

## Synergistic interactions between bFGF and a TGF- $\beta$ family member may mediate myogenic signals from the neural tube

Howard M. Stern, Jennifer Lin-Jones and Stephen D. Hauschka\*

University of Washington, Department of Biochemistry, Box 357350, Seattle, WA 98195, USA

\*Author for correspondence (e-mail: haus@u.washington.edu)

### SUMMARY

Development of the myotome within somites depends on unknown signals from the neural tube. The present study tested the ability of basic fibroblast growth factor (bFGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and dorsalin-1 (*dsl-1*) to promote myogenesis in stage 10-14 chick paraxial mesoderm utilizing 72 hour explant cultures. Each of these factors alone and the combination of bFGF with *dsl-1* had limited to no myogenic-promoting activity, but the combination of bFGF with TGF- $\beta$ 1 demonstrated a potent dose-dependent effect. In addition, bFGF enhanced the survival/proliferation of somite cells. 98% of stage 10-11 caudal segmental plate explants treated with bFGF plus TGF- $\beta$ 1, exhibited myosin heavy chain (MHC)-positive cells (avg.=60 per explant), whereas only 15% of similarly treated somites responded with an average of 5 MHC-positive cells. Thus at stage 10-11, there are rostrocaudal differences in myogenic responsiveness with the caudal (more 'immature') paraxial mesoderm being more myogenically responsive to these factors than are somites. It was also discovered that 17% of stage 10-11 caudal segmental plate explants exhibited several MHC-positive

cells even when cultured without added growth factors, further demonstrating a different myogenic potential of the caudal paraxial mesoderm. Stage 13-14 paraxial mesoderm also exhibited a myogenic response to bFGF/TGF- $\beta$ 1 but, unlike stage 10-11 embryos, both somites and segmental plate exhibited a strong response. A two-step mechanism for the bFGF/TGF- $\beta$ 1 effect is suggested by the finding that only TGF- $\beta$ 1 was required during the first 12 hours of culture, whereas bFGF plus a TGF- $\beta$ -like factor were required for the remainder of the culture. The biological relevance of the findings with bFGF is underscored by the observation that a monoclonal antibody to bFGF inhibited myogenic signaling from the dorsal neural tube. However, a monoclonal antibody that can neutralize the three factors TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 did not block myogenic signals from the neural tube, raising the possibility that another TGF- $\beta$  family member may be involved in vivo.

Key words: somite, neural tube, fibroblast growth factor, FGF, transforming growth factor, TGF, myogenesis, chick embryo

### INTRODUCTION

Numerous studies have shown that the neural tube promotes myogenesis in paraxial mesoderm. In ovo studies have demonstrated that neural tube (or in some cases notochord) is critical for myotome development within somites of day-2 chick embryos (Muchmore, 1951; Strudel, 1955; Teillet and Le Douarin, 1983; Rong et al., 1992; Christ et al., 1992; Pownall et al., 1996; Bober et al., 1994; Spence et al., 1996). This tissue interaction has also been demonstrated in various culture systems (Avery et al., 1956; Kenny-Mobbs and Thorogood, 1987; Vivarelli and Cossu, 1994; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995; Spence et al., 1996; Gamel et al., 1996). Depending upon the specific experimental system, relatively 'stronger' myogenic signals have been demonstrated for both dorsal and ventral portions of the neural tube/notochord complex. For example, in vivo and in vitro studies by Fan and Tessier-Lavigne (1994), Stern et al. (1995) and Spence et al. (1996) have indicated that myogenic signals are localized primarily to the dorsal half of the chick neural tube. A key role for the dorsal

neural tube is further illustrated by the mouse open brain mutation which disrupts normal development of the dorsal neural tube. This mutation prevents formation of the myotomal (epaxial) muscles even though the limb (hypaxial) muscles develop normally (Spörle et al., 1996). However, other studies indicate that the myogenic response of somites to dorsal neural tube is enhanced by the ventral neural tube and notochord (Münsterberg and Lassar, 1995; Stern et al., 1995), and both in vivo and in vitro studies have demonstrated a critical role for the ventral neural tube/notochord (Buffinger and Stockdale, 1995; Pownall et al., 1996). Together, these data suggest a combinatorial mechanism involving signals from the dorsal and ventral neural tube/notochord (Stern et al., 1995; Münsterberg and Lassar, 1995).

