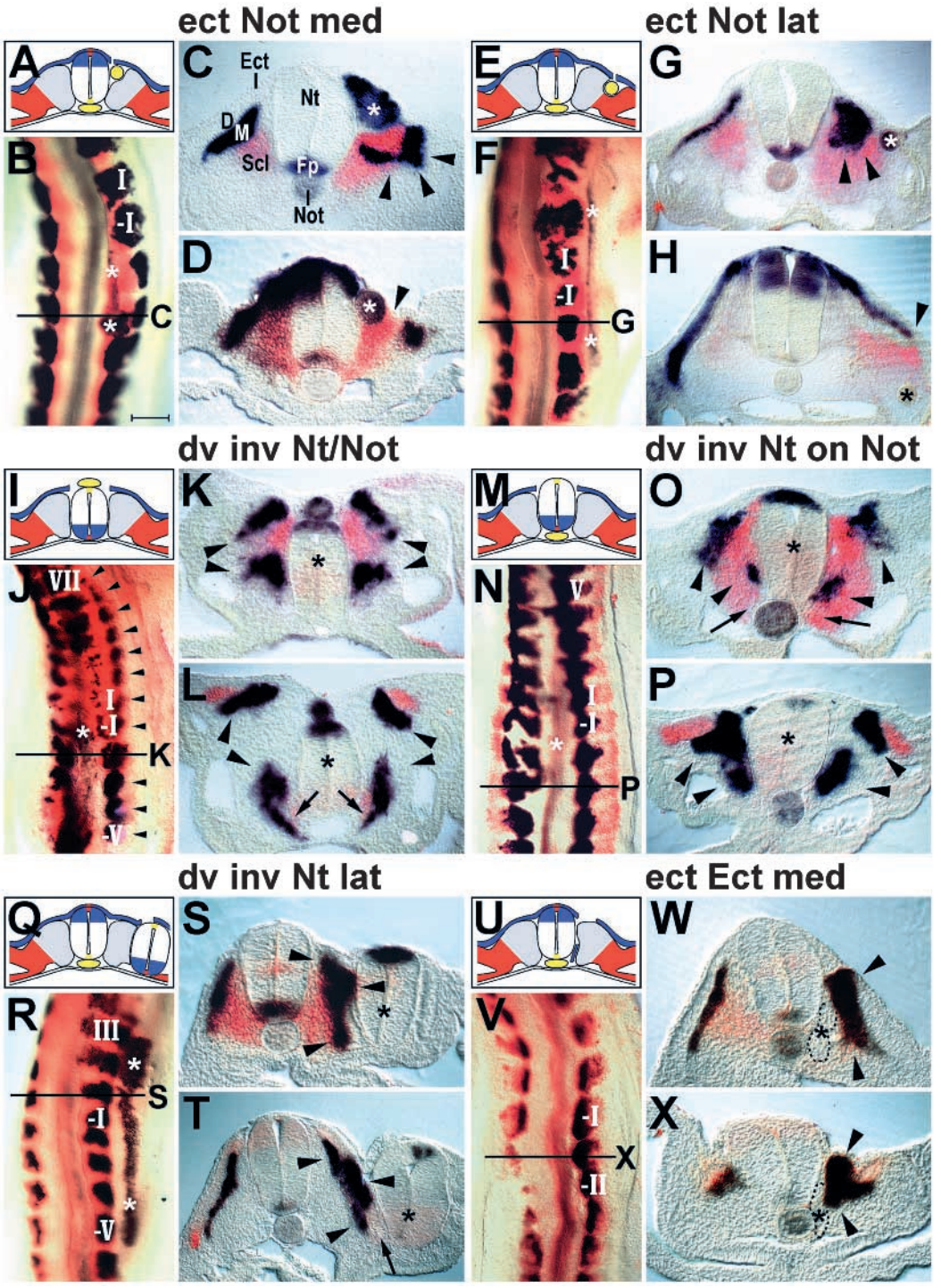

Fig. 3. Heterotopic grafting of dorsalising and ventralising structures. (A,E,I,M,Q,U) Schemes of operations performed at the level of the anterior segmental plate and youngest somites; colour code as before. (B,F,J,N,R,V) Dorsal views of the operated areas 24 hours postsurgery. The developmental age of the paraxial mesoderm and the level of the cross sections (C,G,K,P,S,X) are indicated as before. (C,D,G,H,K,L,O,P,S,T,W,X) Cross sections. The position of the grafts is indicated by stars. The scale bar in B represents 67 μm on the whole-mounted embryos and 50 μm on the sections. Normal expression patterns are found on the left sides of unilaterally operated embryos in B-D,F-H,R-T,W. Abbreviations: dv, dorsoventral; ect, ectopic; inv, inverted; l, lateral; med, medial; others as before. (A-D) Insertion of ectopic notochord medially (ect Not med). The whole-mounted embryo (B) and the corresponding section (C) display expression of *Shh*/*MyoD* in blue and *Pax1* in red. (D) *Shh*/*Pax3* in blue and *Pax1* in red. Note that the ectopic notochord represses *Pax3* (D, arrowhead), induces *Pax1* close by (C, D, arrowhead), and, at some distance, induces *MyoD* close to dorsal neural tube and ectoderm (C, arrowheads). (E-H) Insertion of ectopic notochord laterally (ect Not lat); same marker combination as in (B-D). Similar to medial insertions, the laterally placed notochord represses *Pax3* (H, arrowhead), induces *Pax1* close by (G; H, arrowhead), and, at some distance, induces *MyoD* close to the ectoderm (G, arrowheads). (I-L) Dorsoventrally inverted neural tube and notochord (dv inv Nt/Not). Whole-mounted embryo (J) and section (K) are stained for *Shh*/*MyoD* (blue) and *Pax1*/*Pax6* (red). (L) *Shh*/*MyoD* expression in blue and *Pax3* in red. Notochord and floor plate of the graft induce *Pax1* (K) and repress *Pax3* (L) dorsally. The previously dorsal neural tube induces *Pax3* ventrally (L, arrows). The graft, in combination with the ectoderm induces two domains of *MyoD* (K,L, arrowheads) up to somite VII (J, arrowheads). (M-P) Neural tube rotation on top of the notochord (dv inv Nt on Not). Whole-mounted embryo (N) and section (P) show *Shh*/ *MyoD* signals in blue and *Pax3* in red. (O) *Shh*/*MyoD* in blue and *Pax1*/*Pax6* in red. Similar to the neural tube and notochord rotation experiments, two *MyoD* domains are present (O,P, arrowheads), and the dorsally located floor plate ectopically induces *Pax1* (O). The dorsal neural tube fails to induce *Pax3* ventrally (P) where the endogenous notochord activates *Pax1* (O, arrows). (Q-T) Inverted neural tube lateral to the paraxial mesoderm (dv inv Nt lat); same set of markers as in J-K. Note the dorsomedial-like dermomyotomal lip close to the previously dorsal neural tube (T, arrow). Besides the endogenous myotome, additional *MyoD* domains are located at this lip and within the dermomyotome (S,T, arrowheads). (U-X) Transplantation of ectoderm between neural tube/notochord and paraxial mesoderm (ect Ect med); same marker combination as in (N-P). In addition to the endogenous myotome, a further *MyoD* domain is induced (W,X, arrowheads).

