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Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos

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SUMMARY

Microsurgical, tissue grafting and in situ hybridization techniques have been used to investigate the role of the neural tube and notochord in the control of the myogenic bHLH genes, *QmyoD*, *Qmyf5*, *Qmyogenin* and the cardiac *α-actin* gene, during somite formation in stage 12 quail embryos. Our results reveal that signals from the axial neural tube/notochord complex control both the activation and the maintenance of expression of *QmyoD* and *Qmyf5* in myotomal progenitor cells during the period immediately following somite formation and prior to myotome differentiation. *QmyoD* and *Qmyf5* expression becomes independent of axial signals during myotome differentiation when somites activate expression of *Qmyogenin* and *α-actin*. Ablation studies reveal that the notochord controls *QmyoD* activation and the initiation of the transcriptional cascade of myogenic bHLH genes as epithelial somites condense from segmental plate mesoderm. The dorsal medial neural tube then contributes to the maintenance of myogenic bHLH gene expression in newly formed somites. Notochord grafts can activate ectopic *QmyoD* expression during somite formation, establishing that the notochord is a necessary and sufficient source of diffusible signals to initiate *QmyoD* expression. Myogenic bHLH gene expression is localized to dorsal medial cells of the somite by inhibitory signals produced by the lateral plate and ventral neural tube. Signaling models for the activation and maintenance of myogenic gene expression and the determination of myotomal muscle in somites are discussed.

Key words: notochord, bHLH, somite, myogenesis, quail, neural tube, *myf5*, *myoD*

INTRODUCTION

The myoD family of transcription factors (myoD, myf5, myogenin and MRF4) are dominant regulators of skeletal myogenesis (Weintraub et al., 1990; Emerson, 1990; Olson, 1991). Gene knockout studies in mice establish that *myoD* and *myf5* have partially redundant functions in the upstream processes of myogenic determination in embryos, whereas *myogenin* and *MRF4* control downstream processes in myogenic cell differentiation (Rudnicki et al., 1993; Weintraub, 1993; Emerson, 1993a). Consistent with their regulatory functions in early myogenesis, vertebrate myogenic bHLH genes are activated in somites, which are the source of progenitor cells for skeletal muscle differentiation (for review, see Christ et al., 1986; Wachtler and Christ, 1992). Activation of the myogenic regulatory genes occurs immediately following somite formation and well before myotomal muscle differentiation (Sassoon et al., 1989; Charles de la Brousse and Emerson, 1990; Ott et al., 1991; Pownall and Emerson, 1992; Emerson, 1993b). In the stage 12 quail embryo, *QmyoD* and *Qmyf5* are expressed in the somites along the anterior to posterior axis of the embryo, including the posterior, newly formed somites, but not the in segmental plate mesoderm. *Qmyogenin* is expressed in more anterior somites which then activate expression of contractile protein genes and undergo myotome differentiation. Thus, the early regulatory events of somite determination involve a transcriptional cascade of myogenic bHLH gene expression, leading to the coordinated activation of contractile protein genes during myotome differentiation (Pownall and Emerson, 1992).

The expression of *myoD* genes is localized to somite cells positioned immediately adjacent to the neural tube, suggesting a role of axial signals in the control of *myoD* gene activation and determination of the myotomal cell lineage (Pownall and Emerson, 1992). Lineage marking and chick-quail chimera studies have shown that the medial and lateral halves of early somites arise from distinct regions of the early gastrula (Selleck and Stern, 1991) and give rise to distinct muscle lineages: the medial cells of the early somite differentiate as myotomal muscle in situ, whereas the lateral somite cells migrate to form the limb musculature (Ordahl and LeDouarin, 1992). The ventral somite cells differentiate into the sclerotome, which forms the vertebral cartilage and ribs. Dorsal lateral somite cells give rise to the dermatome, which forms the dermis. The differentiation fates of cells in the newly formed somite, however, are plastic and can be altered, as when somites are reversed in their medial-lateral or their

dorsal-ventral orientations relative to the axis (Ordahl and LeDouarin, 1992; Christ et al., 1992). These experiments suggest that the newly formed somite responds to positional axial signals that determine their myogenic, sclerotome and dermatome differentiation fates.

Grafting and surgical ablation studies have provided evidence for the existence of neural tube/notochord signals involved in somite differentiation in chick embryos. Dorsal grafts of notochord and floor plate promote ectopic sclerotome formation and inhibit myotome formation in chick somites (Pourquie et al., 1993; Brand-Saberi et al., 1993; Goulding et al., 1994). The notochord also inhibits the expression of *pax3*, a marker of the dermomyotome (Goulding et al., 1993, 1994) and activates the expression of *pax1* (Brand-Saberi et al., 1993), indicating ventral floor plate and notochord control sclerotome differentiation. Sonic hedgehog (Shh), which is expressed by notochord and floor plate, has been implicated as a signal in the molecular pathway leading to *pax1* expression and sclerotome differentiation and as an inhibitor of *pax3* and dermomyotome formation (Fan and Tessier-Lavigne, 1994; Fan et al., 1995). Signals from the notochord and neural tube have also been shown to regulate myotome differentiation in ovo (Rong et al., 1992; Christ et al., 1992) and in somite cultures (Packard and Jacobson, 1976; Vivarelli and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Buffinger and Stockdale, 1994, 1995; Stern and Hauschka, 1995); there also is evidence that axial signals control myoD expression in somite culture (Munsterberg and Lassar, 1995). However, the in vivo sources and molecular nature of signals required for the determination and differentiation of the myotomal muscle lineage remain to be established.

We have utilized surgical ablation and tissue grafting techniques to investigate the role of axial signals in the regulation of myogenic bHLH genes and muscle differentiation genes in somites of quail embryos. These experiments were undertaken specifically to examine the temporal and spatial control mechanisms that regulate the transcriptional cascade of myogenic bHLH regulatory genes that lead to the determination of the myogenic lineage in somites of the developing embryo (Pownall and Emerson, 1992; Rudnicki et al., 1993). We have also utilized the expression of myogenic bHLH genes as molecular markers to localize the sources of axial signals for skeletal muscle determination and differentiation. Our findings reveal that the notochord provides the essential signals to initiate the cascade of *QmyoD* and *Qmyf5* activation that determines somite cells to the muscle lineage. Subsequently, neural tube signals contribute to the maintenance of high level *QmyoD* and *Qmyf5* expression in myotomal progenitors until they differentiate, at which time the expression of the myogenic bHLH genes becomes independent of axial signals.

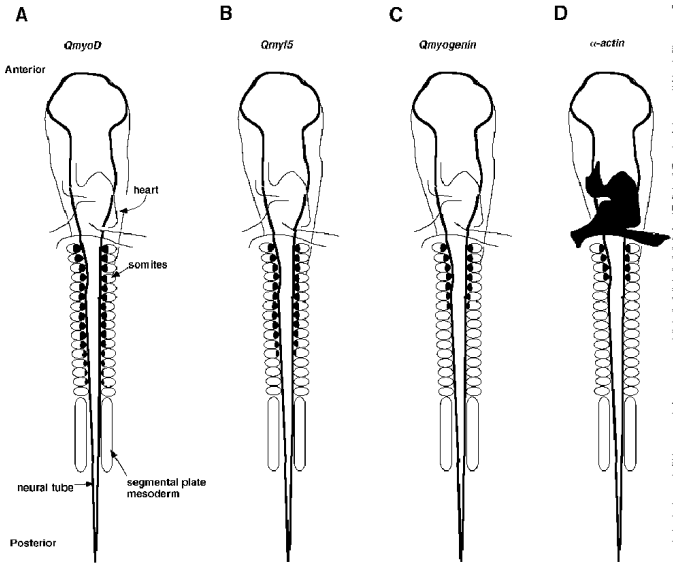


Fig. 1. Summary of myogenic bHLH and *cardiac α-actin* gene expression in stage 12 quail embryos. (A) *QmyoD*, (B) *Qmyf5*, (C) *Qmyogenin*, and (D) the muscle differentiation gene, *cardiac α-actin* are activated along the anterior to posterior axis of somite formation, as determined by in situ hybridization using anti-sense DIG probes. The myogenic regulatory genes are expressed in the dorsal medial somite cells that are progenitors of myotomal muscle. *cardiac α-actin* is expressed in the 4 anterior-most somites of myotome muscle cells, as well as in the heart muscle. Sites of gene expression in somites and heart are shown in black.

MATERIALS AND METHODS

Embryo preparation

Quail embryos were collected after 45 hours of egg incubation (Hamburger and Hamilton (1951), stage 12). Operated embryos had an average of 16 somites, with arange of 14-18 somites. Embryos were removed from the yolk using paper rings (Whatman no. 1) to encircle the blastodisc. Embryos were rinsed in PBS to free the yolk, and microsurgical procedures were done on sylgard plates with electrolitically sharpened tungsten knives. Surgical procedures performed in this study are summarized diagrammatically along side each figure of the resulting in situ hybridization. After surgical manipulation, the embryos were transferred to a pool of thin albumin on a plate of 2% agarose in PBS and incubated at 39°C in a humidified incubator. Neural tube and notochord tissues were isolated surgically from the posterior region of somite formation in donor embryos and grafted at varied sites in host embryos. Cell aggregates were prepared by scraping tissue culture cells from plates, centrifuging cells at 1000 ***g*** for 5 minutes in 15 ml medium, resuspending cells in 1 ml medium, then re-centrifuging cells at 2000 ***g*** for 2 minutes. In some experiments, tissues to be grafted were exposed to rhodamine to distinguish donor grafts from responding host tissue. After culture, the embryos were fixed overnight in 4% paraformaldehyde (pH 7.4) in CMF saline at 4°C in preparation for the whole-mount digoxigenin (DIG) in situ hybridization protocol.