At present, little is known about the identity of the signaling pathways that promote myogenesis. Previous studies of candidate factors capable of eliciting a somite myogenic response suggested that *Wnt-1*, *Wnt-3* and *Wnt-4* can partially substitute for the dorsal neural tube signal, because cells expressing these *Wnts* promote paraxial mesoderm myogenesis (Stern et al., 1995; Münsterberg et al., 1995). Nevertheless,

it is possible that additional dorsal factors are required to promote a full complement of myotomal cells, because *Wnt-1*-expressing cells were not as effective at promoting myogenesis as was dorsal neural tube (Stern et al., 1995). Several investigators have also demonstrated that Sonic Hedgehog is a candidate factor for the ventral neural tube notochord myogenic signal (Johnson et al., 1994; Münsterberg et al., 1995; Borycki, Hart and Emerson, personal communication).

The aim of the present study was to assess the myogenic-inducing ability of several additional candidate factors (or related homologs), which are expressed within the dorsal neural tube during the developmental periods when somitic cells are becoming myogenic. Of particular interest were FGF and TGF- $\beta$  family members. FGFs play a role in a number of relevant tissue interactions, e.g., the induction of mesoderm and muscle tissue in *Xenopus* animal caps (Slack et al., 1987; Kimelman and Kirschner, 1987). In culture, bFGF is a mitogen for myoblasts and it represses the onset of terminal muscle differentiation (Gospodarowicz et al., 1976; Linkhart et al., 1980, 1981). In addition, a population of muscle colony-forming cells in the chick limb bud is dependent on bFGF to enter and/or continue the myogenic program (Seed and Hauschka, 1988). FGF family members may play a similar role in promoting somite myogenesis.

TGF- $\beta$  family members such as activin and Vg1 also induce mesoderm (Smith et al., 1990; Thomsen et al., 1990; Asashima et al., 1990; Thomsen and Melton, 1993) and, although TGF- $\beta$ 1 cannot induce mesoderm on its own in *Xenopus* animal cap assays, it acts synergistically with bFGF (Kimelman and Kirschner, 1987). Recent studies have also demonstrated a delay in the differentiation of C2C12 myoblasts expressing a truncated type II TGF- $\beta$  receptor, suggesting a positive role for TGF- $\beta$ s in muscle development (Filvaroff et al., 1994). For these reasons, we tested TGF- $\beta$ 1 for myogenic activity despite the fact that it is not known to be expressed in the dorsal neural tube. In addition, *dsl-1* was of specific interest in the present study, because it is a TGF- $\beta$  family member expressed in the dorsal portion of stage 10 chick neural tube (Basler et al., 1993), a region that exhibits potent myogenic-promoting activity (Fan and Tessier-Lavigne, 1994; Stern et al., 1995; Spence et al., 1996).

Our study demonstrates that bFGF and TGF- $\beta$ 1 act cooperatively to promote paraxial mesoderm myogenesis in a dose-dependent manner and that antibodies to bFGF block the myogenic response of paraxial mesoderm to neural tube signals. Consequently, members of these families should be considered candidates for mediating myogenic signals from the neural tube. Interestingly, although *dsl-1* is expressed at the right time and place to play a role in somite myogenesis, it does not have myogenic activity in our single somite/segmental plate assay system when tested alone or in combination with bFGF. It was also found that bFGF promotes paraxial mesoderm cell survival/proliferation, supporting the possibility that the mechanism of myogenic signaling could, at least in part, involve maintenance/proliferation of the myogenic lineage.

## MATERIALS AND METHODS

### Somite numbering

Somites are referred to by Roman numerals increasing in a caudal-to-

rostral manner, starting with 'I' for the most recently formed somite as previously described (Ordahl, 1993).

### Dissections

White Leghorn chicken eggs (H and N International) were incubated in a 38°C forced draft incubator at 100% humidity for 2 days. Stage 10-14 embryos (Hamburger and Hamilton, 1951) were utilized for dissections. The somites and segmental plates were removed as previously described (Stern et al., 1995). Whole segmental plates were cut into rostral and caudal halves and then each half was divided transversely into four approximately equal pieces. In some cases, somite-sized fragments of dorsal sections of the neural tube were obtained as previously described (Stern et al., 1995).

