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Myogenesis in paraxial mesoderm: preferential induction by dorsal neural tube and by cells expressing *Wnt-1*

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SUMMARY

Previous studies have demonstrated that the neural tube/notochord complex is required for skeletal muscle development within somites. In order to explore the localization of myogenic inducing signals within the neural tube, dorsal or ventral neural tube halves were cultured in contact with single somites or pieces of segmental plate mesoderm. Somites and segmental plates cultured with the dorsal half of the neural tube exhibited 70% and 85% myogenic response rates, as determined by immunostaining for myosin heavy chain. This response was slightly lower than the 100% response to whole neural tube/notochord, but was much greater than the 30% and 10% myogenic response to ventral neural tube with and without notochord. These results demonstrate that the dorsal neural tube emits a potent myogenic inducing signal which accounts for most of the inductive activity of whole neural tube/notochord. However, a role for ventral neural tube/notochord in somite myogenic induction was clearly evident from the larger number of myogenic cells induced when both dorsal neural tube and ventral neural tube/notochord were present. To address the role of a specific dorsal neural tube factor in somite myogenic induction, we tested the ability of *Wnt-1*-expressing fibroblasts to promote paraxial mesoderm myogenesis in vitro. We found that cells expressing *Wnt-1* induced a small number of somite and segmental plate cells to undergo myogenesis. This finding is consistent with the localized dorsal neural tube inductive activity described above, but since the ventral neural tube/notochord also possesses myogenic inductive capacity yet does not express *Wnt-1*, additional inductive factors are likely involved.

Key words: somite, neural tube, notochord, *Wnt*, myogenesis, induction, chick embryo

INTRODUCTION

The localized development of muscle within somites depends on signals from the adjacent neural tube and in some experimental systems from the notochord. This interaction has been demonstrated both in vivo (Muchmore, 1951; Strudel, 1955; Teillet and Le Douarin, 1983; Rong et al., 1992; Christ et al., 1992; Bober et al., 1994; Pownall, Strunk and Emerson, personal communication) and in vitro (Avery et al., 1956; Kenny-Mobbs and Thorogood, 1987; Vivarelli and Cossu, 1986; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995). In vivo, the medial half of each somite normally gives rise to the myotome, the precursor to axial muscles (Ordahl and Le Douarin, 1992). However, if the neural tube/notochord complex is removed from a chick embryo, myotomes do not form. Similarly, when tested in explant or organotypic culture, the most immature somites do not exhibit muscle differentiation unless the neural tube (or in some cases the notochord) is present.

Somites form by pinching off from the rostral end of the segmental plate mesoderm once every 1.5 hours during stages 7-21 of chick development (Menkes et al., 1961; Primmett et al., 1989). Thus, rostral somites are more developmentally advanced than caudal somites, resulting in a rostrocaudal gradient of myogenic differentiation. Throughout this paper, somites will be numbered according to their maturity as suggested by others (Ordahl, 1993; Christ and Ordahl, 1995). The most recently formed somite is roman numeral I and the number increases by one for each somite more rostral in the gradient.

Explant cultures of single somites at chick stages 8-11 (4-13 total somites) demonstrate that the caudal-most somites (I-V) do not give rise to myosin heavy chain (MHC)-positive cells unless they are cultured with neural tube (or notochord for somites II and older, see below) (Stern and Hauschka, 1995). In contrast, more rostral somites produce MHC-positive cells in the absence of neural tube; however, the number of MHC-positive cells is at least four times greater when neural tube is included. This result indicates a dependence on neural tube even in older somites which already contain cells committed to myogenesis (Stern and Hauschka, 1995). The neural tube induction of myogenesis does not require prior segmentation of paraxial mesoderm into somites since MHC-positive cells can also be induced in explants of segmental plate (Buffinger

and Stockdale, 1994; Stern and Hauschka, 1995). The inductive interaction between neural tube and paraxial mesoderm requires close proximity (Rong et al., 1992; Stern and Hauschka, 1995; Pownall, Strunk, and Emerson, personal communication). Nevertheless, the neural tube signal can cross a 0.05 μm Millipore filter (Fan and Tessier-Lavigne, 1994; Buffinger and Stockdale, 1995). These results suggest that short-range diffusible factors mediate the inductive interaction.

The notochord has also been shown to promote somite myogenesis both in vivo (Rong et al., 1992) and in vitro (Buffinger and Stockdale, 1994; Stern and Hauschka, 1995). When tested in vitro, the notochord promotes myogenesis in somites II and older, but not in somite I or the segmental plate, indicating that the notochord signal might be qualitatively different than that from the neural tube. However, some studies have not demonstrated a positive myogenic role for the notochord (Avery et al., 1956; van Straaten and Hekking, 1991; Christ et al., 1992), and others have suggested that the notochord inhibits myogenesis by recruiting cells to the sclerotomal lineage (Pourquie et al., 1993; Brand-Saberi et al., 1993; Fan and Tessier-Lavigne, 1994). Thus, the role of the notochord in somite myogenesis is still unclear.

The focus of the present study was to determine if a defined neural tube factor could mimic the induction of somite myogenesis. Since dorsal/ventral localization of the myogenic inducing signal(s) in the neural tube would aid in the identification of likely candidate factors, the initial portion of our study involved the culture of single somites and segmental plate pieces with either dorsal or ventral portions of the neural tube. Our results indicate that paraxial mesoderm exhibits a much stronger myogenic response to dorsal than to ventral neural tube. Based on the greater inductive activity of the dorsal neural tube, we tested whether the dorsally expressed factor *Wnt-1* could induce paraxial mesoderm myogenesis. We show that cells expressing *Wnt-1* can induce myogenesis when co-cultured in vitro with somite or segmental plate explants.

MATERIALS AND METHODS

Eggs

Stage 8-15 (Hamburger and Hamilton, 1951) White Leghorn chick embryos were obtained by incubating eggs (H and N International) at 38°C for 2 days.

Dissections

Tissues were dissected as previously described (Stern and Hauschka, 1995). Somites I-IV from stage 8-11 embryos (4-14 total somites) or somites I-III from stage 12-13 embryos (15-19 total somites) were individually removed along with the rostral half of each segmental plate which was then cut into four approximately somite-sized pieces. The neural tube/notochord from the region between the segmental plates and somites I-IV was either cut into pieces roughly corresponding to the rostrocaudal position of the paraxial mesoderm explants, or it was first divided into dorsal/ventral halves which were then divided into rostrocaudal segments. The notochord served as a marker for the ventral side (Fig. 1). In some cases the notochord was removed after the dorsal and ventral portions of the neural tube were separated. In cases where somites with cells already committed to myogenesis were utilized (Stern and Hauschka, 1995), they were obtained from chick stages 12-15, somites V-XI. Tissues were transferred to 96 well gelatin-coated plates (Corning 25860) containing 200 μl of medium per well.

Tissue culture

Tissue explants were cultured in Ham's F10 supplemented with 0.8 mM Ca^{2+} (F10C), 0.05 mg/ml gentamicin (Sigma), 1% chick embryo extract (Konigsberg, 1968), 250 ng/ml amphotericin B (Sigma), 30 nM sodium selenite (Sigma), 100 μM putrescine (Calbiochem), 100 μg/ml chick transferrin (Sigma, conalbumin), and 6 μg/ml insulin (Collaborative Biomedical Products), as described by Stern and Hauschka (1995). The culture time was extended to 3 days from the 2 day period used previously. The longer culture period reduced variability in the number of MHC-positive cells generated in paraxial mesoderm-neural tube co-cultures. The myogenic commitment status of the somites and segmental plates did not change with increased times of culture as noted in a prior study (Stern and Hauschka, 1995).