hosts at the level of somites X to –VI (Fig. 3A). As described previously (Brand-Saberi et al., 1993; Pourquié et al., 1993; Goulding et al., 1994), *Pax1* was activated around the graft at high levels after 24 hours (Fig. 3B,C; 3D, arrowhead), at the expense of both *MyoD* (Fig. 3B,C) and *Pax3* (Fig. 3D, arrowhead). Close to the transplanted notochord, the somite fully de-epithelialised. However, at some distance, and in proximity to the ectoderm or dorsal neural tube, *MyoD* was strongly activated in a ventrally expanded domain, in one embryo leading to an ectopic, ring-like expression pattern below the graft (Fig. 3B,C, arrowheads).

(B) Notochord grafts lateral to paraxial mesoderm (n=8)

When the ectopic notochord was placed laterally to the paraxial mesoderm in the region of somites III to –IV (Fig. 3E), after $6\frac{1}{2}$ hours, *Pax3* was repressed and *MyoD* expression induced close to the graft, provided the graft was located near the ectoderm (not shown). After 24 hours, similar to the dorsomedial grafts, close to the graft, *Pax1* was strongly expressed (Fig. 3F-H), *MyoD* (Fig. 3F,G) and *Pax3* (Fig. 3H, arrowhead) were repressed, and the region appeared mesenchymal. At a greater distance, an intense and ventrally expanded *MyoD* signal was found (Fig. 3F,G, arrowheads). However, this *MyoD*-positive domain still resided in a dorsal territory close to the ectoderm. The more ventrally the transplanted notochord was located, the less dramatic was its effect on the expression of *Pax3* and *MyoD*, and only *Pax1* was activated ectopically. These findings support the idea that the myotome is induced by the combinatorial action of dorsalising signals, here provided by the ectoderm, and weak ventralising signals. Stronger ventralising signals induce the sclerotome, thereby suppressing the dermomyotome.

(2) Dorsoventral inversion of the axial structures

(A) Rotation of the neural tube and notochord (n=9)

To characterise the dorsalising potential of the neural tube, we excised the neural tube and notochord at the level of somites XI to –VI as described above, and back-grafted it in dorsoventrally inverted orientation (Fig. 3I). After $6\frac{1}{2}$ hours, the newly formed somites expressed *Pax1* dorsally, close to notochord and floor plate, suggesting that the dorsalising influence of the ectoderm was overridden by the ventralising signals. Ventrally, a strong *Pax3* signal was found, adjacent to the *Pax3*-positive region of the neural tube. In addition, distant to notochord and floor plate, the normal *Pax3* expression was found below the ectoderm. The *MyoD* gene was expressed in two independent domains, one located dorsally close to the ectoderm and one located ventrally, neighbouring the *Pax6* expression domain in the neural tube (data not shown). After 24 hours, while the dorsal somite appeared mesenchymal and expressed *Pax1* (Fig. 3J,K), the ventral region was epithelially organised and *Pax3*-positive (Fig. 3L, arrows). Two rows of *MyoD* signals traversed the operated area longitudinally (Fig. 3J, arrowheads), residing in the same dorsal and ventral position as seen after $6\frac{1}{2}$ hours (Fig. 3K,L, arrowheads). These observations support the idea that the somite becomes dorsalised by signals emanating from ectoderm and dorsal neural tube, and ventralised by signals from notochord and floor plate. Where the paraxial mesoderm is exposed to the appropriate level of both signals, myotome formation is induced even in ectopic locations.