Probe preparation

cRNA probes were prepared from Bluescript KS^+ cDNA constructs that have been described previously (Charles de la Brousse and Emerson, 1990; Pownall and Emerson, 1992). Briefly, the *QmyoD* cDNA (cC509) and *Qmyogenin* cDNA, (cC527) were linearized with *Hin*dIII, while the *Qmyf5* cDNA (cC528) was linearized with *Xho*I. T7 RNA polymerase was used to transcribe cRNA probes. The *cardiac α-actin* (cC156) cDNA was linearized with *Bam*HI and a cRNA probe was transcribed using T3 RNA polymerase. The digoxigenin UTP analogue was incorporated into the cRNA transcripts, which were hydrolyzed to approximately 150-200 bp in bicarbonate buffer at 60°C, as described in the Boehringer Mannheim protocols.

In situ hybridization analysis

Fixed embryos were washed 3 times for 5 minutes with phosphate-buffered saline (PBS) plus 0.3% Triton X-100 (PBT.3) and treated with proteinase K at 37°C for 5-7 minutes. The embryos were then rinsed 3 times for 5 minutes in PBT.3 and prehybridized at 63°C for

16-24 hours in hybridization buffer without probe (50% formamide, 4× SSC, 1× Denhardts, 0.05% dextran sulfate, 50 mg/ml single-stranded DNA, 25 mg/ml tRNA). Probe (25 ng) was added to fresh hybridization buffer, and embryos were incubated for 24-28 hours at 63°C. Embryos were washed twice for 24-48 hours in fresh hybridization buffer without probe at 63ºC. Embryos were then washed at room temperature for 20 minutes each in 20% PBT.3/80% hyb buffer, 40% PBT.3/60% hyb buffer, 60% PBT.3/40% hyb buffer, 80% PBT.3/20% hyb buffer, and twice in PBT.3, for 45 minutes each. The embryos were treated with 2% Boehringer Mannheim blocking solution for an hour and then embryos were incubated in conjugated anti-DIG antibody diluted 1:1000 in 2% Boehringer Mannheim blocking solution for 2.5 hours at room temperature. The embryos were rinsed three times in PBT.3 and then washed twice for 20 minutes each in PBT.5, overnight in PBT.5, and twice in Boehringer Mannheim buffer 3 in 0.3% Triton X-100 for 15 minutes each. The color reaction proceeded overnight at room temperature, as determined empirically, and was stopped by the addition of TE (10 mm Tris-HCl, 1 mM EDTA, pH 8.0). Embryos were stored in 70% ethanol at 4°C.

Whole-mount DIG-labeled embryos were photographed in 80% glycerol/20% PBS, using a Leica WILD M420 photomacroscope. Embryos were then embedded in 4% low melt agarose in TE. 100 μm sections of DIG-labeled embryos were made using the Vibratome 2000. Sections embedded in 4% agarose/TE were mounted in Aquamount and then photographed using Leica DMRBE upright microscope at 400× magnification with DIC optics.

RESULTS

Axial signals control the activation and maintenance of myogenic bHLH genes during somite formation

The myogenic bHLH genes are activated in myotomal progenitor cells during the anterior-posterior progression of somite formation (Sassoon et al., 1989; Charles de la Brousse and Emerson, 1990; Bober et al., 1991; Hinterberger et al., 1991; Ott et al., 1991; Pownall and Emerson, 1992). In this current study, whole-mount DIG in situ hybridization methods (Coutinho et al., 1992) were utilized to assay myogenic bHLH gene regulation during somite formation in 14-18 somite (stage 12) quail embryos following surgical manipulation. Whole-mount in situ analyses of gene expression largely confirmed our earlier findings (see Fig. 1 for summary); Borycki, Strunk, Savary and Emerson, unpublished data. *QmyoD* is the first gene in the transcriptional cascade of the myogenic bHLH genes to be activated during somite formation. During the period between the 14-18 somite stages *QmyoD* becomes expressed in all but the most posterior somites formed along the anterior to posterior axis and is undetected in the segmental plate. Expression is restricted to a small cluster of dorsal medial somite cells that are the progenitors of myotomal muscle (Fig. 2A, B) and expression

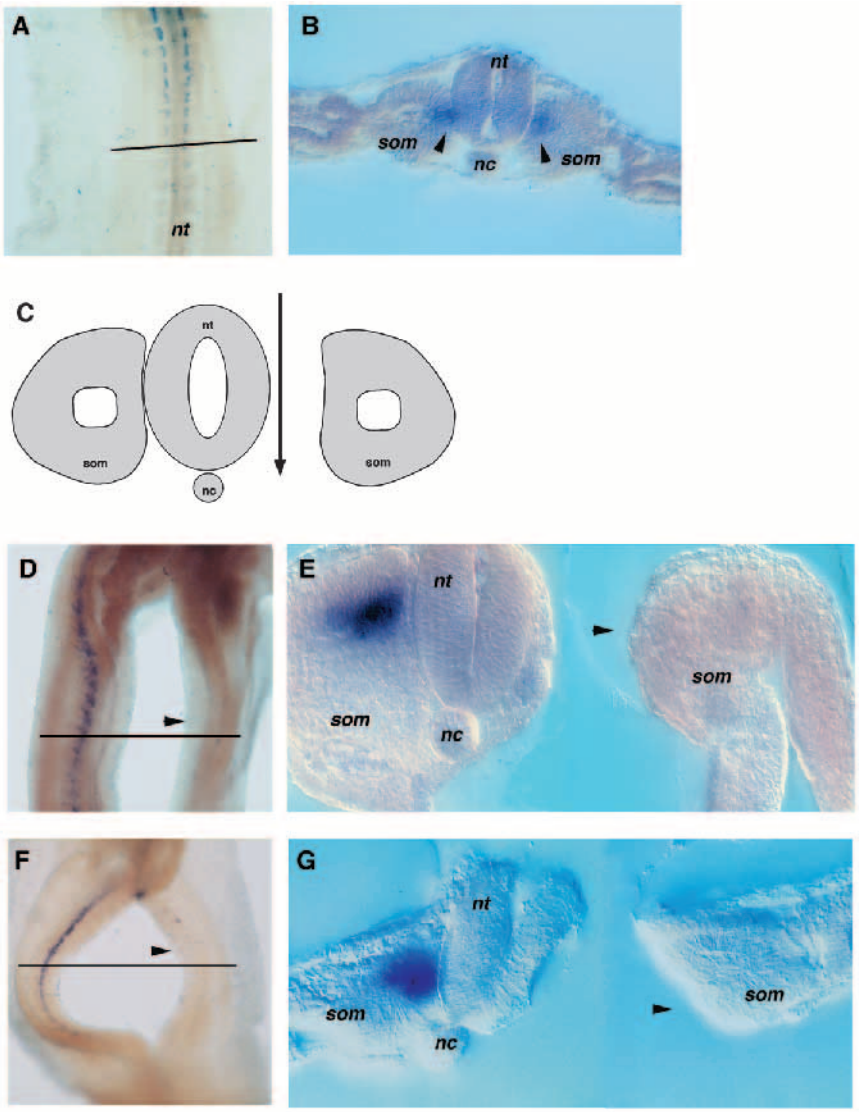


Fig. 2. *QmyoD* expression is extinguished in somites separated from neural tube/notochord. (A) Whole mount of a control stage 12 quail embryo hybridized in situ with DIG labeled *QmyoD* anti-sense probe. *QmyoD* expression is localized to the medial somite and increases along the posterior to anterior axis, detected first at low levels in somite II. (B) Transverse section of embryo in A, through somite V, anterior to segmental plate. *QmyoD* expression (arrowheads) is localized to a restricted group of dorsal medial cells of the epithelial somite. (C) Diagram of neural tube/notochord – somite separations. Somites and segmental plate mesoderm were separated from the neural tube/notochord complex by a dorsal/ventral incision traversing all three germ layers. (D) QmyoD DIG in situ hybridization 16 hours after surgical separation of somites and segmental plate from the neural tube/notochord complex. (E) Transverse section through embryo in D, showing loss of *QmyoD* expression in segmented somites (arrowhead). (F) QmyoD DIG in situ hybridization 2.5 hours after surgical separation of somites from the neural tube/notochord complex. Separated somites rapidly lose *QmyoD* expression (arrowhead), whereas somites on the unoperated side maintain expression. (G) Transverse section of embryo in F, showing loss of *QmyoD* expression in the somites separated from the neural tube/notochord complex (arrowhead). Whole-mount embryos are viewed dorsally and are oriented with anterior at the top. Bars in A, D and F indicate level of transverse sections of embryos shown in B, E and G respectively. nc, notochord; nt, neural tube; som, somite.

levels increase along the posterior to anterior axis (Fig. 2A). *Qmyf5* expression first occurs 2-3 somites anterior to the posterior border of *QmyoD* expression. *Qmyogenin* is detected in the 4-8 anterior-most somites; and *α-actin* expression is detected in the 4 most anterior somites, but is not detected in embryos with less than 16 somites. The anterior to posterior progression of myogenic bHLH activation and myotome differentiation occurs rapidly in embryos between the 14-18 somite stages (Borycki, Strunk, Savary and Emerson, unpublished data).