Wnt-1-expressing fibroblasts were obtained by infecting cell lines (Rat-2, NIH3T3, and Rat-B1a) with the replication-defective retrovirus MV*Wnt-1*, as described previously (Jue et al., 1992). The infected cells were selected in G418 and 30-100 clones pooled. The control fibroblast populations were obtained in the same manner but were infected with the parent MV7 vector without *Wnt-1* (Jue et al., 1992). Rat-2 and NIH3T3 fibroblasts were cultured in DMEM (Sigma) plus 10% heat-inactivated fetal bovine serum (Life Technologies) and 0.05 mg/ml gentamicin. Rat B1a cells (Parkin et al., 1993) were cultured in 2.5% fetal bovine serum plus 7.5% bovine calf serum (Hyclone). For paraxial mesoderm-fibroblast co-culture experiments, five to ten thousand fibroblasts were added per well to 96 well gelatin-coated plates and were cultured for 1-2 days until they reached confluency. At that time, the cells were rinsed two times with F10C prior to switching to explant culture medium.

Fibroblast growth factor bioassay

Conditioned media obtained from Rat-2/MV7 and Rat-2/*Wnt-1* cultures were assayed for the presence of fibroblast growth factor (FGF) via the MM14 mouse myoblast bioassay procedures described previously (Clegg et al., 1987; Seed et al., 1988).

Immunocytochemistry

Cultures were stained for myosin heavy chain (MHC) using the MF20 monoclonal antibody (Bader et al., 1982) as previously described (Stern and Hauschka, 1995), except that 2% nonfat dry milk was used as the blocking agent.

Statistics

When comparisons were made with respect to the number of MHC-positive cells generated in response to different inducing tissues, the data was analyzed using the Students *t*-test. The statistical significance (*P* value) of any differences is indicated in the text.

RESULTS

Dorsal/ventral polarity of neural tube myogenic activity

As an initial test of dorsal/ventral differences in the myogenic influence of neural tube on paraxial mesoderm, single somite or segmental plate explants were cultured in contact with either the dorsal or ventral side of intact segments of the neural tube/notochord as depicted in Fig. 2. The contralateral somite/segmental plate explants were cultured alone to verify that they did not contain cells that were previously committed to myogenesis (Stern and Hauschka, 1995). None of the 61 control somite or segmental plate explants cultured alone exhibited MHC-positive cells (Fig. 2B, column 1), whereas 100% of the explants cultured in contact with the dorsal side of the complete neural tube/notochord complex exhibited

muscle, with an average of 45 (somite) and 70 (segmental plate) MHC-positive cells per explant (Fig. 2B,C, column 2). In contrast, only 42% of the explants cultured in contact with the ventral side of the complete neural tube/notochord complex exhibited MHC-positive cells (Fig. 2B, column 3). Furthermore, the cultures that did respond to the axial structures in the ventral orientation contained an average of only about 23 MHC-positive cells (Fig. 2C, column 3). This result suggests that ventral myogenic signals are weaker than dorsal signals but does not distinguish signaling from ventral neural tube versus notochord (see below). As observed previously, segmental plate and recently formed somite explants cultured alone (column I) did not exhibit MHC-positive cells (Stern and Hauschka, 1995), and hence most of our subsequent experiments did not include explants cultured alone.

The above data demonstrate potent inductive signals from the dorsal neural tube, and raise the question of whether signals from dorsal neural tissue are sufficient to induce somite myogenesis in the absence of ventral neural tube and notochord. To test this hypothesis, the neural tube/notochord was divided into dorsal and ventral halves, using the notochord as a marker for the ventral half (Fig. 1). One of each pair of paraxial mesoderm explants was cultured with dorsal neural tube, whereas the contralateral tissue was cultured with ventral neural tube with or without notochord (see below). When somite and segmental plate explants were cultured with just the dorsal half of the neural tube (Fig. 2B,C, column 4; Fig. 3A), the percentage of responsive co-cultures was slightly lower than was observed with the whole neural tube/notochord in the dorsal orientation (Fig. 2B, compare column 2 with 4). As a control to verify that MHC-positive cells did not originate from the dorsal neural tissue, dorsal neural tube segments were cultured alone. They did not give rise to any myosin-positive cells. These experiments demonstrate that induction of myogenesis by dorsal neural tube accounts for most, but not all, of the inductive activity emanating from whole neural tube/notochord.

Interestingly, the average number of MHC-positive cells was significantly higher (P = 0.001) when somites were cultured with whole neural tube/notochord in the dorsal orientation (70 MHC-positive cells, Fig. 2C column 2) compared to somites cultured with dorsal neural tube alone (20 MHC-positive cells, column 4). Thus, although dorsal signals can act autonomously, a more robust response is obtained when ventral neural tube/notochord is included in somite-dorsal neural tube co-cultures. In contrast, segmental plate explants exhibited the same number of MHC-positive cells regardless of whether whole neural tube/notochord (45 MHC-positive cells, column 2) or dorsal neural tube alone (48 MHC-positive cells, column 4) was the inducing agent. These observations suggest that fewer cells respond exclusively to dorsal neural tube in somites than in similarly sized segmental plate explants and that the ventral neural tube and/or notochord is directly or indirectly responsible for inducing some somite cells to undergo myogenesis.

To verify that the quantitative difference in MHC-positive cells exhibited by somites was caused by removal of the ventral neural tube/notochord and not by perturbations related to cutting the neural tube in half, dorsal and ventral portions were recombined to mimic a whole neural tube/notochord. These were then cultured such that the dorsal neural tube was adjacent to the somite or segmental plate explant (Fig. 2B,C, column 5). The response rate and average number of MHC-positive cells for such cultures was virtually the same as for cultures of whole neural tube/notochord in the dorsal orientation (column 2), indicating that cutting the neural tube had no adverse effect on myogenic signals.

To determine whether exposure to larger numbers of dorsal neural tube segments increases the number of MHC-positive cells generated in paraxial mesoderm cultures, somites and segmental plate pieces were surrounded with four segments of dorsal neural tube. In these cultures, the average number of MHC-positive cells in both somite and segmental plate explants was about two-fold higher (P = 0.01) than in cultures with only one segment of dorsal neural tube (compare Fig. 2B,C; columns 4 and 6). Interestingly, while the myogenic response of segmental plates to 4 dorsal neural tube segments was greater than their response to the complete neural tube/notochord, the response of somites I to IV was less than with complete neural tube/notochord. Somite cultures also exhibited only about half the number of MHC-positive cells observed in segmental plate cultures (P = 0.03).

In parallel with the dorsal neural tube experiments described above, the contralateral somites and segmental plate pieces were cultured with segments of the ventral neural tube alone or with notochord (Fig. 2B,C, columns 7 and 8, Fig. 3B). Cultures containing the ventral neural tube with or without notochord (Fig. 2B, columns 7 and 8) were much less responsive than cultures with dorsal neural tube alone (column 4) or with the whole neural tube/notochord in the dorsal orientation (column 2). The number of MHC-positive cells generated in the few cultures responding to ventral neural tube (Fig. 2C, columns 7 and 8) was also significantly less than in cultures with dorsal neural tube alone (column 4, P = 0.005) and less than with whole neural tube/notochord in either the dorsal (column 2, P = 0.003) or ventral (column 3, P = 0.01) orientation. In addition, some paraxial mesoderm explants were cultured with notochord alone (column 9). None of the segmental plate explants exhibited MHC-positive cells when co-cultured with notochord, whereas 50% of somites I to IV exhibited an average of 15 MHC-positive cells, in agreement with previous results (Stern and Hauschka, 1995). These data clearly demonstrate that the ventral structures only weakly promote somite myogenesis. Although Fig. 2 presents data on embryo stages 10-13, the low myogenic activity of ventral structures compared to dorsal neural tube was also observed with embryo stages 8 and 9. In such early embryos, 61% of paraxial mesoderm cultures with dorsal neural tube exhibited MHC-positive cells (n = 23), whereas only 30% of cultures with ventral neural tube exhibited a myogenic response (n = 23). The average number of MHC-positive cells per responding explant was 24±6 with dorsal neural tube and 14±4 with ventral neural tube.