(B) Ablation of notochord and rotation of neural tube (n=8)

When the notochord was removed and only the neural tube reinserted in dorsoventrally rotated orientation in the region of somites VII to –IV, we obtained the same results as for the neural tube/notochord inversions, underlining the redundant function of notochord and floor plate (data not shown).

(C) Transplantation of dorsoventrally inverted neural tube on top of notochord (n=7)

To analyse the direct competition of dorsalising and ventralising signals, we inverted the neural tube leaving the notochord in place in the region of somites X to –VI (Fig. 3M). The inverted neural tube resisted the ventralising influence underneath as monitored by the expression of *Bmp4*, *Wnt4* (not shown), *Pax3* (Fig. 3N,P), *Pax6* (Fig. 3O) and *Shh* (Fig. 3N-P). In the paraxial mesoderm, after $6\frac{1}{2}$ hours the marker gene expression resembled the pattern observed in our previous inversion experiments. After 24 hours, however, *Pax1* was expressed both adjacent to the ectopic floor plate, and around the notochord (Fig. 3O, arrows), at the expense of *Pax3* (Fig. 3N,P). *MyoD* remained active in two separate domains in agreement with Spence et al. (1996), with the ventrally located domain appearing smaller and shifted dorsally compared with the embryos where both neural tube and notochord were rotated (Fig. 3N-P, arrowheads). In one embryo, at the anterior end of the operated area, the inverted neural tube resided at some distance from the notochord, with the gap filled by paraxial mesoderm. The originally dorsal neural tube was surrounded by a thin layer of *Pax3*-expressing cells, while the notochord was enclosed by a massive ring of mesenchymal cells, possibly positive for *Pax1* (not tested). Between the axial structures, and at the ventral border of the *Pax3* domain, an intensely *MyoD*-labelled zone was found (data not shown). Together, these findings suggest that the dorsalising signals provided by the neural tube in combination with the signals derived from both ventralising centres were sufficient to induce the ventral *MyoD* domain. Close to the endogenous notochord, the ventralising information overrode the dorsalising signals, thereby repressing *Pax3* and *MyoD*.

(D) Transplantation of dorsoventrally inverted neural tube lateral to paraxial mesoderm (n=2)

As the medial and the lateral half of the paraxial mesoderm have been shown to respond differently to myogenic inducers in vitro (Gamel et al., 1995; Cossu et al., 1996), we transplanted the dorsoventrally inverted neural tube lateral to the paraxial mesoderm in the region of somites III to –IV (Fig. 3Q). After 24 hours, the normal expression patterns of *MyoD*, *Pax1* and *Pax3* were established medially, under the control of the endogenous axial structures (Fig. 3R-T). Laterally, the strong *Pax3* signal typical for the lateral dermomyotome was absent. However, *Pax3* was expressed at lower levels in ventral locations close to the graft, and a lip characteristic of the dorsomedial dermomyotome had formed (Fig. 3T, arrow). From this lip, a second, ventrolateral *MyoD*-positive cell population emerged, fusing with the expanding endogenous myotome (Fig. 3R-T, left arrowheads). Dorsolaterally, a third *MyoD* signal was found below the ectoderm at the expense of *Pax3* (Fig. 3R-T, right arrowhead). Here, the somite had retained its epithelial characteristics and did not express *Pax1* (Fig. 3R,S),

presumably due to the distance of the floor plate from the paraxial mesoderm. These findings suggest in line with Ordahl and Le Douarin (1992) that, similar to the medial half, the lateral somite is capable of activating the medial dorsoventral program, responding to signals in this case derived from ectoderm, dorsal neural tube and floor plate.

(3) Transplantation of ectoderm fragments to ectopic sites (*n*=8)

To directly test the dorsalising capacity of the ectoderm, we separated ectoderm and segmental plate as described before, cut off a strip of ectoderm about 3 somites long, and immediately slid it into the groove between neural tube and segmental plate with the basal epithelial surface facing the segmental plate (Fig. 3U). After 24 hours, marker gene expression resembled the pattern observed after neural tube inversion on top of the notochord. *MyoD* signals were found in a second, ventrally located, small domain in immediate proximity to a dorsal, normal looking myotome (Fig. 3V-X, arrowheads), supporting the idea of a dorsalising and myotome-inducing function of the ectoderm.