Surgical experiments were performed on stage 12 embryos to examine whether contact between somites and neural tube/notochord is required for the determination of somite cells to the myogenic lineage. The expression of *QmyoD* and the other myogenic bHLH genes during somite formation provides early molecular markers of undifferentiated cells in the muscle lineage. In situ hybridization was used to assay *QmyoD* expression in experiments where somites and segmental plate on the right side of 14-18 somite embryos were surgically separated from the neural tube/notochord (see Fig. 2C). Somites on the left side served as unoperated, internal controls. Embryos were cultured on a pool of thin albumin, during which time new somites continue to form from the segmental plate at a rate of one somite pair per 2.5 hours. *QmyoD* expression was undetected in all but the 2 (±2) most anterior somites when separated from axial contact during a 16-hour culture period (Fig. 2D,E). *QmyoD* was not activated in the 6 somite pairs that form from segmental plate during a 16-hour culture following neural tube/notochord separation, and furthermore, somites that had already activated *QmyoD* expression at the time of the operation failed to maintain expression in the absence of neural tube/notochord. In contrast, somites formed on the contralateral, unoperated side both activated and maintained normal expression of *QmyoD*. *QmyoD* expression was very rapidly and completely extinguished in the posterior, newly formed somites that had already initiated expression at the time of surgery, and extinction occurred within 2.5 hours following surgical separation (Fig. 2F,G). The rapid loss of expression indicates that *QmyoD* transcripts are very unstable and turn over quickly in the absence of neural tube/notochord signals. As discussed later (see Fig. 3A), the anterior-most differentiated somites maintain *QmyoD* expression when surgically separated from the neural tube/notochord, indicating that in mature somites *QmyoD* expression has been stabilized.

It was unexpected to see extinction of *QmyoD* expression in separated somites that had previously expressed *QmyoD*, given the autoregulation of *myoD* expression demonstrated in tissue culture cells (Thayer et al., 1989). We know, however, that this is not due to cell death or damage during the operation or poor viability of separated somite cells because of several observations. Firstly, the anterior-most somites do maintain expression of *QmyoD* when separated from the axial tissues (Fig. 3A). To ensure that somite damage did not affect gene expression, and to examine whether the structure of the somite was required for proper signaling of *QmyoD* expression, a cut was made through somites leaving the medial part of the somites attached to the axial tissues (see Fig. 3B). When the entire ventral and lateral region of segmental plate or newly formed somites was ablated, we found that the narrow band of somite cells adherent to the neural tube activated and maintained expression of *QmyoD* (Fig. 3C,D). We also found that expression of *Qmyf5* and *Qmyogenin*, and *α-actin*, were expressed in somite cell remnants associated with the neural tube (data not shown). This demonstrates that the myogenic bHLHs can be expressed in somite cells associated with the neural tube/notochord in spite of the damage done to the somites as a whole. Furthermore, the ventral and lateral somite does not contribute to the signaling processes required to activate and maintain myogenic bHLH gene expression and to initiate differentiation. Another observation was that *QmyoD* extinction is observed in separated somites of embryos that are cultured either on thin albumin or on a complete muscle cell tissue culture medium containing 15% horse serum and 10% embryo extract (Konigsberg, 1963), indicating that loss of gene expression was not a response of somites to nutrient deprivation (data not shown). We also found that somites separated from axial tissues responded (6/10 cases) to medial neural tube/notochord grafts by activation of *QmyoD* expression (Fig. 3F), indicating that the surgical separation procedures do not damage the responsiveness of somites to axial signals. Finally, somites continue to form from segmental plate in the absence of contact with neural tube/notochord, and the epithelial integrity of separated somites is maintained for at least 16 hours in the absence of axial contact (Fig. 3G).

We also examined the expression of *Qmyf5*, *Qmyogenin* and *α-actin* in separated somites (Fig. 4A; see also Table 1). The expression of *Qmyf5* was extinguished in all but the 6 (±2) anterior-most of the 12 separated somites that had activated expression at the time of the surgery (Fig. 4B,C). The posterior boundaries of stable *Qmyogenin* and *α-actin* also include the 6 anterior-most separated somites. Since on average, 8 anterior somites express *Qmyogenin* at the time of surgery, this expression is stably maintained in almost all *Qmyogenin*-expressing, separated somites, within experimental error, given the rapid changes in expression in embryos at these stages (Fig. 4D,E). Similarly, since *α-actin* expression is restricted to the 4 anterior-most somites, all separated somites expressing *α-actin* at the time of surgery stably maintain high levels of expression (Fig. 4F,G). However, somites fail to activate new *Qmyogenin* expression in the absence of axial contact, except perhaps at very low levels. The results of these somite separation and gene expression studies, therefore, establish that axial signals regulate both the activation and the maintenance of *QmyoD* and *Qmyf5*, which are the upstream regulators of myogenic determination (Rudnicki et al., 1993). In contrast, maintenance of *Qmyogenin* and *α-actin* expression is independent of axial signals, which likely reflects the fact that the somites that express these genes at stage 12 have initiated myotome differentiation. However, the anterior to posterior progression of myotome differentiation is blocked in separated somites because expression of the upstream regulators, *QmyoD* and *Qmyf5*, is extinguished in the absence of the neural tube and notochord.

The results of these somite separation studies, as summarized in Fig. 5 and Table 1, identify three phases in the regulation of *QmyoD* expression: activation of expression during somite formation; maintenance of expression during somite maturation; autonomous expression during myotomal differentiation. Fig. 5 illustrates a dynamic progression whereby myogenic fate is stabilized during somitogenesis. Neural tube/notochord contact is required for both the activation and

Table 1. Gene expression in somites separated from the neural tube/notochord complex

Gene	*n*	Somites/ embryo	Somites showing gene expression: Unoperated side	Somites showing gene expression: Operated side
QmyoD	10	22	III-XXII	XX-XXII
Qmyf5	9	23	V-XXIII	XVII-XXIII
Qmyogenin	11	23	X-XXIII	XVII-XXII
Cardiac α-actin	10	22	VII-XXII	XVI-XXII

n, number of embryos examined in each set.

Roman numerals denote somite number along the anterior to posterior axis of the embryo, with I being the most posterior somite (Ordahl, 1993). Anterior to posterior boundaries of gene expression in somites of individual embryos varied ±2 somites from the averages.

See Fig. 4 legend and text for experimental details.

Table 2. *QmyoD* expression in somites after various experimental procedures

Tissue	*QmyoD* expression: –	+/–	+	?	Total
A. Neural tube separations					
Somites I-IX (16 hour)	5	5	3	1	14
Somites I-IX (2.5 hour)	1	0	7	0	8
Segmental plate	14	1	0	4	19
See Fig. 6 legend and text for experimental details.					
B. Notochord ablations					
Somites I-IV	5	3	10	3	21
Segmental plate	26	2	1	4	33
See Fig. 7 legend and text for experimental details.					
C. Notochord and control cell grafts					
Notochord medial graft	4	0	3	0	7
Notochord lateral graft	2	0	4	1	7
MV7-3T3 medial graft	9	0	0	0	9
$10T\frac{1}{2}$ lateral graft	10	0	0	0	10

See Fig. 8 legend and text for experimental details.

As controls, aggregates of MV7-3T3 and $10T\frac{1}{2}$ cells were grafted laterally and medially to the segmental plate.

QmyoD expression was assayed in whole mount and transverse sections of DIG labeled embryos. (–) no detectable expression; (+/–) low level expression; (+) high level of expression; (?) uninterpretable expression. (*n*) number of embryos examined.

maintenance of *QmyoD* expression in newly formed somites, until the time of myotome differentiation, when expression becomes autonomous.

Ventral neural tube/notochord controls activation and dorsal medial neural tube controls maintenance of *QmyoD* and *Qmyf5* expression

In order to characterize the in vivo sources of signals required for activation and maintenance of myogenic bHLH expression, the ventral neural tube and notochord were separated from the segmental plate and somites by an oblique surgical incision through the dorsal aspect of the neural tube (see Fig. 6A). This ventral ablation operation leaves somites and segmental plate on one side of the axis in contact with the dorsal and lateral neural tube, and somites and segmental plate on the other side in contact with the notochord, and the ventral, lateral and dorsal neural tube. We found that *QmyoD* is not activated in somites that form from segmental plate that is associated only with lateral and dorsal neural tube, whereas activation was normal in the contralateral somites that formed in contact with the notochord and the ventral, lateral and dorsal neural tube (Fig. 6B,C; Table 2A). Thus, the process of *QmyoD* activation in newly forming somites requires ventral signals.

In contrast to our finding that ventral neural tube and notochord are required for activation of *QmyoD* during somite formation, we found that already-formed somites in contact with only the lateral and dorsal neural tube (see Fig. 6D) can maintain high level expression for at least the first 2.5 hours following surgery (Table 2A; Fig. 6E,F), although after 16 hours, *QmyoD* expression diminishes significantly (Fig. 6G,H; Table 2A). In a few cases (3/14), somites maintain high level expression of *QmyoD*, perhaps because the surgery did not ablate all ventral tissue (see below). *α-actin* expression is also activated, albeit at much reduced levels, in somites when left in contact with the lateral and dorsal neural tube (Fig. 6I,J). These data, therefore, indicate that the ventral neural tube and notochord control the activation of *QmyoD* during somite formation, but the neural tube alone can maintain *QmyoD* expression.