### Tissue culture

Chick tissue explants were placed in gelatin-coated 96-well plates containing 200  $\mu$ l supplemented Ham's F10 medium with 1% chick embryo extract (CEE) (Stern and Hauschka, 1995). Cultures were incubated for 3 days at 37°C, 5% CO<sub>2</sub> and 100% humidity. Stock and serially diluted solutions of human recombinant bFGF and acid-activated human recombinant TGF- $\beta$ 1 (kindly provided by Bristol-Myers Squibb) were made in explant culture medium at 600 ng/ml and 450 ng/ml, respectively, and used within 3 weeks. Appropriate volumes of the stock or serially diluted solutions were added to culture wells to achieve a particular dose. In some cases, the factors were removed from explant cultures by rinsing three times with F10C followed by three rinses in fresh explant culture medium. During the rinsing process, 50  $\mu$ l of fluid was always left in the well to avoid cell loss due to surface tension effects. Following addition of the fresh culture medium, this rinsing procedure should achieve about a 16,000-fold reduction of the initial growth factor concentration. A monoclonal antibody that neutralizes bFGF activity (Sigma) and a single monoclonal antibody that neutralizes TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 (Genzyme) were each provided in 0.01 M PBS, pH 7.4 and were utilized at final medium concentrations of 40  $\mu$ g/ml to test for inhibition of myogenic signals from the neural tube. The concentration of bFGF in chick embryo extract was determined via the MM14 mouse myoblast bioassay as 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 et al., 1995). The color reaction utilized diaminobenzidine as the peroxidase substrate, which stains immunopositive cells brown. Cells were counted as MHC-positive only if they stained intensely brown. Many cultures also contained a subpopulation amounting to no greater than 1% of the cells that exhibited gradations of much lighter tan staining. These cells, whose identity is unclear, tended to appear at the periphery of the cultures. Since the relative proportions of such cells were not correlated with any of the experimental manipulations or dose-response studies, their exclusion from the tabulated MHC-classification does not unduly bias the data.

### Production of dorsalin-1 (*dsl-1*)

COS cells were transfected with the expression plasmid pMT21 (kindly provided by Dr Thomas Jessell) containing the cDNA for myc-tagged *dsl-1* (Basler et al., 1993). COS cells were also mock transfected without any DNA constructs for use as a negative control. The lipofectamine method was utilized according to the manufacturers recommendations and it yielded 20-24% transfected cells based on control transfections with *lacZ*. COS cells were cultured in 150 mm dishes and maintained in DMEM plus 10% FCS and 0.05 mg/ml gentamicin. After transfection, the medium was changed to the explant culture medium described in a previous section and the transfected COS cells were allowed to condition this medium for 2 days. The conditioned media were tested at various concentrations (1%-

100%) for myogenic activity either alone or in combination with 30 ng/ml bFGF.

Dsl-1 was isolated from conditioned medium as described previously (Basler et al., 1993). 25 ml of the myc-tagged *dsl-1*-containing medium and 25 ml of mock-transfected conditioned medium were incubated with 9E10 epitope myc antibody-conjugated agarose beads (Santa Cruz Biotechnology) followed by rinsing in F10C and then elution with 0.1 M glycine (pH 2.5). The eluate was then concentrated to a final volume of 100-130  $\mu$ l in a Centricon-10 microconcentrator (Amicon).

The presence of *dsl-1*-myc in the conditioned medium and affinity-purified eluate was verified by western blot utilizing 9E10 epitope myc antibodies (Santa Cruz Biotechnology). The clonal, mouse MC3T3-E1 osteoblast cell line has been shown to exhibit increased proliferation and expression of alkaline phosphatase activity when treated with bone morphogenetic proteins (BMPs) such as OP-1/BMP-7 (Asahina et al., 1996). Because *dsl-1* exhibits homology with BMPs, the MC3T3-E1 cell line was used to verify that the *dsl-1* that we produced was active by comparing induction of alkaline phosphatase activity in MC3T3-E1 osteoblast cultures treated with *dsl-1*-conditioned medium versus the mock controls. The methods for this assay were as previously described (Asahina et al., 1996). (Dorsalin assays in MC3T3-E1 cells were kindly performed by Dr P. Hauschka and E. Skazkina, Children's Hospital, Boston.)

### Statistics

Statistical analysis of differences in the mean number of MHC-positive cells per explant was conducted via the Student's *t*-test. *P* values for such comparisons are indicated in the text.