Ectopic paraxial mesoderm

(1) Replacement of the axial structures by mature somites (*n*=20)

To test whether the patterning function of the axial structures can be substituted by mature somites, we ablated the neural tube/notochord at the level of somites VI to –VI, leaving the ectoderm in place. We then inserted somites XXV to VII obtained from HH 13-17 donors (Fig. 4B), maintaining the dorsoventral and inverting the anteroposterior orientation such that the most mature somites in the graft were brought into the proximity of the youngest somites and segmental plates of the host (Fig. 4A). After $6\frac{1}{2}$ to $9\frac{1}{2}$ hours, the host embryos had developed 4 to 6 further somites, none of which expressed *MyoD* (Fig. 4A,C) or *Pax1* (not shown). Also the youngest somites present at the time of operation were deprived of both markers, while *Pax3* was expressed as soon as the paraxial mesoderm was covered with ectoderm (Fig. 4A,C, arrowhead). After 24 hours, the host failed to express *Pax1* and *MyoD* in the entire operated field, and the signals of these genes faded in the graft as well (not shown). Thus, the embryos exhibited a phenotype typical for ablations of the neural tube and notochord (S. Dietrich, F. Schubert, S. C. Chapman and A. Lumsden, unpublished observations; Teillet and Le Douarin, 1983; Rong et al., 1992; Pownall et al., 1996), suggesting that the presence of the axial structures is essential for the induction and maintenance of sclerotome and myotome.

(2) Anteroposterior rotation of segmental plates (*n*=11)

In our study, we changed the dorsoventral patterning of the paraxial mesoderm by manipulating ectoderm and neural tube, which provided dorsalising signals, and notochord and floor plate, releasing ventralising signals. However, the *Pax1* and *MyoD* genes did not respond prior to somitogenesis. To test whether this response depended on the developmental age of the inducers or the paraxial mesoderm itself, we inserted segmental plates including the recently formed somite from HH 10-13 donors between the neural tube/notochord and the host paraxial mesoderm at the levels of somites IV to –IX (Fig. 4E). The anteroposterior orientation of the grafts was inverted, so that the previously anterior, segmenting area was exposed to a younger neural tube and notochord, while the posterior end faced a more mature neural tube and notochord (Fig. 4D). After $6\frac{1}{2}$ hours, both graft and host had developed 4-5 additional somites. At the operated side, the host paraxial mesoderm failed to express *MyoD* (Fig. 4D,F) and *Pax1* (not shown) presumably because it lacked contact with the axial structures. The

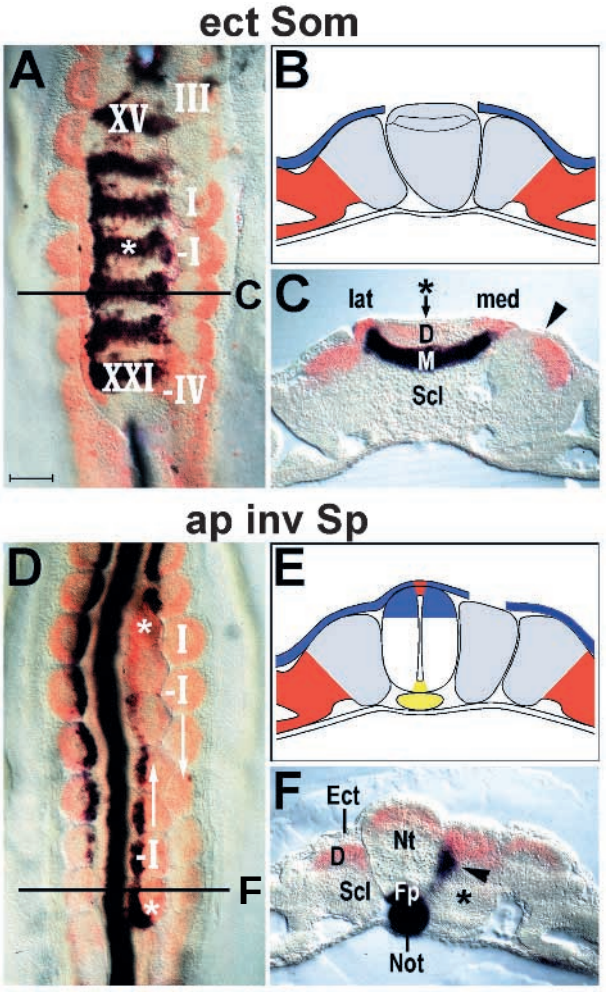

Fig. 4. Heterotopic transplantation of mature somites and segmental plates. (A,D) Dorsal views of the operated areas $6\frac{1}{2}$ hours after surgery. The developmental age of the paraxial mesoderm and the level of the cross sections (C,F) are indicated as before. (B,E) Schemes of operations performed at the level of the anterior segmental plate and youngest somites; colour code as before. (C,F) Cross sections of the embryos in A,D. The position of the grafts is indicated by stars. The scale bar in A represents 90 μm in A,D and 50 μm in C,F. Abbreviations: ap, anteroposterior; Som, somites; others as before. (A-C) Somites XV to XXI replacing neural tube/notochord (ect Som). The embryo is hybridised with *Shh/MyoD* in blue and *Pax3* in red. In the host somites, *MyoD* expression is lacking. *Pax3* is active where the paraxial mesoderm is covered by ectoderm (C, arrowhead). (D-F) Anteroposteriorly rotated segmental plate grafted lateral to neural tube and notochord (ap inv Sp), labelled for *Shh/MyoD* in blue and *Pax3* in red. On the operated side, the orientation of the paraxial mesoderm is indicated by arrows. In the graft, *MyoD* is activated according to the developmental age of the paraxial mesoderm (F, arrowhead).