The notochord provides necessary and sufficient signals for the activation of *QmyoD* expression

Notochord ablation experiments were performed to investigate the role of the notochord as a specific tissue source of ventral signals for *QmyoD* activation. Segments of notochord (200-400 μm) were ablated from stage 12 embryos, either in the region of segmental plate (see Fig. 7A) or in the region of the 4 posterior-most somites (somites I-IV) (see Fig. 7C). We observed that somites located in the central region of the ablation which had formed from segmental plate in the absence of notochord failed to activate *QmyoD* (Table 2B; Fig. 7B). Therefore, the notochord is required for activation of *QmyoD* during somite formation. In these studies, we frequently observed that *QmyoD* was hyper-expressed in somites located adjacent to the cut notochord, just within the region of the notochord ablation. Transverse sections revealed that hyper-expressing somites show high levels of *QmyoD* transcripts ectopically in ventral somite cells as well as in the dorsal medial cells that normally express it (Fig. 7B1). We propose that these hyper-expressing somites adjacent to the notochord ablation are receiving abundant signals for activation from the intact notochord just anterior to the site of ablation, and also are escaping inhibitory signals, possibly from the floorplate, that normally repress ventral somite (i.e. future sclerotome) expression of *QmyoD*.

In contrast to the results of notochord ablation in the region of segmental plate, ablation of the notochord beneath posterior somites (see Fig. 7C) in a majority of cases (10/18) did not affect high level *QmyoD* expression, while expression was diminished in the other cases. Given the earlier result that dorsal and lateral neural tube alone are not sufficient to maintain high level *QmyoD* expression in somites, the loss of expression in these cases is most likely due to damage to the ventral neural tube during surgery (Fig. 7D, D1, D2; Table 2B). Therefore, at this point of somite determination, the ventral neural tube can provide signals to maintain *QmyoD* expression in somites in the absence of the notochord. These results

demonstrate that the ventral neural tube is a source of signals for maintenance of *QmyoD* expression in the posterior somites, but that the notochord is essential for *QmyoD* activation as somites condense from segmental plate mesoderm.

To examine whether the notochord alone is sufficient for the activation of *QmyoD* expression during somite formation, explants of notochord were removed from the segmental plate region of donor embryos and grafted onto the medial aspect of segmental plate that had been separated from the neural tube/notochord complex (see Fig. 8A). As shown earlier, somites forming from segmental plate do not activate *QmyoD* in the absence of axial tissues, however, *QmyoD* activation was observed in newly formed somites in response to medially located notochord grafts (Fig. 8B,C; Table 2C). In control experiments, aggregates of MV7-3T3 tissue culture cells implanted medially in separated segmental plate did not activate *QmyoD* (Table 2C). These results, therefore, demonstrate that notochord alone is sufficient for the activation of *QmyoD* during somite formation.

As in situ hybridization assays of *QmyoD* gene expression are not quantitative, we cannot conclude whether notochord grafts induce a quantitatively complete inductive response in somites. Furthermore, this experiment does not address the ability of the notochord to maintain *QmyoD* expression, since expression was analyzed soon after placement of the experimental graft. However, it is important to note that the responding somite cells expressing *QmyoD* in this experiment are immediately adjacent to the notochord graft (Fig. 8C), indicating that the posterior notochord produces inducers of *QmyoD*, and not inhibitors. Interestingly, we have also found that grafts of posterior, but not anterior notochord induce *QmyoD*, suggesting that anterior notochord either has lost inducing activity and/or produces myogenic inhibitors. Since we also show that the neural tube in the anterior region is sufficient for maintenance of *QmyoD* (Table 2A; Fig. 6), the anterior notochord may not be required to produce activating signals once somites have formed.

Somite rotation studies established that newly formed somites are not committed to myogenesis at the time of somite formation when *QmyoD* is activated medially, but rather, they

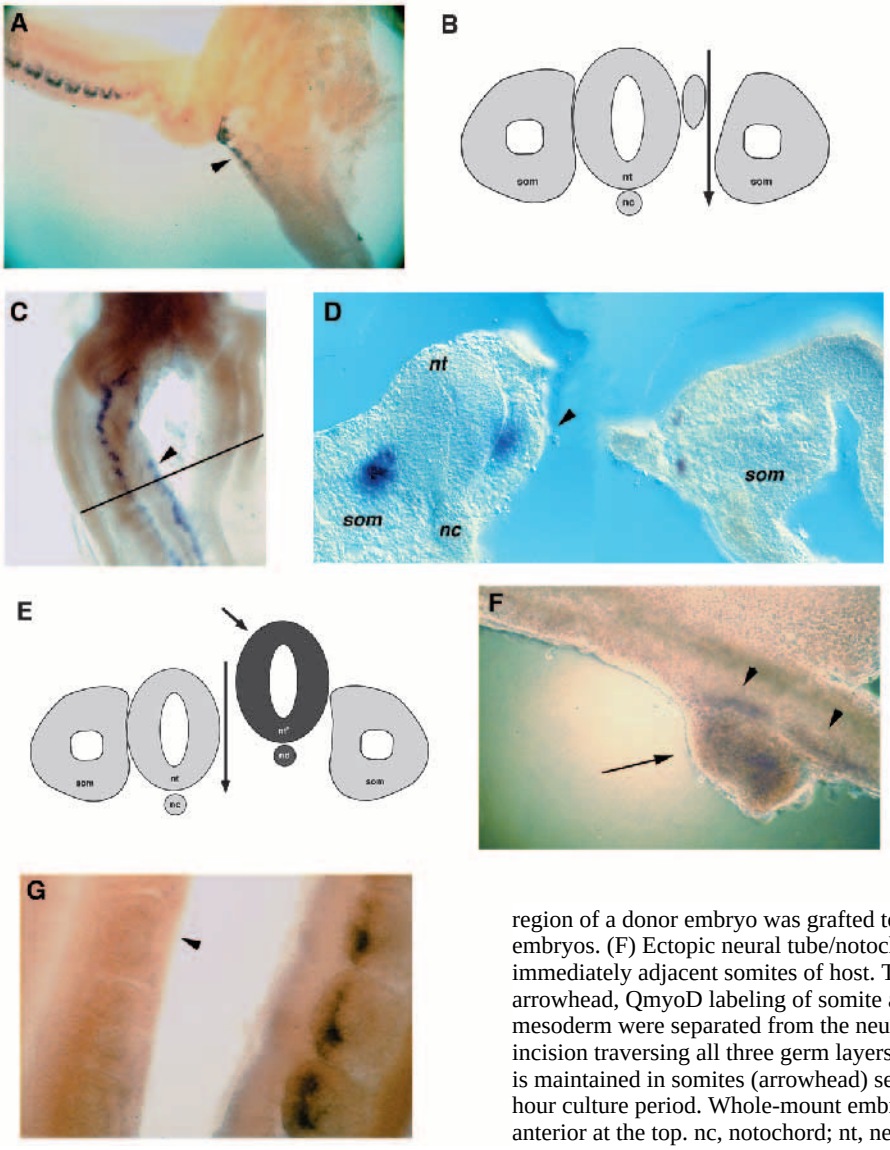


Fig. 3. Myogenic bHLH gene expression in surgically manipulated embryos. (A) Dorsal view of a quail embryo subject to surgical separation of somites from the neural tube/notochord along the entire anterior to posterior axis (see Fig. 2C), followed by culture for 16 hours. *QmyoD* expression is maintained only in the anterior-most four separated somites (arrowhead). (B) Diagram illustrating partial somite separations. Somites and segmental plate mesoderm were separated by a dorsal/ventral incision displaced slightly lateral to the neural tube to leave a residue of somitic cells associated with the neural tube/notochord. (C) QmyoD DIG in situ hybridization of an embryo after partial surgical separation of somites, 16 hours after surgery. (D) Transverse section (at level indicated by the bar) through the embryo in C, showing continued expression of *QmyoD* in residual somitic cells adherent to the neural tube/notochord (arrow). (E) Diagram of neural tube/notochord graft to separated segmental plate. Segmental plate was separated from neural tube/notochord by a dorsal/ventral incision, and neural tube/notochord from the same region of a donor embryo was grafted to a medial site on the segmented plate of host embryos. (F) Ectopic neural tube/notochord graft maintains *QmyoD* expression in two immediately adjacent somites of host. The arrow indicates the grafted tissue, and the arrowhead, QmyoD labeling of somite adjacent to graft. (G) Somites and segmental plate mesoderm were separated from the neural tube/notochord complex by a dorsal/ventral incision traversing all three germ layers. *Qmyf5* expression is lost, but epithelial integrity is maintained in somites (arrowhead) separated from the neural tube/notochord for the 16-hour culture period. Whole-mount embryos are viewed dorsally and are oriented with anterior at the top. nc, notochord; nt, neural tube; som, somite.

have the capacity to regulate their potential to form myotomal muscle, depending on the position of somite cells relative to the axial tissues (Ordahl and LeDouarin, 1992; Christ et al., 1992). Based on these observations, we grafted neural tube/notochord explants from donor embryos to the side of segmental plate mesoderm (that is, between the lateral plate and the segmental plate of host, stage 12 embryos), and we observed the ectopic activation of *QmyoD* in lateral somite cells (data not shown). We then performed a series of notochord grafts to determine whether the notochord alone was sufficient to signal ectopic *QmyoD* activation in lateral somite cells.