The foregoing results suggest that the dorsal neural tube is a strong inducer of somite/segmental plate myogenesis, whereas the ventral neural tube/notochord is a weak inducer. These data leave open the possibility that the ventral structures could also emit an inhibitory signal as previously suggested by in vivo experiments (Pourquie et al., 1993; Brand-Saberi et al., 1993; Fan and Tessier-Lavigne, 1994). To examine this possibility, somite and segmental plate explants were cultured between dorsal and ventral neural tube halves with and without

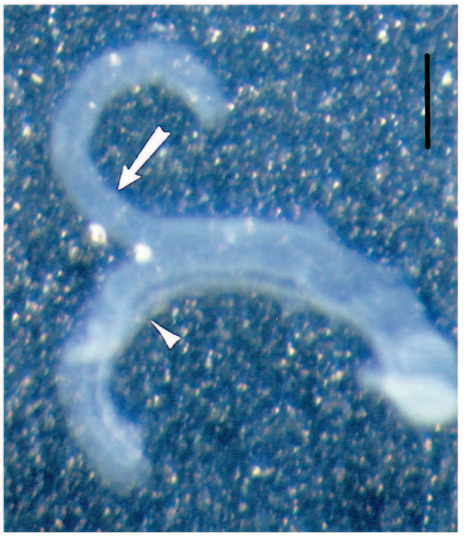

Fig. 1. Lateral view of a complete neural tube/notochord complex being divided into dorsal (arrow) and ventral (arrowhead) halves. During manipulations, the notochord serves as a marker for the ventral side. Scale bar, 200 μm.

notochord (Fig. 2B,C, columns 10 and 11). Most of these cultures exhibited MHC-positive cells. The average number of MHC-positive cells exhibited by somites cultured with both dorsal and ventral neural tube halves was greater than in co-cultures with only the dorsal neural tube (Fig. 2C compare column 4 with 10, $P < 0.001$; and 11, $P = 0.02$). For segmental plate explants cultured with dorsal and ventral neural tube (column 11), the average number of MHC-positive cells was somewhat lower than when only the dorsal neural tube was the inducing tissue (column 4). When notochord was included in such cultures, there was no difference compared to cultures with dorsal neural tube alone (compare columns 4 and 10). These results suggest a positive role for ventral neural tube and notochord in somite myogenesis, and are consistent with a negative role for ventral neural tube on segmental plates but not on somites.

To determine whether the presence of additional ventral neural tube fragments would potentiate any inhibition of myo-

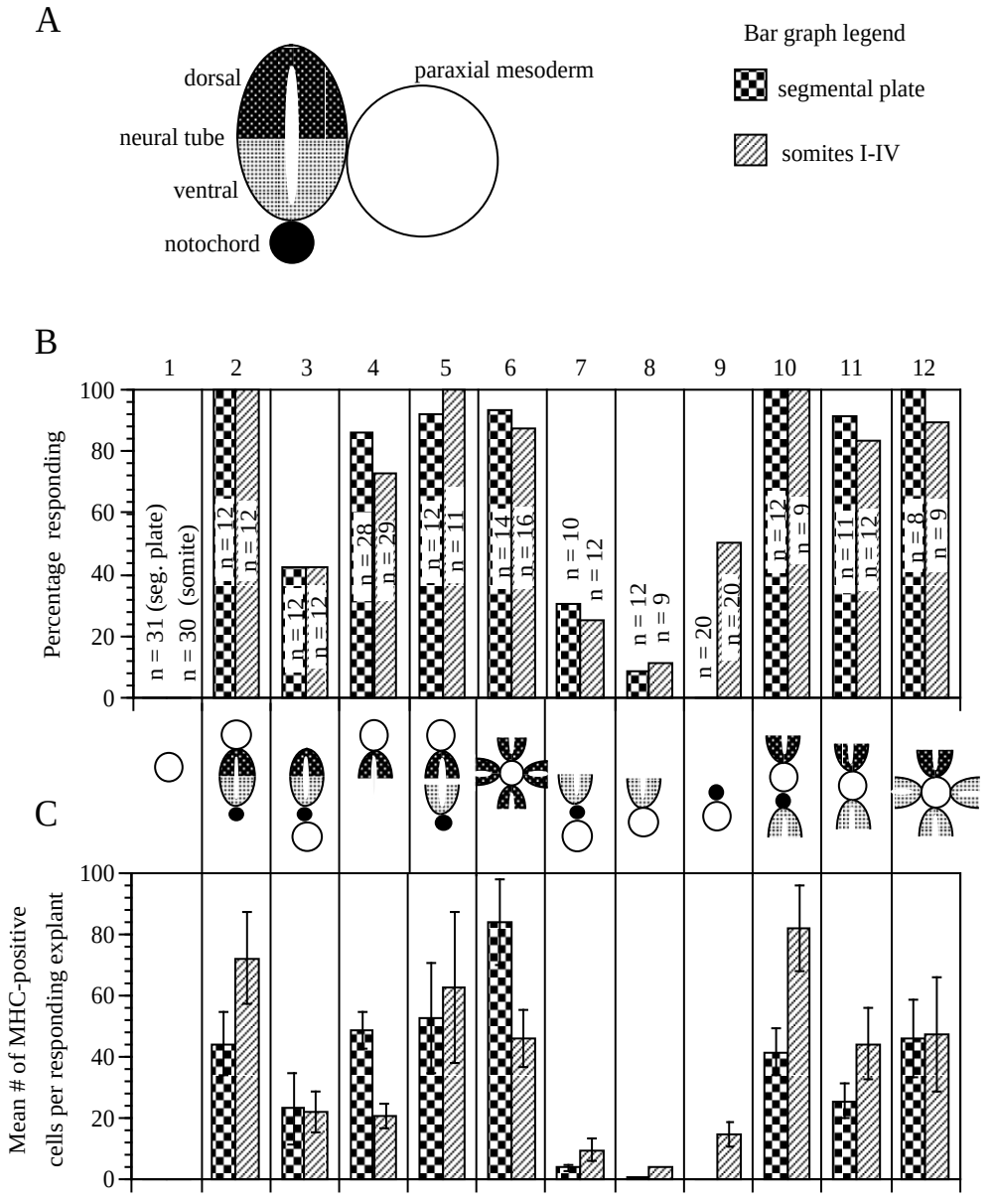

Fig. 2. Various combinations of dorsal neural tube, ventral neural tube and notochord segments were cultured with individual pieces of segmental plate mesoderm or a somite from levels I to IV of stage 10 and 11 chick embryos or levels I to III of stage 12 and 13 embryos. The legend in A provides a key to the tested tissue combinations which are depicted between the bar graphs (B,C). The data in the bar graphs has been grouped such that results from somite and segmental plate explants are indicated separately (see bar graph legend, A). The top bar graph (B) indicates the percentage of somite and segmental plate explants exhibiting one or more MHC-positive cells for each of the tissue combinations. The lower bar graph (C) indicates the average number of MHC-positive cells that were generated in the responding explants (error bars = standard error of the mean). To help refer the reader to the appropriate data for a particular tissue combination, the results are presented in numbered columns. The data in B and C are lined up such that each column contains the data for the tissue combination indicated between the graphs. The number of samples in each experiment is indicated within the bars in B. No differences were apparent in the data for the various embryo stages tested (10-13). For experiments of the type depicted in columns 4 and 8, similar results were obtained with stage 8 and 9 embryos (see text).