somites derived from the graft had activated both genes (Fig. 4D,F, arrowhead), while the still unsegmented area remained negative (Fig. 4D). This finding suggests that the paraxial mesoderm gains the competence to activate the sclerotomal and myotomal programs after segmentation. After 24 hours, when the graft was fully segmented, it expressed *Pax1* and *MyoD* along its entire length. Compared to the control side, the expression of both genes was laterally expanded, at the expense of the intense lateral expression of *Pax3*. By contrast, the host somites in the operated region showed a medial extension of this *Pax3* domain, but lacked expression of *Pax1* and *MyoD* (data not shown).

DISCUSSION

A critical step in the development of the paraxial mesoderm is its dorsoventral patterning, as it leads to the formation of all the three derivatives, the ventral sclerotome, the dorsal dermomyotome and the intermediate myotome (reviewed in Christ and Ordahl, 1995). Ventralisation of the paraxial mesoderm depends entirely on the action of notochord or floor plate (Brand-Saberi et al., 1993; Dietrich et al., 1993; Koseki et al., 1993; Pourquié et al., 1993; Goulding et al., 1994). In contrast, little is known about the expression of dorsal properties (Fan and Tessier-Lavigne, 1994; Kuratani et al., 1994; Brill et al., 1995; Fan et al., 1995), and the studies on myotome formation are contradictory (reviewed in Molkentin and Olson, 1996). Therefore, in this study, we characterised the dorsoventral patterning of the somite, assaying in particular for dorsalising inducers, and for redundant, antagonistic and cooperative signals.

The paraxial mesoderm is dorsalised by the dorsal neural tube and surface ectoderm

The initial expression of the dorsal, dermomyotomal marker *Pax3* throughout the dorsoventral perimeter of the chick midsegmental plate or anterior mouse segmental plate had led to the suggestion that the paraxial mesoderm is dorsally predisposed (Dietrich et al., 1993; Williams and Ordahl, 1994). In vitro however, this marker is activated upon coculture of segmental plates with dorsal neural tube or surface ectoderm (Fan and Tessier-Lavigne, 1994; Fan et al., 1995), suggesting that the paraxial mesoderm is able to respond to dorsalising signals. In vivo, the expression of the dermomyotomal marker *MHox* depends on the presence of the ectoderm (Kuratani et al., 1994), while the release of dermis progenitors from the dermomyotome requires contact with the dorsal neural tube or exposure to the neurotrophic factor *NT-3* (Brill et al., 1995). Our results confirm that neural tube and surface ectoderm play a crucial role in the initiation and maintenance of the dorsal pathway in the paraxial mesoderm in vivo: when both the neural tube and the surface ectoderm have been removed, the segmental plate fails to activate *Pax3*. In addition, neural tube/ectoderm removal at the level of the anterior segmental plate and young somites expels expression of *Pax3* from the paraxial mesoderm. In both cases, the somites appear completely mesenchymal and express *Pax1* throughout their dorsoventral perimeter, indicating that they have been fully ventralised. In line with previous studies, the absence of the neural tube (Teillet and Le Douarin, 1983; Bober et al., 1994; Spence et al., 1996) or ectoderm alone does not interfere with the dorsoventral patterning of the medial somite territory, suggesting that neural tube and surface ectoderm provide redundant dorsalising signals. This interpretation is supported by heterotopic grafting experiments: neural tube transplants induced mesodermal *Pax3* expression ectopically, close to the previously dorsal, *Pax3*- and *Wnt4*-positive region of the grafted neural tube. Similarly, when dorsalisation of the neural tube is promoted by the ablation of the newly formed and nascent notochord, the extension of the *Pax3*-positive somite epithelium precisely corresponds with the extension of the *Pax3*/*Wnt4* expression domain in the neural tube, in line with Goulding et al. (1994). In mouse notochord mutants, the position of dorsalising signals provided by surface ectoderm and neural tube seems to determine the expression of dorsal identities in the paraxial mesoderm in a similar fashion (Dietrich et al., 1993; Koseki et al., 1993; S. Dietrich, F. Schubert and A. Lumsden, unpublished observations). Our findings suggest that the paraxial mesoderm does not possess any dorsal identity by default. It is dorsalised by signals provided by the surface ectoderm and the neural tube, similar to the neural tube itself (Liem et al., 1995).