In the first of these experiments, notochord removed from the segmental plate region of donor embryos was grafted between segmental and lateral plate mesoderm (see Fig. 8D), and embryos were cultured for 16 hours to allow somites to form in the region of the graft. We found that *QmyoD* expression was activated ectopically throughout to the lateral extent of the dermamyotome and immediately adjacent to the notochord graft itself (Fig. 8E,F; Table 2C). Our findings are consistent with those of earlier studies, which show that notochord grafts placed lateral to newly forming somites do not initially disrupt the dermamyotome or inhibit *myoD*

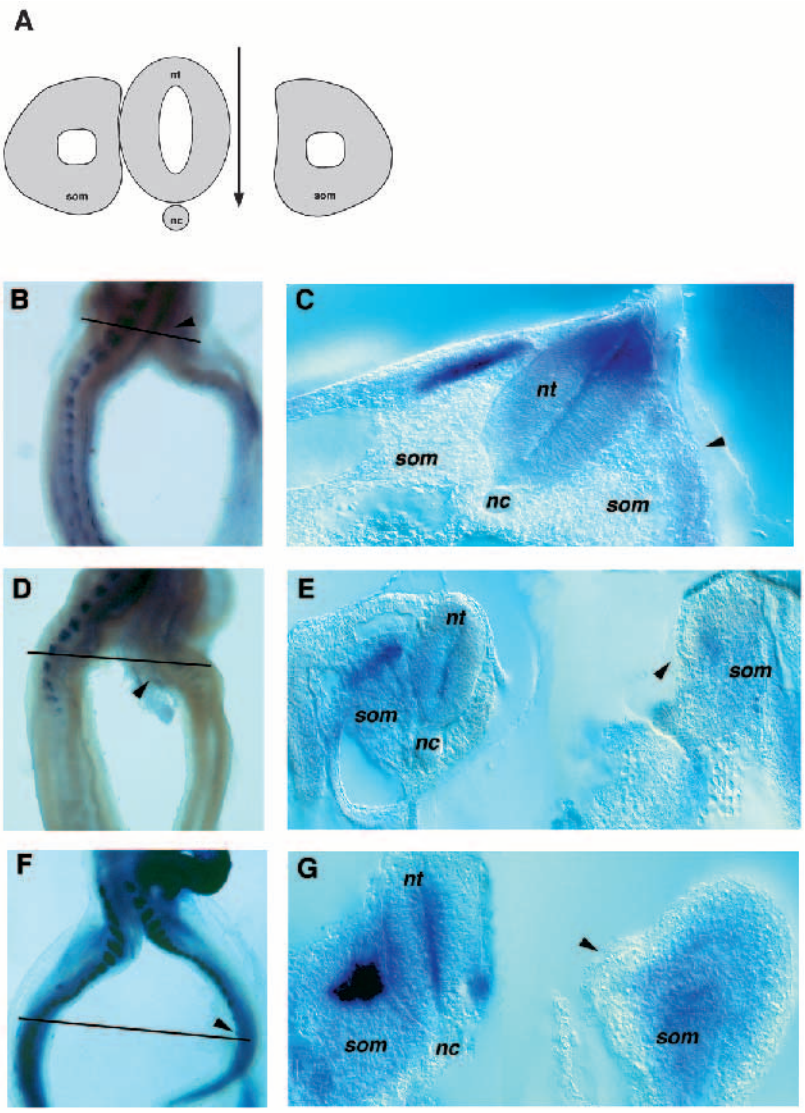


Fig. 4. *Qmyf5*, *Qmyogenin* and *cardiac α-actin* expression in embryos where somites and segmental plate mesoderm were separated from the neural tube/notochord complex by a dorsal/ventral incision traversing all three germ layers. Subsequent to the operation, embryos were cultured for 16 hours followed by fixation and whole-mount in situ hybridization. (A) Diagram of experimental procedure. Embryos hybridized with DIG probes *Qmyf5* (B,C), Q*myogenin* (D,E) and *cardiac α-actin* (F,G). Whole-mounts are shown in B, D, F and the transverse sections (as indicated by bars) of these embryos are shown in C, E and G, rerspectively. Note that the *Qmyf5* probe (B and C) hybridizes to dorsal neural tube (Tajbakhsh et al., 1994). Whole-mount embryos are viewed dorsally and oriented with the anterior to the top. nc, notochord; nt, neural tube; som, somite.

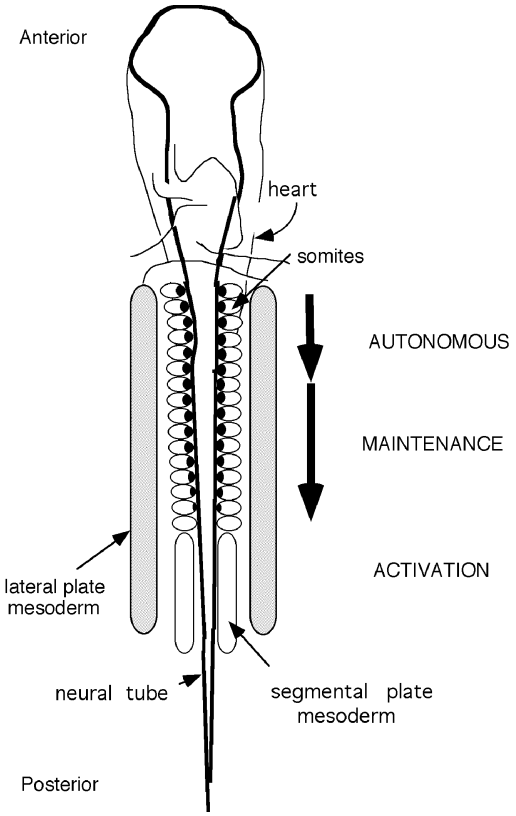


Fig. 5. Schematic representation of *QmyoD* gene regulation in somites of stage 12 quail embryos. Shaded arrows to the right depict the anterior to posterior wave of *QmyoD* activation and maintenance as somites form in stage 12 embryos. *QmyoD* activation occurs as somites form in an anterior-posterior progression and expression is maintained by neural tube/notochord signals, except in the anterior-most somites, which initiate myotome differentiation independent of axial contact. Activation of expression occurs as segmental plate epithelializes and also requires axial tissue contact. *Qmyf5* and *Qmyogenin* expression in somites respond to similar activation and maintenance controls provided by axial tissue interactions.

expression (Bober et al., 1994b). In contrast, medial notochord grafts can disrupt dermamyotome formation and stability (Goulding et al., 1994), although disruption of the dermamyotome is not always immediate (Pourquie et al., 1993). We have observed that medial grafts of posterior notochord have variable effects on dermamyotome formation over a 16-hour period following introduction of the graft into the region of newly forming somites. In some cases, dermamyotome architecture was disrupted immediately adjacent to the graft, but in other cases, dermamyotome structures were present adjacent to the grafted notochord (data not shown). These variations may be related to the precise spatial arrangement of the graft relative to the somite and neural tube and to induction of ectopic floor plate, which, if formed, may disrupt dermamyotome stability over time (Pourquie et al., 1993).

In addtional control experiments, we also show that aggregates of 10T½ tissue culture cells grafted laterally (see Fig. 8G; Table 2C) do not induce ectopic expression of *QmyoD* in the lateral dermamyotome (Fig. 8H,I; Table 2C). Therefore, the lateral activation of *QmyoD* by notochord is not likely the result of removal or interference with lateral plate inhibitory signals, which has been shown to exist, by surgical separation of lateral plate (see Fig. 8J). Such lateral plate separations lead to ectopic activation of *QmyoD* in lateral dermamyotome (Fig. 8K,L; Pourquie et al., 1995). These inhibitory signals likely function to restrict *QmyoD* expression to medial somite cells, which activate *QmyoD* in response to inductive signals from the notochord. However, we do not exclude the possibility that lateral notochord grafts also repress the expression of myogenic inhibitors produced by lateral plate cells. Taken together, however, the results of our medial and lateral notochord grafting studies together with our notochord ablation studies provide strong evidence that the notochord provides both necessary and sufficient signals for *QmyoD* activation during somite formation.

DISCUSSION

These experiments have investigated the role of the neural tube and notochord in regulating myogenic bHLH gene expression and myotome differentiation in the somites of developing quail embryos. Our results show that signals from the notochord and neural tube control the upstream regulatory processes required for both the activation and the maintenance of expression of *QmyoD* and *Qmyf5*, the myogenic bHLH genes that are essential for myogenic lineage determination (Rudnicki et al., 1993). The notochord provides the primary signals regulating the activation of the myogenic bHLH genes when somites form from segmental plate mesoderm. Signals from the neural tube then maintain high level expression of *QmyoD* and *Qmyf5* in newly formed somites until the time of *Qmyogenin* activation and the initiation of myotome differentiation.

A role for axial tissues in the processes of somite myotome differentiation has been described previously (Rong et al., 1992). Our work now clarifies that the axial tissues regulate myotome differentiation by controlling the activation and maintenance of expression of the myogenic bHLH genes, which are essential for myogenesis (Rudnicki et al., 1993). Therefore, signals from axial tissues are the molecular regulators of the determination of the myotomal muscle lineage. Contradictory to our findings, Bober et al. (1994b) report that *myoD* expression and myotome differentiation are autonomous of axial tissues. We do not offer an explanation for this discrepancy, although, in contrast to their study, we have examined gene expression using contralateral somite controls, which have allowed us to unambiguously evaluate the quantitative impact of surgical interventions on gene expression and differentiation.

Somite cell culture studies also have shown that the notochord and neural tube can promote myotome differentiation (Packard and Jacobson, 1976; Vivarelli, and Cossu, 1986; Kenny-Mobbs and Thorogood, 1987; Munsterberg and Lassar, 1995; Buffinger and Stockdale, 1994, 1995; Stern and Hauschka, 1995). In significant ways, however, it is difficult to compare cell culture results with our embryo studies. In cell culture, tissue interactions are disrupted, the extracellular environment is artificial, and morphological and temporal aspects of somite formation and maturation are minimal. Our approach is to dissect mechanisms that regulate both the activation and the maintenance of expression of myogenic bHLH genes during somite formation, as well as mechanisms that regulate timing and spatial determination of myotomal lineages during later somite morphogenesis, within the context of the developing embryo.