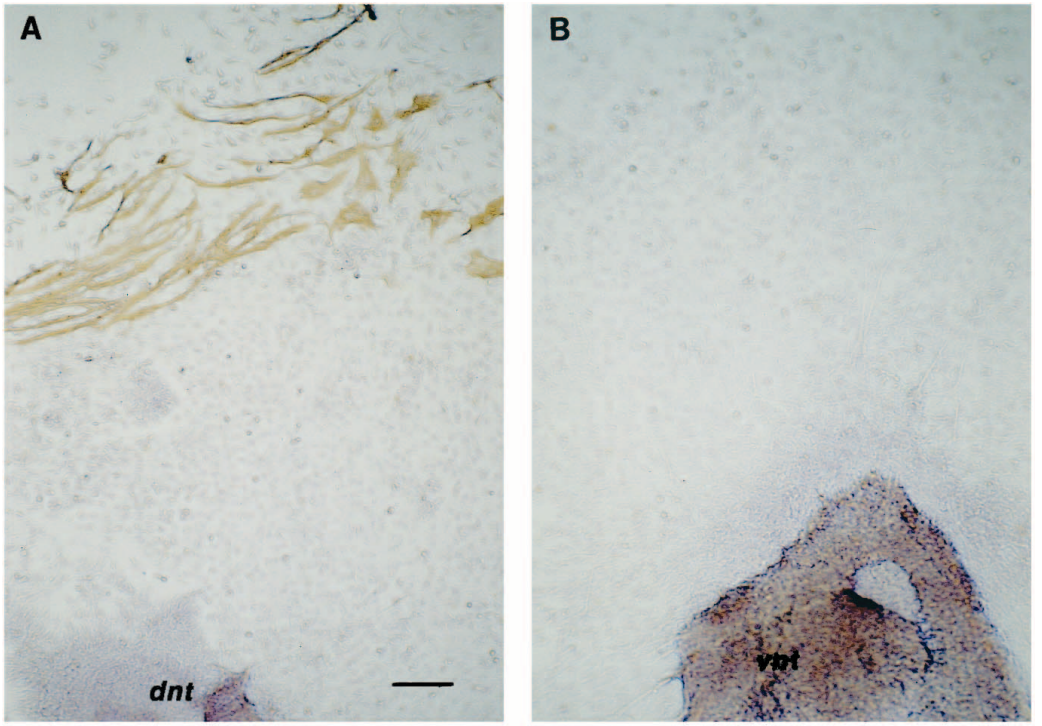

Fig. 3. Tissues from the middle of both segmental plates from a stage 12 embryo were cultured either with the dorsal or the ventral half of the neural tube. The culture was immunostained with a monoclonal antibody to MHC (MF20), such that immunopositive cells appear brown. (A) Segmental plate plus dorsal neural tube. (B) Contralateral segmental plate plus ventral neural tube (without notochord). dnt, dorsal neural tube; vnt, ventral neural tube. Scale bar, 100 μm.

genesis, experiments were performed in which somite or segmental plate explants were cultured in contact with one dorsal neural tube segment and three ventral neural tube segments (without notochord) (Fig. 2B, C, column 12). The myogenic response in segmental plate cultures was not significantly different than with dorsal neural tube alone, indicating that increasing the amount of ventral neural tube tissue does not reveal an inhibitory myogenic signal. In fact, somites exhibited about a two-fold increase (P = 0.05) in the number of MHC-positive cells (column 12) compared to cultures with just dorsal neural tube (column 4). Thus, these experiments do not support a negative role for the ventral neural tube in somite or segmental plate myogenic induction, at least in this in vitro system.

Several of the experiments described above also relate to inductive activities of the notochord. For cultures in which somites were placed between dorsal and ventral neural tube halves, inclusion of the notochord with the ventral half produced a two-fold increase in the number of MHC-positive cells (Fig. 2B,C, compare column 10 with 11; P = 0.05). In contrast, there was no significant difference in the number of MHC-positive cells in segmental plate when notochord was present (compare column 10 with 11; P = 0.12). These experiments in combination with the data for notochord alone (column 9) suggest that the increased somite myogenic effect caused by including the notochord with dorsal and ventral neural tube is at least in part due to direct myogenic signals from the notochord.

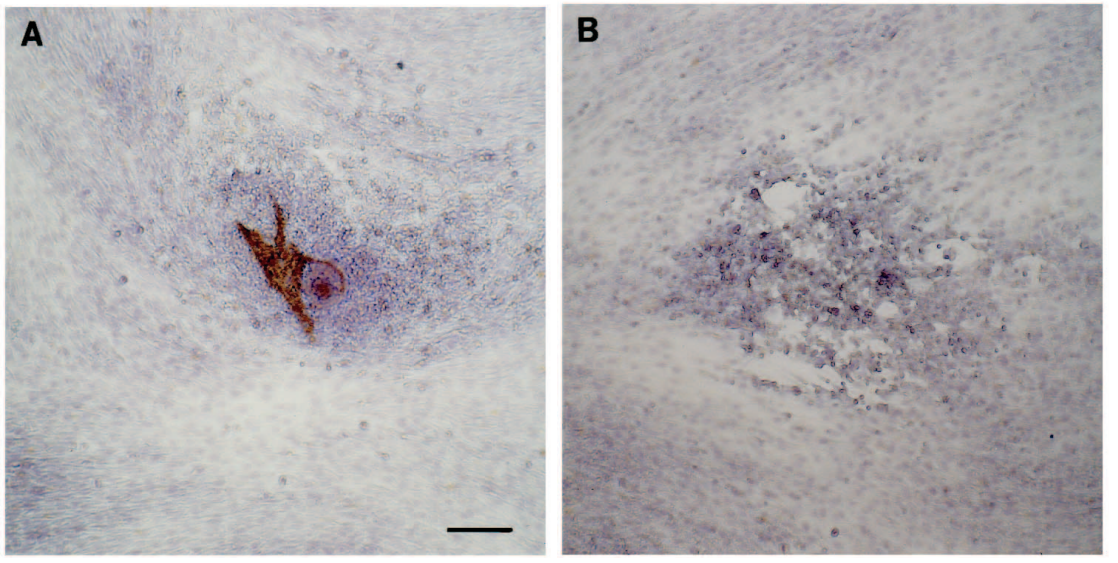

Fig. 4. Level IV somites from a stage 10 embryo were cultured on monolayers of Rat-2/*Wnt-1* fibroblasts (A) or a monolayer of control Rat-2 fibroblasts (B). The cultures were immunostained with a monoclonal antibody to MHC (MF20). Note the brown MHC-positive cells from the explant on the *Wnt-1* cells (A) but not from the contralateral somite on the control fibroblasts (B). Scale bar, 100 μm.