Dorsalising and ventralising signals are antagonistic

As the paraxial mesoderm is fully ventralised upon neural tube/ectoderm removal and dorsalised when both notochord and floor plate are absent, the spatial organisation of sclerotome and dermomyotome seems to result from an antagonistic function of the dorsalising and ventralising signals. However, dorsalisation fails and *Pax1* is expressed at the expense of *Pax3*, when the dorsalising factors have to compete with strong ventralising signals. This situation occurs when notochord or floor plate is placed dorsally, close to dorsal neural tube or surface ectoderm (Brand-Saberi et al., 1993; Pourquié et al., 1993; Goulding et al., 1994; Xue and Xue, 1996; this study), when ectoderm grafts reside ventrally, or when the neural tube is dorsoventrally inverted above the notochord. Corresponding results have been obtained in vitro, where dorsalising factors provided by the neural tube can be overridden by the exogenously applied ventral signalling molecule *Sonic hedgehog* (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). These findings suggest that the relative levels of the dorsalising and ventralising signals determine whether a mesodermal cell adopts a dorsal or a ventral identity.

Dorsalising and ventralising signals synergise in myotome induction

In the medial aspect of chick somite I or II, *MyoD* as the earliest myotomal marker is initiated in intermediate locations at the ventral border of the *Pax3* expression domain (Williams and Ordahl, 1994; Molkentin and Olson, 1996). A similar expression profile has been shown for the earliest myotomal marker in the mouse, *Myf5* (Ott et al., 1991). In both species, the myotome is later displaced dorsally, probably because of the strong mitotic activity of the sclerotome (reviewed in Christ and Ordahl, 1995). However, its initial positioning suggests that it belongs to neither the dorsal nor the ventral program of the somite, but rather represents a third developmental pathway. This idea is supported by our ablation experiments: when either all dorsalising or all ventralising structures were ablated, myotomal markers were neither activated nor main-

tained, suggesting that dorsalising as well as ventralising signals are crucial for the formation of the myotome.

Our results are consistent with observations in other organisms. The mouse mutant *open brain* represents the category of dorsal aberations: its dorsal neural tube deficiencies coincide with reduction and subsequent loss of the dermomyotomal marker *Pax3*, the myotomal markers *Myf5* and *Myogenin*, and later a strong reduction of the epaxial musculature (Spörle et al., 1996). Ventral midline phenotypes affecting myotome development are evident in mouse notochord mutants (S. Dietrich, F. Schubert and A. Lumsden, unpublished observations). In *T/Brachyury curtailed* and in *truncate*, some activation of the myotomal, but not the sclerotomal program (Dietrich et al., 1993) takes place, probably due to the presence of notochord precursors. In these mutants as well as in *Danforth's short tail* and *Pintail*, which exhibit notochord degeneration, the myotomal markers decline when the all ventralising factors are lost. Correspondingly, in the zebrafish notochord mutants *momo* and *Not-1/floating head* lacking notochord precursors, only a few cells in the neural tube express *Shh* as a floor plate marker, and the induction of adaxial muscle pioneers as well as maintenance of lateral musculature is prevented (reviewed in Odenthal et al., 1996; Stemple et al., 1996). In *T/no tail* mutants, where notochord precursors are generated but fail to differentiate, adaxial muscle pioneers are absent and lateral *MyoD* expression reduced.

We directly tested the combinatorial action of dorsalising and ventralising signals by heterotopic grafting experiments: when dorsal neural tube or ectoderm and notochord or floor plate are combined in ectopic locations, ectopic myotomes are induced, reminiscent of the results of previous studies (Bober et al., 1994; Pownall et al., 1996; Spence et al., 1996; Xue and Xue, 1996). The maximal number of myotomes was found after insertion of a dorsoventrally inverted neural tube lateral to the segmental plate: in addition to the myotome induced by the endogenous axial structures, two myotomes formed laterally, presumably induced by the ectopic dorsal neural tube/floor plate, and the ectopic floor plate co-operating with the endogenous ectoderm. These findings suggest that, contrary to the situation in vitro (Gamel et al., 1995; Cossu et al., 1996), the medial and lateral aspects of the paraxial mesoderm exhibit the same competence to respond to external signals in vivo, in line with the mediolateral switch-graft experiments by Ordahl and Le Douarin (1992). In addition, as for results obtained in vitro by Münsterberg and colleagues (1995) and Stern and co-workers (1995), the combinatorial action of ventralising and dorsalising signals induces and maintains the myotome in vivo.

The ventralising signals act in a dose dependent fashion

In our heterotopic grafting experiments, the response of the paraxial mesoderm to notochord or floor plate depended on the duration of the ventralising signals: $6\frac{1}{2}$ and $9\frac{1}{2}$ hours after the operation *MyoD* was induced close to notochord or floor plate and *Pax3* was not yet repressed. After 24 hours, this region lacked *MyoD* and *Pax3* signals, but expressed *Pax1* intensely. Now, the *MyoD*-positive domain resided at some distance from the graft, where *Pax1* had not been activated, but *Pax3* was repressed. The latter remained active far from the ventralising centres and, in proximity to the dorsal neural tube or ectoderm, suggesting that the ventralising signals exert two different effects: at long range and low concentrations, myotome is induced and dermomyotome repressed, in line with Bober et al. (1994), Pownall et al. (1996), and Xue and Xue (1996); at close range and high concentrations, myogenesis is blocked, and sclerotome formation is promoted, as suggested by Brand-Saberi et al. (1993), Pourquié et al. (1993), Goulding et al. (1994), and Xue and Xue (1996).