The notochord is necessary and sufficient to activate the myogenic bHLH genes in newly forming somites

Our study establishes that the notochord is the necessary and sufficient source of signals that activate the myogenic bHLH genes and initiate the cascade of regulatory processes required for myotome differentiation. Our results also clarify previous conflicting views of the role of the notochord in somite morphogenesis and myotome differentiation. On the one hand, the notochord has been shown to act as an inhibitor of myotome differentiation and an inducer of ventral sclerotome determination when grafted dorsally adjacent to the neural tube (Pourquie et al., 1993; Brand-Saberi et al., 1993; Goulding et al., 1993). Our work, however, provides evidence that notochord grafts, placed laterally and away from the neural tube, induce *QmyoD* expression in newly formed somites. Therefore, inhibitory effects of the notochord likely reflect the placement of the notochord adjacent to the neural tube. This type of graft leads to the induction of ventral floor plate which produces locally acting

Fig. 7. Notochord ablations. Notochord was surgically ablated in a 200-400 μm region of the segmental plate or newly formed posterior somites. (A) Diagram showing the ablation of the notochord in the region of the segmental plate. (B, B1 and B2) Whole-mount in situ hybridization of *QmyoD* expression; lines 1 and 2 in B show extent of notochord ablation. (C) Diagram of the notochord ablation in the region of the posterior somites. (D, D1 and D2) Whole-mount in situ hybridization of *QmyoD* expression; lines 1 and 2 in D show extent of notochord ablation. Embryos were cultured for 16 hours prior to fixation and in situ hybridization with QmyoD DIG probes. Transverse sections (B1, B2 and D1, D2) were made through somites, as shown by the bars. Note that *QmyoD* expression does not occur in the somites in the ablation region, more distant from the notochord. However, hyperactive *QmyoD* expression that spreads ventrally is in somites near to the notochord ablation (B1).

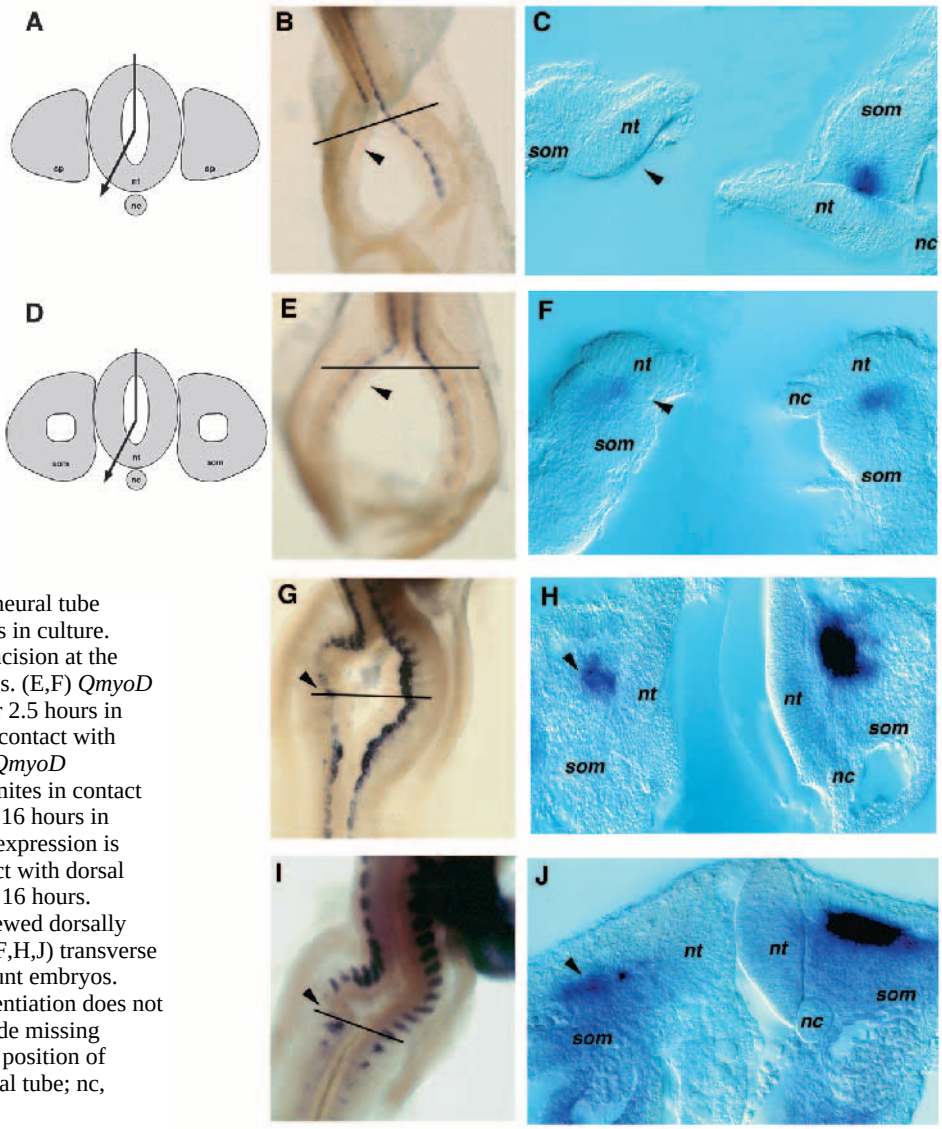


Fig. 6. The neural tube of embryos was separated from the neural tube/notochord complex by an oblique dorsal and lateral incision in the region of segmental plate or in the region of newly formed somites, followed by culture and in situ hybridization analysis. (A) Diagram showing the incision at level of segmental plate. (B,C) Failure to activate *QmyoD* expression in somites formed in association with neural tube (arrowhead) during 16 hours in culture. (D) Diagram showing the incision at the level of the posterior somites. (E,F) *QmyoD* expression is maintained for 2.5 hours in somites I-VII that maintain contact with neural tube (arrow). (G,H) *QmyoD* expression is reduced in somites in contact with neural tube alone after 16 hours in culture (G,H). (I,J) *α-actin* expression is reduced in somites in contact with dorsal neural tube (arrowhead) for 16 hours. (B,E,G,I) Whole mounts viewed dorsally with anterior to the top; (C,F,H,J) transverse sections through whole-mount embryos. Note that neural tube differentiation does not progress on the separated side missing ventral tissue. Bars indicate position of vibrotome sections; nt, neural tube; nc, notochord; som, somite

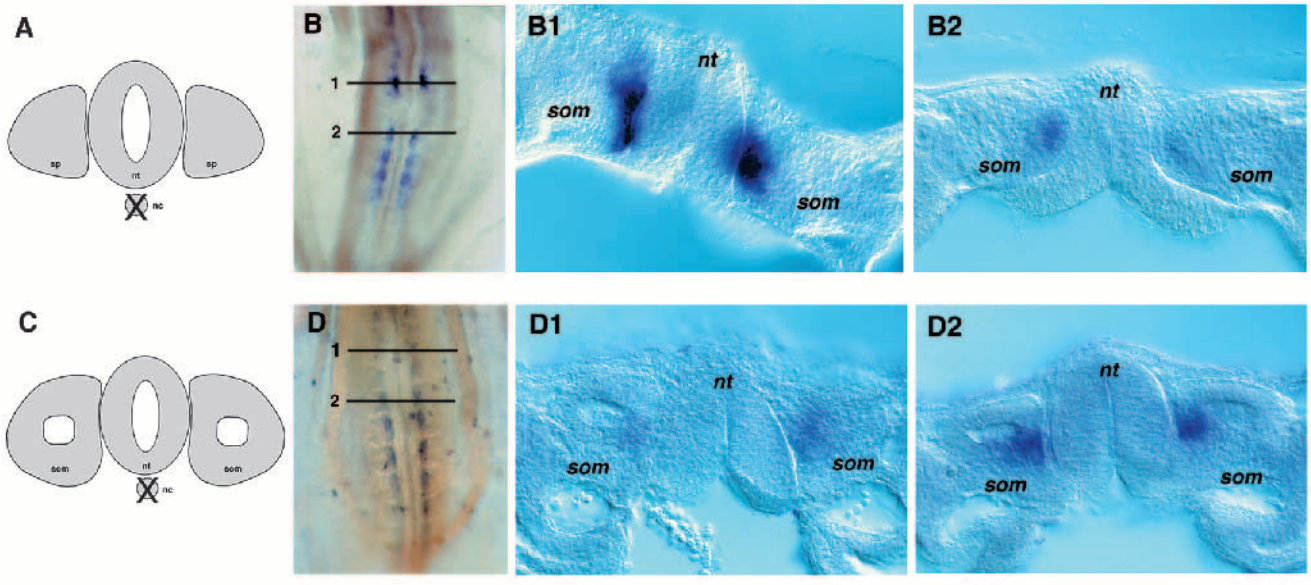


Fig. 7

inhibitors of myogenesis and inducers of sclerotome formation (Placzek et al., 1990). Consistent with this interpretation, we observed that *QmyoD* is hyper-activated ectopically in the dorsal and ventral aspects of somites forming adjacent to the sites of notochord ablations, but that activation does not occur in somites forming in a large ablation region at a distance from the cut notochord. Goulding et al. (1994) made a similar observation, and earlier embryological studies have shown that muscle differentiation occurs ventrally in embryos in the regions of small notochord ablations (Van Straaten and Hekking, 1991). These observations and our findings support the possibility that the notochord produces a long range signal that can activate bHLH genes over an anterior/posterior distance of at least a somite length and that the notochord also induces the ventral neural tube to produce short inhibitors of myogenesis. The ventral neural tube, and not the notochord, must be the source of such inhibitors since we also show that the notochord alone can induce myogenic bHLH genes in the absence of neural tube. An alternative possibility is that the notochord and ventral neural tube produce concentration-dependent signals that activate *QmyoD* in medial somite cells when these signal(s) are at lower concentrations at a distance from their source of production in the notochord and ventral neural tube. At higher concentrations in the ventral somite, these same signal(s) would inhibit myogenesis and promote sclerotome formation. In either case, however, our data provide evidence for the sufficiency of notochord signal(s) for the earliest events of somite myogenesis; that is, for the activation of *QmyoD*. Although our experiments do not address whether the notochord provides quantiatively complete signals for *QmyoD* activation, previous embryo surgery and tissue culture studes have established the notochord alone is a sufficient source of signals to promote the later events of myogenic differentiation (Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995). Together, these findings provide compelling evidence for the involvement of notochord in all aspects of somite myogenesis. As discussed later, the neural tube also has a function in somite myogenesis, but its role appears modulatory and involves maintenance of myogenic gene expression.