Cells expressing *Wnt-1* promote paraxial mesoderm myogenesis in vitro

Since the neural tube is known to express a number of secreted signaling factors, one or more of these may play a role in promoting somite/segmental plate myogenesis. The secreted glycoprotein Wnt-1 was of particular interest because of its localized expression by the dorsal neural tube in chick embryos (Bally-Cuif and Wassef, 1994; Dickinson et al., 1995; Hollyday et al., 1995) and because *Wnt* family members act as modifiers of mesodermal fate in other systems (Christian and Moon, 1993; Herzlinger et al., 1994; Parr and McMahon, 1994). Since purified Wnt-1 protein is not yet available, we used fibroblasts expressing *Wnt-1* cDNA as a means of testing the capacity of *Wnt-1* to promote myogenesis in paraxial mesoderm. These cells have been shown to elicit *Wnt-1*-dependent biological effects in a paracrine manner and to secrete Wnt-1 proteins which are mostly associated with extracellular or pericellular matrix (Jue et al., 1992).

Somite and segmental plate explants were cultured on monolayers of Rat-2, NIH3T3, or Rat-B1a cells infected with the retroviral vector MV*Wnt-1*, and the contralateral somite/segmental plate explants were cultured on control cells infected with the MV7 expression vector alone. Although the number of MHC-positive cells was low (see below), 61% of somite explants and 72% of segmental plate explants exhibited MHC-positive cells in the presence of Rat-2/*Wnt-1* fibroblasts (Table 1 and Fig. 4A), whereas none of the cultures on control Rat-2/MV7 fibroblasts exhibited any MHC-positive cells (Table 1 and Fig. 4B). The myogenic effect of the fibroblasts was therefore dependent on their expression of *Wnt-1*. In further support of this conclusion, somites and segmental plates cultured on NIH3T3/*Wnt-1* and Rat-B1a /*Wnt-1* monolayers also exhibited MHC-positive cells, but no myogenic response was observed on the control MV7-infected cells (Table 1). Conditioned medium from Rat-2/*Wnt-1* or control fibroblasts did not exhibit any myogenic inducing capacity when added to paraxial mesoderm cultures.

A dose response analysis was performed by diluting Rat-2/*Wnt-1* cells with control Rat-2 cells to create mixed monolayers. The percentage of somite and segmental plate explants exhibiting MHC-positive cells increased as the percentage of *Wnt-1* cells increased in the monolayers (Fig. 5). Furthermore, the average number of MHC-positive cells generated in *Wnt-1*-responding cultures increased with increasing percentage of *Wnt-1*-expressing cells (Fig. 5). These results suggest a relationship between *Wnt-1* dose and myogenesis.

Although a majority of explants exhibited a myogenic

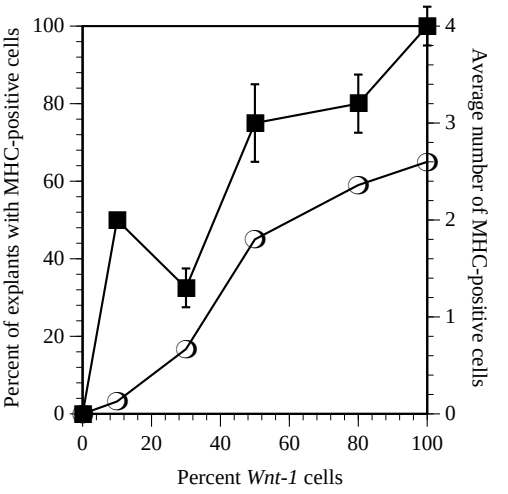

Fig. 5. A dose response analysis of chick stage 9 to 13 paraxial mesoderm myogenesis due to Rat-2/*Wnt-1* fibroblasts. The dose of *Wnt-1* was varied by diluting the Rat-2/*Wnt-1* fibroblasts with control fibroblasts. The total number of cells in the monolayer was similar in each case, but the percentage of cells expressing *Wnt-1* varied as indicated on the x-axis. The percentage of paraxial mesoderm explants exhibiting MHC-positive cells (circles, left y-axis) increases as the percentage of *Wnt-1*-expressing cells increases. Similarly, the average number of MHC-positive cells per responding explant also increases (squares, right y-axis). Error bars represent standard error of the mean.

Table 1. Cells expressing *Wnt-1* promote paraxial mesoderm myogenesis

Parent cell line for monolayer	Retrovirus vector tested*	Chick tissue†	Number of explants tested	Number of explants with MHC-positive cells
Rat-2	MV*Wnt-1*	somites	59	36 (61%)‡
		segmental plate	36	26 (72%)‡
	MV7	somites	55	0
		segmental plate	32	0
NIH3T3	MV*Wnt-1*	somites	23	10 (43%)§
		segmental plate	32	9 (28%)§
	MV7	somites	24	0
		segmental plate	32	0
Rat-B1a	MV*Wnt-1*	somites	28	19 (68%)¶
		segmental plate	36	25 (69%)§
	MV7	somites	28	0
		segmental plate	36	0

*In each case, the parent cell line was infected with the replication-defective retrovirus vector indicated. MV7 is the parental vector and MV*Wnt-1* is MV7 containing *Wnt-1* cDNA.

†Somite explants consisted of somites I-IV from stage 9-11 chick embryos and somites I-III from stage 12 and 13 embryos. Segmental plate explants were from the rostral half of the segmental plate of stage 9-13 embryos

‡The average number of MHC-positive cells in these cultures was 4.

§The average number of MHC-positive cells in these cultures was 2.

¶The average number of MHC-positive cells in these cultures was 3.

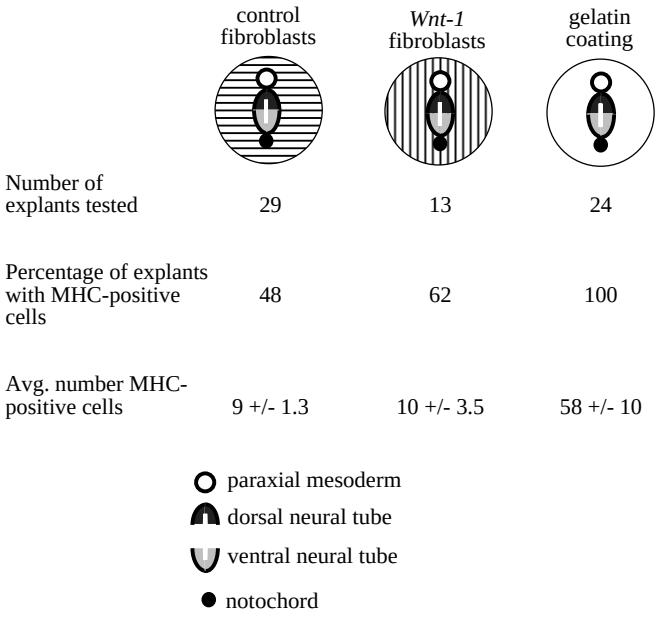

Fig. 6. Test for myogenic inhibition by fibroblasts. Paraxial mesoderm explants from stage 10-13 embryos were cultured next to the dorsal side of the neural tube/notochord on monolayers of control Rat-2/MV7 fibroblasts or Rat-2/*Wnt-1* fibroblasts. For comparison, data from similar cultures on gelatin is included (taken from Fig. 2, column 2). Paraxial mesoderm explants on either of the fibroblast cultures were much less responsive than those on gelatin.