This interpretation is consistent with observations on notochord-deprived chick embryos, and notochord mutants in both mouse and fish: the reduction of ventralising signals interferes primarily with the development of sclerotomal, and in the fish adaxial fates, while myotomal/lateral fates are less strongly affected (Dietrich et al., 1993; Koseki et al., 1993; Pourquié et al., 1993; Goulding et al., 1994; Odenthal et al., 1996; Stemple et al., 1996; S. Dietrich, F. Schubert and A. Lumsden, unpublished observations). In the mouse mutants, we saw a differential effect even within the myotome: the medial, *Myf5*-expressing myotome (Smith et al., 1994; Braun and Arnold, 1996; Cossu et al., 1996) is more sensitive to the loss of ventralising factors than the lateral, MyoD-positive myotome (S. Dietrich, F. Schubert and A. Lumsden, unpublished observations).

Does the paraxial mesoderm represent a naïve tissue?

Rotation experiments and ectopic transplantation of somite halves have indicated that – in contrast to the rostrocaudal polarity – the dorsoventral and mediolateral organisation of the paraxial mesoderm is yet undetermined in segmental plate and young somites, as the grafts develop 'ortsgemäß' rather than 'herkunftsgemäß' (Aoyama and Asamoto, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992; reviewed in Christ and Ordahl, 1995). Our experiments show that moreover external dorsalising and ventralising signals are strictly required for the establishment of all three somitic derivatives, dermomyotome, myotome and sclerotome. The paraxial mesoderm may therefore represent an entirely naïve tissue. Indeed, when contact to the axial as well as the lateral structures is blocked, or when segmental plates are grafted between the contralateral paraxial mesoderm after neural tube/notochord ablation, none of the mesodermal markers is expressed, as long as the operated field is shielded from ectoderm (S. Dietrich, F. Schubert, S. C. Chapman and A. Lumsden, unpublished observations). The sclerotomal and myotomal programs are not rescued by mature somites grafted into the position of the neural tube/notochord, in agreement with the in vitro experiments of Fan and Tessier-Lavigne (1994). Thus all the inductive events patterning the paraxial mesoderm are extrinsic, appositional and instructive.

Paraxial mesoderm acquires competence to respond to the external signals

While the fate of somitic cells is determined extrinsically, the paraxial mesoderm intrinsically controls its competence to respond: activation of *MyoD* and *Pax1* expression was never found before somitogenesis, even when the immature region of the segmental plate was exposed to mature axial signals. However, in mouse segmentation mutants, the expression of these genes follows the same time course as in wild-type animals (S. Dietrich, P. Tremblay, A. Faisst, B. Kammandel, F.

Schubert, A. Lumsden and P. Gruss, unpublished observations). This suggests that a timing mechanism intrinsic to the paraxial mesoderm controls its responsiveness to external cues, independent from segmentation.

Factors for somite dorsalisation and ventralisation

Our study shows that surface ectoderm, neural tube, floor plate and notochord account for the dorsoventral patterning of the paraxial mesoderm, providing redundant signals that act in an antagonistic as well as in a synergistic manner. What candidate dorsalising signals are produced by the surface ectoderm and the dorsal neural tube, and what ventralising signals are provided by the floor plate and the notochord?

The ventralising function of notochord and floor plate seems to be carried out in part by the signalling molecule *Sonic hedgehog* (SHH). It is appropriately expressed in both structures in all vertebrates. In vivo and in vitro, sclerotomal/adaxial and to lesser extent myotomal/lateral markers are activated upon ectopic expression of *Shh*, of *Indian hedgehog* (*Ihh*; not active in the ventral midline), or of a dominant negative form of the general *hh* antagonist cAMP-dependent *Protein kinase A* (*PKA*), while a constitutively active form of *PKA* suppresses *Shh*-inducible structures (Johnson et al., 1994; Fan et al., 1995; Concordet et al., 1996; Hammerschmidt et al., 1996; Weinberg et al., 1996). In addition, the putative *hh* receptor *patched* is expressed in the sclerotome and adaxial muscle pioneers of zebrafish embryos, and in the sclerotome of mouse and chick embryos in a gradient that is highest close to the notochord (Concordet et al., 1996; Goodrich et al., 1996; Marigo and Tabin, 1996). Similarly, the zinc finger transcription factors *Gli-2* and *Gli-3*, mouse homologues of the *Drosophila hh* target *cubitus interruptus*, are expressed in the sclerotome, suggesting that a *Shh*-dependent signalling mechanism could ventralise the paraxial mesoderm (Mo et al., 1997). However, in *Shh* knock-out mice, *Pax1* still is weakly expressed, *Myf5* at reduced levels and *MyoD* in wild-type fashion, and ribs and epaxial musculature develop (Chiang et al., 1996). In addition, in the zebrafish embryo, the formation of the adaxial muscle pioneers requires besides SHH another HH molecule, *Echidna hedgehog* (EHH), which is released from the notochord and acts primarily on the ventral myotome (Currie and Ingham, 1996). This suggests that factors other than *Shh* also contribute to the ventralisation of the paraxial mesoderm.