Significantly, the notochord from the segmental plate region can activate *QmyoD* when grafted adjacent to segmental plate as it condenses to form epithelial somites. This not only demonstrates that the notochord alone is sufficient for the activation of *QmyoD* expression, but also leads to some other important conclusions. First, activation of *QmyoD* has not been observed when grafts of notochord, taken from more anterior regions of the embryo, where somites have formed and are maturing, were used. This suggest that the posterior notochord in the segmental plate region is more active in the production of signals. The notochord is known to express several developmentally important genes, all of which are potential candidates for membership in a signaling pathway regulating the initiation of myotome determination, such as Shh (Riddle et al., 1993; Roelink et al., 1994), cNot (Stein and Kessel, 1995), and Brachyury (Ch-T) (Kispert et al., 1995). None of these molecules, however, has been shown to be expressed exclusively in the posterior notochord where this activity is localized. A prediction is that a secreted factor with this activity will be found to be localized in the posterior notochord in avians, as eFGF has been shown to be expressed in the posterior notochord in *Xenopus* (Isaacs et al., 1995). Secondly, our observations indicate that segmental plate cells become competent to respond to notochord signals as they form the epithelial somite. This is consistent with results of Buffinger and Stockdale (1994), who observed that epithelial somite cells, but not segmental plate, are responsive to notochord induction of myotome differentiation. The product of the *Delta* gene, which is known to be involved in lateral inhibition in *Drosophila*, has been shown to be highly expressed in the unsegmented mesoderm and not in the newly formed somites (Henrique et al., 1995) and may be involved in keeping this region unresponsive to notochord signals. Alternatively, receptors for axial signals may be synthesized or become functional when segmental plate cells form somites. Our results, however, do not exclude the possibility that secondary signals from associated ectoderm, lateral plate, or somite cells themselves also produce signals that act cooperatively with the notochord to activate myogenic bHLH genes. Our study also does not directly address whether activation of myogenic bHLH genes, in the myogenic lineages migrating to the limb (Charles de la Brousse and Emerson, 1990), are subject to similar signal regulation as the myotomal lineage in the somite, although the lateral notochord grafting studies (Fig. 8E,F) and somite rotation studies (Ordhal and LeDourain, 1992) indicate that these limb lineage cells are capable of responding to myogenic axial signals.

Neural tube signals contribute to maintenance of myogenic bHLH gene expression

Our results further show that the neural tube contributes signals to maintain myogenic bHLH gene expression. Previous tissue culture studies on somite differentiation and myoD regulation did not predict that axial tissues would be required for both the activation and the maintenance of myogenic gene expression in newly formed somites. Since the notochord alone is sufficient for myogenesis in embryos (Rong et al., 1992) and in cell culture (Buffinger and Stockdale, 1994), and the notochord is necessary and sufficient to activate the myogenic bHLH genes, the neural tube signal for maintenance could be redundant and produced in response to the notochord signal. The neural tube could be passive in signal production by acting as a physical reservoir for signaling molecules produced by the notochord; or, once somites have formed, the neural tube could be induced by the notochord to actively produce higher levels of a common signaling molecule(s) as part of an amplification mechanism required to maintain high level expression of the myogenic bHLH genes during the period leading up to myotome differentiation. Another possibility is that a notochord signal, in addition to its role in activation of myogenic gene expression, also could induce a secondary signal in the neural tube that functions to maintain and/or amplify *QmyoD* expression in somites. Recent in vitro somite cultures studies indicate that Sonic hedgehog and certain wnt proteins can provide combinatorial signals for maintenance of *QmyoD* expression (Munsterberg and Lassar, 1995; Munsterberg et al., 1995). These notochord and neural tube proteins clearly are candidate signal molecules for the activation/maintenace signaling

pathways of *QmyoD* regulation that have been revealed in this study. It should be emphasized, however, that there is abundant evidence that notochord alone is sufficient to support at least qualitative levels of myotome differentiation, both in ovo and in somite cultures (Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995). Therefore, the neural tube would not be predicted to produce qualitatively distinct signal molecules essential for myogenesis.

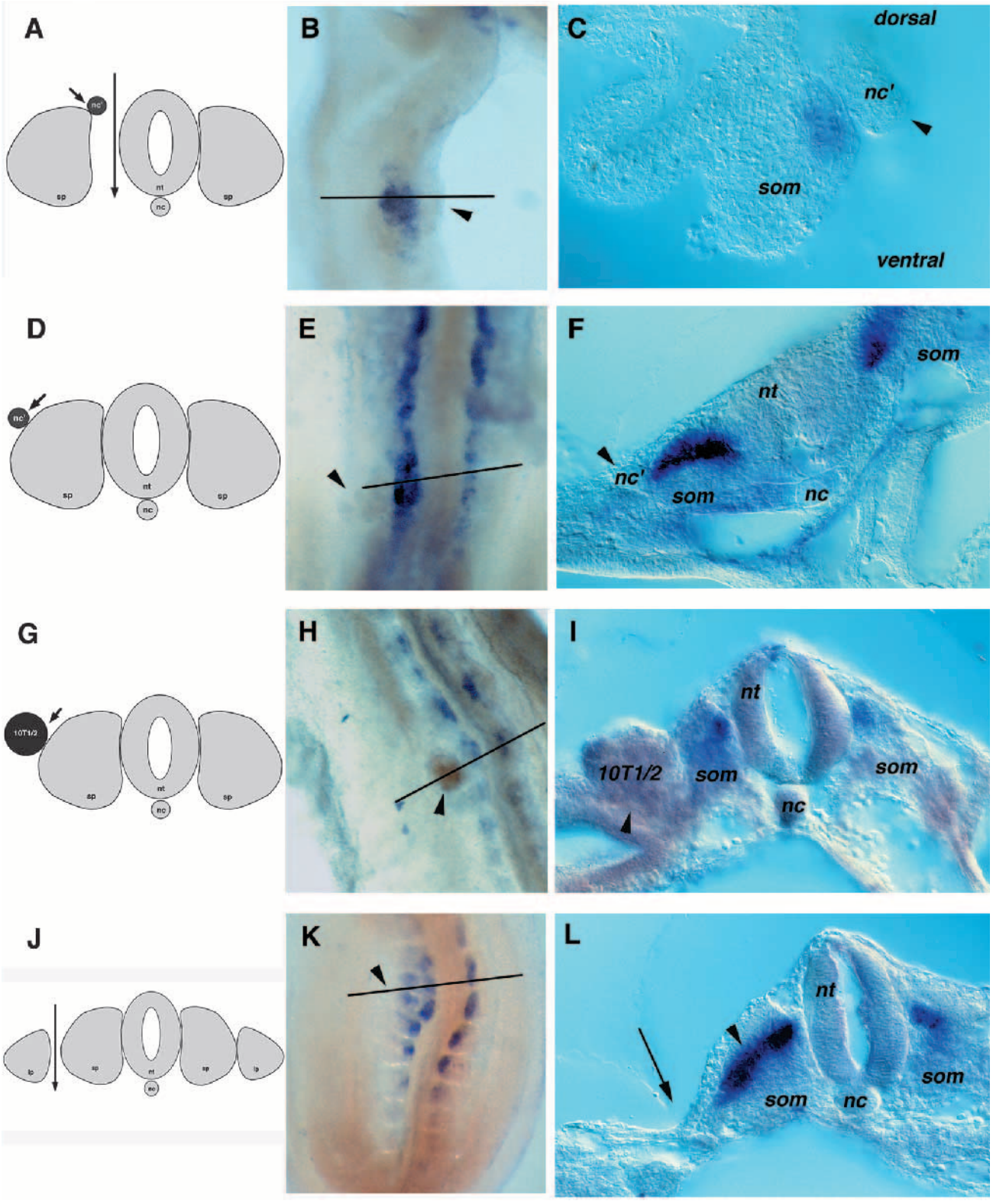
The discovery that axial signals are required to maintain myogenic bHLH gene expression in newly formed somites explains previous embryological results that newly formed somites are plastic in their potential for myotome differentiation (Ordahl and LeDourain, 1992; Christ et al., 1992), even though these somites have initiated *QmyoD* gene expression (Pownall and Emerson, 1992). A maintenance requirement for *QmyoD* expression was unexpected since earlier *myoD* cDNA transfection studies had established that *myoD* genes can be auto- and cross-activated in tissue culture cells (Thayer et al., 1989; Yutzey et al., 1990). In the embryo, however, we show that expression of *QmyoD*, as well as *Qmyf5*, becomes stabilized and independent of axial interactions only later when myotome differentiation initiates in these somites. This lag between activation of expression and stabilization may reflect a requirement for myoD and myf5 proteins to reach hypothetical threshold levels to establish auto- or cross-regulatory mechanisms. Alternatively, this delay may reflect the time required to establish mechanisms for mRNA stabilization that become operative when myotomal cells differentiate or perhaps to activate additional regulatory genes required for myogenic differentiation.