response to Rat-2/*Wnt-1* fibroblasts, the average number of MHC-positive cells generated in cultures with 100% *Wnt-1* fibroblast monolayers was only 4. This response is much lower than the average of 21 and 49 MHC-positive cells achieved when somites and segmental plate explants were cultured with dorsal neural tube (Fig. 2C, column 4). The difference in myogenic activity between dorsal neural tube and cells expressing *Wnt-1* could mean that Rat-2/*Wnt-1* cells are able to induce myogenesis in only a small sub-population of potentially myogenic cells, or that insufficient Wnt-1 protein is produced to induce a robust myogenic response. Alternatively, *Wnt-1* could be a strong myogenic inducer, but the response might be inhibited by the fibroblast monolayer. To test the latter hypothesis, paraxial mesoderm explants were cultured in contact with the dorsal side of whole neural tube/notochord segments on monolayers of control or *Wnt-1*-expressing Rat-2 fibroblasts (Fig. 6). Only 48% of the cultures on control Rat-2 cells and 62% of those on Rat-2/*Wnt-1* cells exhibited a myogenic response. Each responding culture gave an average of only 9 to 10 MHC-positive cells. This result is in sharp contrast to the response of paraxial mesoderm to neural tube when the co-cultures are grown directly on gelatin-coated plastic; under those conditions, 100% of the explants exhibited myogenesis with an average of 58 MHC-positive cells per explant (Fig. 6). Data from co-cultures grown on Rat-2 cells are thus consistent with the hypothesis that the fibroblast monolayer inhibits myogenesis. Preliminary experiments with NIH3T3 fibroblasts, COS cells and chick embryo fibroblasts suggest that monolayers of these cells are also inhibitory to paraxial mesoderm myogenesis (data not shown).

One possible reason for the inhibition of myogenesis could be the secretion of basic fibroblast growth factor (bFGF) by the Rat-2 fibroblasts because bFGF has been shown to inhibit muscle differentiation in a number of systems (Gospodarowicz et al., 1976; Linkhart et al., 1980), and many fibroblasts express FGF (Moscatelli et al., 1986; Winkles et al., 1987). To test this hypothesis, 1-, 2- and 3-day conditioned media from control and *Wnt-1*-expressing Rat-2 fibroblasts were tested for FGF activity using a clonal mouse myoblast (MM14) bioassay (Clegg et al., 1987; Seed et al., 1988). The conditioned media had no mitogenic activity and did not repress differentiation of MM14 cells. Since the MM14 mitogenesis assay can detect as little as 3 pg/ml bFGF, the observed repression of somite/segmental plate myogenesis by Rat-2 fibroblasts is probably not due to secretion of FGF.

Heparin inhibits the myogenic inducing activity of dorsal neural tube

Previous studies have shown that heparin binds Wnt-1 protein and abrogates the biological activity of *Wnt-1* in mammary cell transformation assays (Bradley and Brown, 1990; Jue et al., 1992). If *Wnt-1* is an endogenous inducer of paraxial mesoderm myogenesis, heparin should inhibit the ability of dorsal neural tube to promote myogenesis. This hypothesis was tested by culturing contralateral pairs of somite and segmental plate explants with dorsal neural tube in the presence or absence of 200 μg/ml heparin (Table 2). 83% of the cultures without heparin responded to dorsal neural tube with an average of 45 MHC-positive cells. In contrast, only 43% of the heparin-treated cultures responded with an average of 9 MHC-

Table 2. Heparin inhibits the dorsal neural tube induction of paraxial mesoderm myogenesis

Tissues	Heparin	Number of explants tested	Number of explants with MHC-positive cells	Average number of MHC-positive cells‡
D O*	none	24	20 (83%)	43±12
D O*	200 μg/ml	24	10 (42%)	9±3
⊘†	none	23	23 (100%)	85±12
⊘†	200 μg/ml	23	23 (100%)	83±11

*Dorsal neural tube co-cultured with a somite (I to IV for stage 11 embryos and I to III for stage 12 and 13 embryos) or with an explant from the rostral half of the segmental plate.

†Somites V to XI from stage 12 to 15 embryos. These somites undergo myogenesis in vitro without neural tube/notochord (Stern and Hauschka, 1995).

‡Mean ± standard error of the mean.

positive cells. To verify that the decreased myogenesis in the presence of heparin was due to an inhibition of neural tube signaling and not simply an inhibitory effect on myogenesis itself, an additional experiment was performed. Using stage 12-15 embryos, contralateral pairs of older somites (V-XI), which are committed to myogenesis but do not yet contain MHC-positive cells (Stern and Hauschka, 1995), were cultured with and without 200 μg/ml heparin. The percentage of explants containing MHC-positive cells and the average number of MHC-positive cells per explant were equivalent in the presence and absence of heparin (Table 2). Thus, heparin does not inhibit myogenesis per se, but rather interferes with the dorsal neural tube's induction of myogenesis. Although the interpretation of these experiments is complicated by the potential effects of heparin on other peptide factors, the results are consistent with the notion that *Wnt-1* (or other *Wnt* family members) plays a role in neural tube induction of paraxial mesoderm myogenesis.

DISCUSSION

The present study demonstrates that single somite and segmental plate explants exhibit significant myogenesis when cultured with dorsal neural tube. Although the myogenic inducing activity of ventral structures was minimal when tested alone, combinations of the ventral neural tube/notochord with dorsal neural tube resulted in increased numbers of MHC-positive cells in somites I-IV compared to cultures with dorsal neural tube alone. The inductive activity of dorsal neural tube could be mimicked in part by co-culturing somites and segmental plates with cells expressing *Wnt-1*.

Myogenic signals from the dorsal neural tube

Previous studies have examined the hypothesis that myogenic signals from the neural tube may exhibit dorsal/ventral polarity. Fan and Tessier-Lavigne (1994) cultured segmental plate mesoderm in contact with both dorsal and ventral regions of the neural tube. They demonstrated that segmental plate on the dorsal side exhibited *Pax 3*-positive cells. Because persistent *Pax 3* expression is associated with the dermomyotome (Goulding et al., 1994; Williams and Ordahl, 1994), their experiments demonstrated a positive role for dorsal neural tube in dorsal specification of the somite. Our studies agree with and extend their results by showing that the dorsal neural tube can induce MHC-positive cells, a marker for differentiated muscle. Our studies are also in general agreement with the somite myogenic inductive activity of dorsolateral neural tube demonstrated by Münsterberg and Lassar (1995). One important difference, however, is that our experiments indicate that the dorsal neural tube can induce myogenesis in segmental plate and somites I-IV without additional ventral signals. Our demonstration of a strong dorsal signal is in greater contrast to the work of Buffinger and Stockdale (1995) who detected weak positive myogenic signals from the dorsal neural tube and strong signals from the ventral neural tube. Reasons for the minor and major discrepancies among these studies may reflect multiple differences in experimental design (e.g. medium composition, single vs. groups of somites and location of neural tube-somite contact).

It is unclear whether the dorsal neural tube myogenic signal(s) is permissive (i.e. supports muscle precursor cell survival or proliferation) or instructive (i.e. recruits muscle precursors from a pleuripotent population of somite cells). Experiments in which the neural tube/notochord were removed from day-2 chick embryos resulted in somite cell death, suggesting that the neural tube/notochord supplies factors necessary for somite cell survival (Teillet and Le Douarin, 1983; Rong et al., 1992). Although it is possible that this putative survival factor(s) is necessary to maintain the myogenic lineage, that does not rule out the possibility that the neural tube/notochord provides instructive myogenic signals. In our experiments there does not appear to be any major differences in somite cell survival when comparing cultures with dorsal versus ventral neural tube, yet the dorsal neural tube is much more potent at promoting muscle development. Although this observation may support the possibility of an instructive interaction, it is by no means definitive. If the muscle precursor population were small compared to the total number of cells in the somite, there would not have been any obvious differences in cell health. Because our experiments cannot distinguish clearly between instructive and permissive signals, the term 'induction' encompasses both types of signals when used to describe this tissue interaction.