Candidates for the dorsalisation of the paraxial mesoderm exerted by surface ectoderm and neural tube are members of the *TGFβ* superfamily: for example, the bone morphogenetic proteins BMP4 and BMP7 are capable of dorsalising the neural tube (Liem et al., 1995). However, at the stages critical for somite dorsalisation, weak *Bmp7* expression is restricted to the dorsal neural tube, and *Bmp4* is expressed in the roof plate and its overlying ectoderm. In addition, BMP4 has been shown to mimic the myotome-repressing function of the lateral plate mesoderm (Pourquié et al., 1996). When we inverted the neural tube, the newly induced dermomyotomal lip always pointed away from the neural tube. Thus, while both BMP molecules may be involved in setting the dorsal border of the myotome and the formation of the dermomyotomal lips, they may not be sufficient for the dorsalisation of the paraxial mesoderm and the induction of the myotome.

In contrast to BMP molecules, members of the *wingless* (*wg*) family of signalling molecules seem more appropriately displayed in the embryo to account for the dorsalising and myotome-inducing function of neural tube and ectoderm. In the mouse and chick, *Wnt1*, *Wnt3* and *Wnt3A* are expressed in the roof plate of the neural tube, and *Wnt4* in the dorsal neural tube similar to *Pax3*, while general ectodermal expression has been reported for *Wnt3*, *Wnt4*, *Wnt6* and *Wnt7B* (Parr et al., 1993; Hollyday et al., 1995). In vitro, a dermomyotome-inducing function has been ascribed to *Wnt1* (Maroto et al., 1997). Similarly, a myotome-inducing capacity has been demonstrated for *Wnt1*, *Wnt3* and *Wnt4*, in particular in concert with *Shh* (Münsterberg et al., 1995; Stern et al., 1995), and, in *Drosophila*, *wg* is able to induce the *MyoD* homologue *nautilus* (Ranganayakulu et al., 1996). Thus the positive role of *wg/Wnt* genes for muscle development seems to be conserved during evolution. Interestingly, in the mouse *Wnt4* is additionally expressed in the floor plate, while its *Xenopus* counterpart lacks the dorsal expression domain (reviewed in Moon, 1993). Whether these differences account for the size difference of sclerotome and myotome in amniotes and anamniotes, is unclear.

Model for the dorsoventral patterning of the paraxial mesoderm

In summary, we would like to propose the following model. The paraxial mesoderm is a naïve tissue, which is dorsalised by redundant signals from surface ectoderm and dorsal neural tube. BMP4 or BMP7 may initiate this dorsalisation and, later in development, determine the dorsomedial and dorsolateral border of the myotome. However, the prolonged action of the dorsalising structures crucial for both dermomyotome and myotome development may be instigated primarily by members of the *Wnt* gene family. By contrast, redundant signals from notochord and floor plate ventralise the paraxial mesoderm. Candidate ventralising factors are SHH in concert with other HH family members or with the products of the

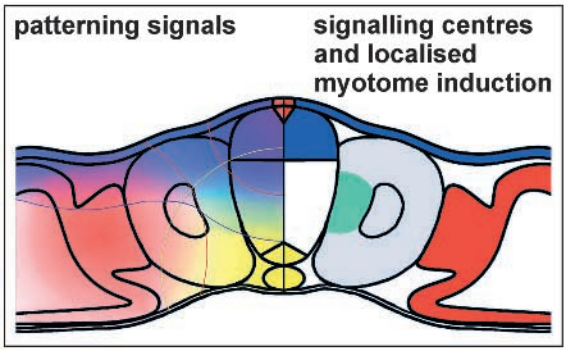

Fig. 5. Model for the localised induction of the myotome. The left side of the scheme shows the possible graded distribution of the signals patterning the somite, the right side shows the position of the signalling structures and the resulting localised induction of the myotome (green). The lateralising signals (BMP4) are shown in red, the dorsalising signals, possibly WNT signalling molecules, are shown in blue, the ventralising signals, possibly SHH in combination with other HH or WNT molecules, are displayed in yellow. The range of the dorsalising, ventralising and lateralising signals is indicated by a blue, yellow and red line, respectively. Beyond these lines, the signals are antagonised and their effects repressed. Where BMP4 is absent, and dorsalising and ventralising signals overlap, *MyoD* (chick) or *Myf5* (mouse) is induced in the somite epithelium.

ventrally expressed *Wnt* genes. The response of the paraxial mesoderm to the dorsalising and ventralising factors depends on their relative levels: close to the signalling source, they antagonise each other, thereby allowing the development of either sclerotome or dermomyotome. At some distance they co-operate, thereby inducing the myotome (Fig. 5).

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