A signaling model for myogenic bHLH regulation and somite determination

Our results are consistent with simple signaling models for the activation and maintenance of myogenic bHLH gene expression and somite myogenesis (Fig. 9). For instance, the localized activation and maintenance of myogenic gene expression could be controlled by a combination of activation and inhibitory signals. Long range activation signals coming from the notochord and traversing the ventral region of the neural tube could activate myogenic bHLH genes in newly formed somites, which become responsive to these signals following epithelialization of the segmental plate mesoderm. Inhibitory signals from the ventral neural tube and lateral plate (Pourquie et al., 1995; Figs 7B, 8K,L) would counterbalance these activation signals to localize expression to dorsal medial somite cells that would become determined to the myotomal cell lineage and initiate differentiation. After a period of maintenance and amplification by neural tube signals, myotomal cells would become stabilized in the expression of the myogenic bHLH genes as a result of the establishment of autoregulatory loops, and myotome differentiation would then initiate. Alternatively, activation and maintenance of myogenic gene expression could be controlled by concentration-dependent signals, produced by the notochord, that diffuse and form a concentration gradient with different activities along the dorsal ventral axis. For instance, at low concentrations and at a distance from the notochord source, such a signal could activate *QmyoD*, whereas at high concentrations near the notochord, the same signal could inhibit *QmyoD* and promote sclerotome formation in the ventral somite. However, we consider such a simple concentration-dependent signaling model unlikely, given our observation that posterior notochord can activate *QmyoD* immediately adjacent to notochord grafts (Fig. 8C) and that the dorsal medial neural tube assumes a role in the maintenance of *QmyoD* expression following somite formation. A third possibility is that *QmyoD* is regulated by a combination of signals including notochord signals involved in activation of expression during somite formation, neural tube signals involved in amplification and/or maintenance of expression during somite maturation, as well as inhibitory signals from ventral and lateral sources, which localize *QmyoD* expression to the medial somite.

The permissive or instructive nature of the axial signals for somite myogenesis is unknown. For two reasons, we favor the interpretation that these signals are instructive and specific. First, we show that neural tube/notochord and notochord alone can activate ectopic expression of *QmyoD* in lateral somite cells. Second, QmyoD and Qmyf5 have highly potent, muscle-specific functions that control the determination of myogenic cells in the somite (Rudnicki et al., 1993). Induction of such a regulatory gene cascade would be expected to require specific signals. The activities of these inducing signals are counterbalanced by inhibitory signals that restrict and localize expression, providing a mechanism to precisely localize cell determination, as has been seen in other systems (Wilkinson et al., 1994). Sclerotome cell determination also requires specific axial signals, which are generated from the notochord and floor plate (Pourquie et al., 1993; Brand-Saberi et al., 1993). Somite cells that give rise to myotome and sclerotome require additional signals for long term cell survival (Teillet and LeDourain, 1983). Whether axial factors for myogenic and sclerotome determination and for survival are the same or different molecules remains to be determined. However, somite cell death appears to be a normal part of somite patterning and neural crest migration (Jeffs and Osmond, 1992), and, therefore, may be an alternative, default fate of somite cells that do not become determined to myotomal or scleratomal fates by specific axial signals.

The molecular nature of the inducing and inhibitory axial signals and somite response systems remains to be determined. It is clear that these signals control a cascade of transcriptional regulatory events (Pownall and Emerson, 1992), including activation of *myoD* transcriptional enhancers (Goldhamer et al., 1992, 1995; Pinney et al., 1995). Our findings indicate that the notochord signals for myogenic gene activation are likely diffusible, consistent with cell culture studies of myotome differentiation (Buffinger and Stockdale, 1995). In avian embryos, there is a precedent for diffusible factors emanating from the neural tube and notochord that influence neural crest differentiation (Kalcheim and LeDouarin, 1986) and *pax* gene expression (Goulding, et al., 1993, 1994). In *Xenopus*, the early embryo is patterned by the actions of a complexity of diffusible growth factors, including FGFs, wnts, and members of the TGF-β family (for reviews, see Smith, 1989; Slack, 1993; Kessler and Melton, 1994). Furthermore, the expression of XmyoD is an early response to mesoderm induction by FGFs and activin (Hopewood et al., 1989). Sonic hedgehog has



been shown to be involved in concentration-dependent patterning the neural tube and limbs of the chick embryo (Riddle et al., 1993; Roelink et al., 1994, 1995). Shh is expressed ventrally in the notochord and the floor plate (Marti et al., 1995), and Shh regulates sclerotome specification and somite patterning (Fan and Tessier-Lavigne, 1994, 1995; see also Bumcrot and McMahon, 1995). Retroviral-mediated Shh expression in segmental plate mesoderm induces *myoD* expression in lateral regions of newly formed somites (Johnson et al., 1994), and Shh in combination with wnt

Fig. 8. Notochord (200 μm), was surgically removed from the segmental plate region of a donor embryo and implanted into stage 12 host embryo as described. (A) Diagram of notochord grafts to segmental plate separated from neural tube/notochord. Segmental plate or newly formed somites of the host embryo were separated from neural tube/notochord by a dorsal/ventral incision through all three germ layers, and notochord (100-200 μm) removed from segmental plate region of a donor embryo was grafted to a medial site on the separated segmental plate. (B,C) *QmyoD* expression is activated in response to notochord grafts (arrowhead) in somites formed after separation of the segmental plate from neural tube/notochord complex. (D) Diagram of the experiment where notochord grafts were implanted in an incision between segmental plate mesoderm and lateral plate mesoderm of a stage 12 host embryo. (E,F) *QmyoD* expression spreads laterally in the dermomyotome in response to the notochord graft inserted between the lateral plate and segmental plate (arrowhead). (G) Diagram of lateral 10T½ grafts. 10T½ cell aggregates were implanted in an incision between the lateral plate and the segmental plate. (H,I) *QmyoD* expression is unaffected and remains localized medially in response to lateral grafts of 10T½ cells (arrowhead). (J) Diagram of lateral plate ablations. Lateral plate was surgically separated from segmental plate by a dorsal/ventral surgical incision. (K,L) *QmyoD* expression spreads laterally in somites formed after separation of segmental plate from the lateral plate (arrow). Embryos were cultured for 16 hours following surgery, prior to fixation and in situ hybridization with QmyoD DIG probes. (B,E,H,K) Whole-mount embryos; (C,F,I,L) transverse sections. nc, notochord; nc′, notochord graft; nt, neural tube; som, somite; 10T½, 10T½ cell aggregate graft. Bars indicate sites of transverse sections on whole-mount embryos.

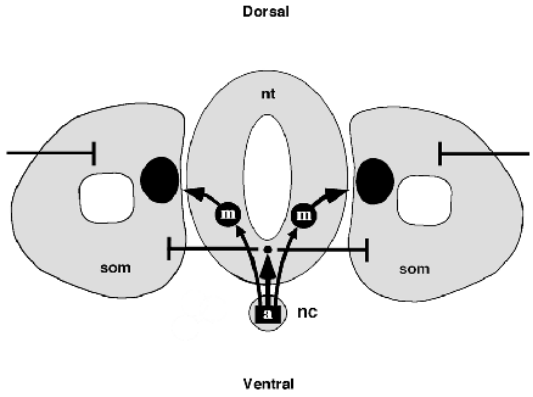


Fig. 9. Signaling model for myogenic bHLH regulation during somite formation. Inductive (→) and inhibitory (⊣) signals cooperate to localize expression of the myogenic bHLH genes to dorsal medial myotomal progenitor cells in newly formed somites. A long range activation signal (a) is produced by the notochord that activates myogenic bHLH genes during somite formation. The dorsal medial neural tube is then induced to produce additional or new signals (m) to amplify and/or maintain this notochord signal in newly formed somites. The notochord also stimulates the ventral neural tube to produce signals that inhibit myogenesis and/or to promote sclerotome differentiation in ventral somite cells (Pourquie et al., 1993). Inhibitory signals are also produced by the lateral plate to repress myogenic bHLH expression in the lateral aspect of the dermomyotome (Pourquie et al., 1995), thereby restricting myogenic bHLH expression in the dermomyotome to the medial somite cell progenitors of myotomal muscle. The molecular nature of the inducing signals for activation and maintenance of myogenic genes, for sclerotome formation, and for inhibition of myogenesis in the ventral and lateral somite are unknown. Each of these processes could be controlled by distinct signaling molecules or by common molecules that induce different concentration-dependent responses. nc, notochord; nt, neural tube; som, somite.

proteins can maintain *myoD* expression in cultured somites (Munsterberg et al., 1995). These findings are consistent with the possibility that Shh is one of a combination of specific molecular signals that regulate activation and maintenance of myogenic gene expression during somite formation and myotome differentiation. Our embryological findings now provide a basis for experimental studies to identify the axial signaling molecules that control the activation and maintenance of *QmyoD* during somite formation and the early gene regulatory processes of somite myogenesis.

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