Myogenic signals from the ventral neural tube and notochord

The present study suggests that positive signals from the ventral neural tube alone or in combination with the notochord can weakly promote myogenesis within segmental plate and somites I-IV (Fig. 2B, C, columns 7 and 8), whereas the notochord tested alone can promote myogenesis only in somites (column 9), specifically somites II and older (Stern and Hauschka, 1995). Furthermore, our data demonstrate that dorsal and ventral neural tube signals may have combinatorial effects on somites I-IV because the number of MHC-positive cells observed with dorsal neural tube alone was 2- to 3-fold lower than when ventral neural tube/notochord was included in the culture (Fig. 2B,C, compare column 4 with columns 2, 5, 10 and 11). The demonstration of combinatorial signals for myogenesis in somites I-IV is in agreement with the results of Münsterberg and Lassar (1995). However, our results do not support combinatorial signaling for the segmental plate since the myogenic response to dorsal neural tube is not increased when ventral axial structures are included.

Some of the previous in vivo studies have demonstrated a negative myogenic signal from the notochord and ventral neural tube when these are placed lateral or medial to the segmental plate and the youngest somites (Pourquie et al., 1993; Brand-Saberi et al., 1993; Fan and Tessier-Lavigne, 1994). However, none of our experiments indicated a negative role for the notochord or a consistent negative effect with ventral neural tube.

When comparing results obtained in different experimental systems, it is important to reiterate that signals from the neural tube and notochord may involve a complex mix of positive and negative factors. The efficiency of factor production and the magnitude of myogenic responsiveness may be affected differentially by the assay conditions. This situation may explain why different experimental paradigms have led to apparently contradictory results. For example, chick limb muscle precursor cells are not equivalent in terms of their medium

requirements for differentiation. Some limb muscle colony-forming cells depend on an early in vitro exposure to conditioned medium or bFGF, whereas other muscle colony-forming cells do not (White et al., 1975; Seed and Hauschka, 1988). Like the limb myogenic precursors, it is possible that the myotomal precursors are not all equivalent in their response to the neural tube/notochord or in their medium requirements for determination and/or differentiation.

Wnt-1 is a candidate dorsal neural tube signal

The experiments reported here demonstrate that expression of *Wnt-1* in Rat-2, NIH3T3, and Rat-B1a fibroblasts promotes myogenesis in somite and segmental plate mesoderm (Table 1 and Fig. 4). Cells expressing *Wnt-1* have previously been shown to secrete Wnt-1 protein, the majority of which is found associated with the extracellular matrix or cell surface (Bradley and Brown, 1990; Papkoff and Schryver, 1990; Jue et al., 1992). The simplest interpretation of our results is that secreted Wnt-1 protein directly induces paraxial mesoderm myogenesis. However, we cannot rule out the possibility that *Wnt-1* acts in an autocrine fashion on the fibroblasts, causing the release of an unknown myogenic factor which is not produced in sufficient quantities by the control fibroblasts.

Although *Wnt-1*-expressing fibroblasts promote myogenesis in paraxial mesoderm explants, the average number of MHC-positive cells is only four, whereas cultures containing dorsal neural tube exhibit 5- to 10-fold more MHC-positive cells. A number of reasons might explain why the response to cells expressing *Wnt-1* is not more robust. First, we have demonstrated that Rat-2 fibroblasts are inhibitory to paraxial mesoderm myogenic induction by neural tube/notochord (Fig. 6). In view of this inhibition, the weak positive response to *Wnt-1*-expressing fibroblasts is probably an underestimate of the ability of *Wnt-1* expression to induce paraxial mesoderm myogenesis. Second, it is possible that the mouse *Wnt-1* used in our experiments is not optimal for chick cells due to subtle species differences. However, this possibility seems unlikely since mouse and *Drosophila Wnt-1* orthologs are functionally redundant in other assays (Ramakrishna and Brown, 1993), and since the sequence of Wnt-1 protein is highly conserved among vertebrates (Nusse and Varmus, 1992). Third, other *Wnt* family members expressed in the dorsal neural tube could be involved in promoting somite myogenesis (see below). Finally, the weak myogenic response to *Wnt-1*-expressing cells may reflect the possibility that *Wnt-1* is only one of many factors that promote paraxial mesoderm myogenesis. A requirement for multiple different factors is observed in other systems. For example, chick limb bud anterior-posterior axis determination is thought to require a number of factors including *Wnt*, FGF, and *hedgehog* family members (Parr and McMahon, 1995; Yingzi and Niswander, 1995). Similarly, mesoderm induction and patterning in *Xenopus* is thought to involve combinations of FGF, transforming growth factor-β (TGF-β), and possibly *Wnt* and *noggin* family members (Cornell et al., 1995). The same could be true for somite patterning and differentiation. In support of this possibility is the finding that combinations of bFGF and TGF-β1 can also promote myogenesis in paraxial mesoderm (Stern and Hauschka, unpublished data). In addition, the neural tube expresses numerous factors other than *Wnts*, such as bFGF, dorsalin-1, and Sonic hedgehog (Kalcheim and Neufeld, 1990; Basler et al., 1993; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). Of these, the TGF-β family member, dorsalin-1, is specifically localized to the dorsal neural tube (Basler et al., 1993).

The demonstration that Rat-2/*Wnt-1*, NIH3T3/*Wnt-1*, and Rat-B1a/*Wnt-1* cells can promote paraxial mesoderm myogenesis in vitro suggests that *Wnt-1*, or other members of the *Wnt* family, could mediate myogenesis in vivo. A number of results support this possibility. First, *Wnt-1* is expressed in the dorsal neural tube (Parr et al., 1993; Bally-Cuif and Wassef, 1994; Dickinson et al., 1995; Hollyday et al., 1995) as are *Wnt-3* and *Wnt-3a* (Parr et al., 1993; Hollyday et al., 1995) which have activity similar to *Wnt-1* in mammary cell transformation (Roelink et al., 1990; Wong et al., 1994). These observations correlate with the potent dorsal myogenic activity demonstrated in Fig. 2. Second, heparin diminishes the ability of the dorsal neural tube to promote paraxial mesoderm myogenesis. Although effects of heparin are not specific to Wnt proteins, this observation is consistent with an endogenous role for *Wnts* since heparin inhibits Wnt-1 activity (Jue et al., 1992; Bradley and Brown, 1995).

Although our evidence suggests that *Wnt-1* could be involved in paraxial mesoderm myogenesis, no muscle effect has been reported in *Wnt-1* or *Wnt-3a* knockout mice (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; Takada et al., 1994). However, since these and other functionally redundant *Wnts* are expressed in dorsal neural tube (Roelink and Nusse, 1991; Parr et al., 1993; Wong et al., 1994; Hollyday et al., 1995), it seems likely that they may substitute for each other in myogenic induction. Furthermore, as discussed above, additional non-*Wnt* factors may mediate the dorsal myogenic inducing signal in vivo.

Wnt-1 signaling in other systems has been shown to modulate steady-state levels of the intracellular adhesion-related proteins β or γ catenin, and in *Drosophila* there is genetic evidence implying that such changes are instrumental in *Wnt* signaling (Bradley et al., 1993; Hinck et al., 1994; Peifer et al., 1994). Although in some cases *Wnt-1* expression results in increased cell-cell adhesion, this may not be universally true, and recent evidence suggests that β-catenin may have a novel signaling capacity independent of its role in adhesive complexes (Funayama et al., 1995). It is unclear whether the induction of myogenesis in somites or segmental plate normally involves changes in cell adhesion. Indeed, the existing evidence suggests that cell-cell adhesion may not be required for this process since dissociation of segmental plate cells by trypsinization can result in spontaneous myogenesis in vitro in the absence of external inducers (George-Weinstein et al., 1994). In certain tissues in vivo, *Wnt-1* expression results in mitogenic effects (Tsukamoto et al., 1988; Dickinson et al., 1994). In the present system, however, there is no evidence of preferential growth of myogenic cells. Currently, therefore, the mechanism by which *Wnt-1* promotes muscle development in vitro remains obscure. These mechanistic questions will be easier to address once purified Wnt-1 protein is available and/or Wnt receptors are identified.

A model for neural tube/notochord induction of paraxial mesoderm myogenesis

Based on the evidence discussed, we propose the following model for axial structure dependent paraxial mesoderm myogenesis. For somites, signals from three sources have a positive

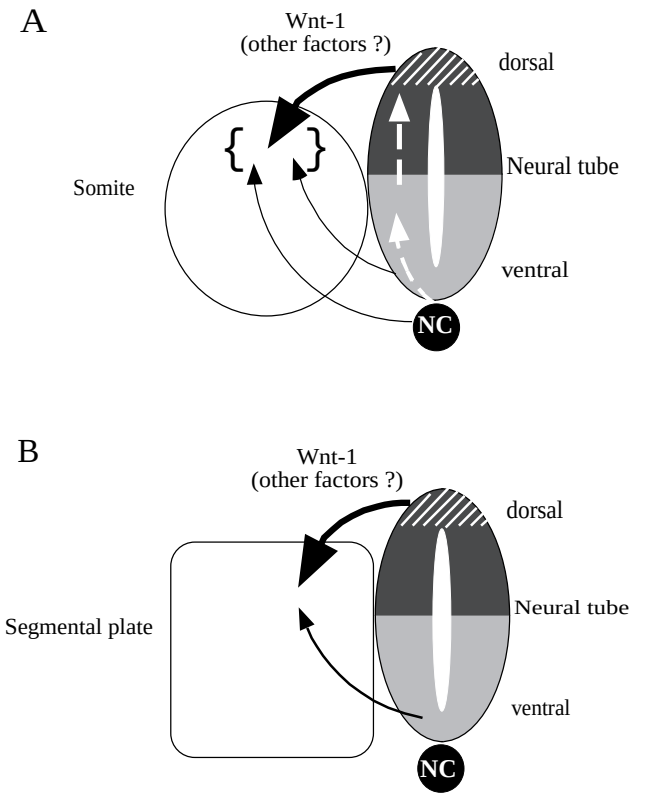

Fig. 7. Proposed models of axial structure-dependent paraxial mesoderm myogenesis. For somites (A) the major signal (thick, solid arrow) comes from the dorsal neural tube and may be mediated in part by *Wnt-1* or other *Wnts* in this region of the neural tube such as *Wnt-3a*. The expression pattern of *Wnt-1* and *Wnt-3a* as determined by Hollyday et al. (1995) is indicated by white hatching. Weaker myogenic signals (thin, solid arrows) emanate from the ventral neural tube and notochord and act directly on somites. These ventral signals may interact in some way (bracket) with the dorsal signal causing a synergistic increase in the number of MHC-positive cells induced. Alternatively, the synergy could be due to intra-axial signaling. For example, the ventral neural tube/notochord might signal the dorsal neural tube to boost the dorsal signal (white broken arrows). For segmental plate tissue (B), the dorsal signal remains the major signal (thick arrow) and could be mediated in part by *Wnt-1* or other *Wnts*. The ventral neural tube may emit a weak positive myogenic signal (thin arrow), but our study shows no evidence for synergy between dorsal and ventral signals for segmental plate myogenesis. NC, notochord.

effect on myogenesis (Fig. 7A). The most potent signal emanates from the dorsal neural tube and could be mediated, at least in part, by *Wnt-1* or by another *Wnt* family member such as *Wnt-3a*. The expression pattern of chick *Wnt-1* and *Wnt-3a* during the stage 10 to 13 period that neural tubes were removed for our studies in Fig. 2 is indicated in Fig. 7A by the white hatching (Hollyday et al., 1995). In addition to the dorsal signal, somites exhibit myogenic responsiveness to the notochord and ventral neural tube. Because the ventral neural tube and notochord have a mild positive myogenic effect when tested alone, the model illustrates signals from the ventral structures (thin solid arrows) that are independent from the dorsal signal. It is of interest that *Wnt-1* and *Wnt-3a* are not expressed in the ventral half of the closed neural tube in stage 9 to 13 embryos. This could suggest another factor is responsible for the ventral inductive signals. However, since *Wnt-7a* and *Wnt-7b* are expressed in the ventral half of the closed neural tube at later stages, (Dealy et al., 1993; Hollyday et al., 1995), it is possible that these *Wnts* are upregulated in vitro sometime during the 3-day culture period. No *Wnts* have been found in the notochord, and therefore other factors are likely responsible for mediating the notochord signal.

Experiments in which both ventral neural tube/notochord and dorsal neural tube are cultured with somites demonstrate a synergistic effect with respect to the number of MHC-positive cells induced. Although this effect could be due to an interaction between the ventral and dorsal signals (brackets, Fig. 7A), it is also possible that the ventral neural tube/notochord influences the dorsal neural tube (white broken arrows) in a manner which increases the potency of the dorsal signal(s). These possibilities are in agreement with the models proposed by Münsterberg and Lassar (1995). Because *Sonic hedgehog* (*Shh*) is expressed in the notochord and ventral neural tube (floor plate) (Yamada et al., 1991; Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994), and since *Shh*-expressing cells may have a positive influence on somite myogenesis (Johnson et al., 1994), it is tempting to speculate that the positive notochord and ventral neural tube signals may be mediated by *Shh*. Consistent with that hypothesis is the observation that the floor plate and notochord continue to express *Shh* after 5 days in culture (Münsterberg and Lassar, 1995). The greater myogenic response when ventral neural tube and notochord are tested together than when ventral neural tube is tested alone could be due to the inability of the floor plate to maintain *Shh* expression in the absence of the notochord.

For segmental plate tissue (Fig. 7B), dorsal neural tube was a more potent myogenic inducer than ventral neural tube, and this dorsal signal may be mediated, at least in part, by *Wnt-1* or perhaps *Wnt-3a*. These observations are similar to results with somite tissue. In contrast, the signals from the notochord and ventral neural tube appear somewhat different when tested with segmental plate. The notochord is unable to promote segmental plate myogenesis (Fig. 2; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995), and cultures of segmental plate with both dorsal neural tube and ventral neural tube/notochord were no more responsive than cultures with just the dorsal neural tube, indicating that there is no interaction between dorsal and ventral signals as was observed with somites. Nevertheless, there was a very mild myogenic response when the ventral neural tube was tested alone or with notochord; consequently a weak positive signal is illustrated by a thin arrow from the ventral neural tube in Fig. 7B.

These models present a potentially complex array of signals involved in paraxial mesoderm myogenic induction. Moreover, it should be noted that this study has focused only on myogenic signals from axial tissues. Additional experiments suggest that non-axial tissues such as the ectoderm might also play a positive role in somite myogenic induction (Kenny-Mobbs and Thorogood, 1987; Fan and Tessier-Lavigne, 1994). The apparent complexity of signaling in paraxial mesoderm myogenesis is not surprising since the interaction may involve spatial components which determine the dorsal/ventral and medial/lateral polarity of the somite as well as inductive components which either instruct or maintain cells in the proper spatial domain to become differentiated muscle.

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