## RESULTS

### Myogenic commitment status of paraxial mesoderm

Since the goal of these studies was to identify neural tube factors that might be involved in myogenic induction, an initial set of experiments was designed to examine the myogenic commitment status of different paraxial mesoderm regions and stages that could be used for subsequent factor assays. Previous

experiments had shown that explant cultures of somites I-IV from stage 8-11 embryos and the rostral half of the segmental plate from stage 10-13 embryos do not contain any myosin heavy chain (MHC)-positive cells after up to 7 days in culture (Stern and Hauschka, 1995; Stern et al., 1995). However, the commitment status of caudal segmental plate explants had not been tested beyond 2 days (Stern and Hauschka, 1995). Surprisingly, we found that 17% of 3-day explants from the caudal segmental plate half of stage 10-11 embryos exhibited a low number of MHC-positive cells (avg.=3, Table 1A, minus factors) and that 33% of explants from the most caudal eighth of the segmental plate contained MHC-positive cells. In contrast and consistent with previous experiments, 3-day cultures of somites I-IV and rostral segmental plate explants did not exhibit any MHC-positive cells when cultured without additional factors (Table 1A). Paraxial mesoderm explants from stage 13-14 embryos exhibited a pattern of myogenic commitment that is similar to that of somite and rostral segmental plate of stage 10-11 embryos, and only a single caudal segmental plate explant showed a trace of MHC-positive cells (Table 1B, minus factors). Thus, at stage 10-11, a small fraction of cells in the segmental plate region that is the least mature with respect to somitogenesis has a 'cryptic' myogenic potential that is not exhibited by cells in more rostral segmental plate and somites I-IV as tested by this *in vitro* assay system. These data suggested that stage 10-11 caudal segmental plate explants might be more responsive in subsequent factor assays.

### Myogenic effects of bFGF on paraxial mesoderm

To test for positive myogenic activity on paraxial mesoderm, somite or segmental plate explants from stage 10-11 embryos were cultured in 10 ng/ml bFGF. This treatment has only a limited effect on myogenesis (Table 1A). Experiments performed at bFGF doses as high as 100 ng/ml or as low as 1 ng/ml did not result in a greater myogenic effect. To determine

**Table 1A. Stage 10-11 paraxial mesoderm myogenesis in the presence and absence of bFGF and TGF- $\beta$ 1**

Tissue	minus factors		10 ng/ml bFGF		10 ng/ml TGF- $\beta$ 1		10 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1					
	<i>n</i>	% <sup>(a)</sup>	$\bar{x}$ <sup>(b)</sup>	<i>n</i>	%	$\bar{x}$	<i>n</i>	%	$\bar{x}$			
		MHC + explants	MHC + cells		MHC + explants	MHC + cells		MHC + explants	MHC + cells	MHC + explants	MHC + cells	
somite <sup>(c)</sup>	20	0	—	32	3	6	24	0	—	20	15	4.7 $\pm$ 2.2
rostral seg. plate	20	0	—	32	6	4 $\pm$ 1	24	0	—	30	53	9.6 $\pm$ 3
caudal seg. plate	24	17	3 $\pm$ 1	32	25	6 $\pm$ 1	24	21	3 $\pm$ 1	45	98	59.6 $\pm$ 6.8

**Table 1B. Stage 13-14 paraxial mesoderm myogenesis in the presence and absence of bFGF and TGF- $\beta$ 1**

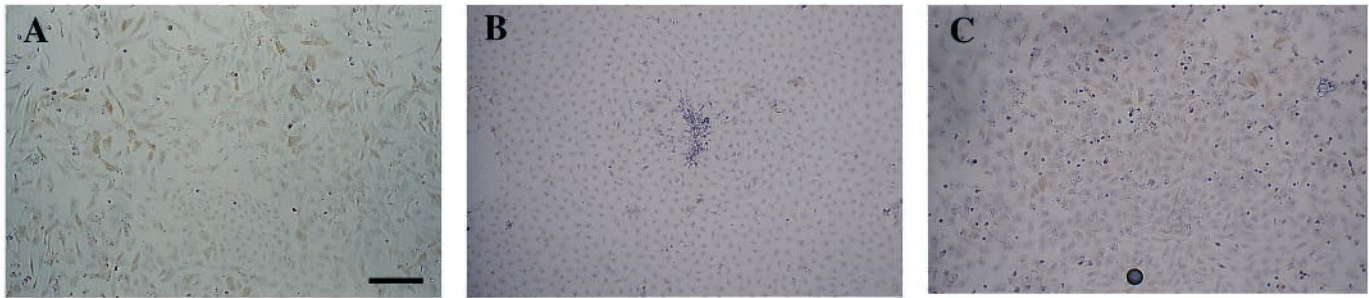
Tissue	minus factors		10 ng/ml bFGF		10 ng/ml TGF- $\beta$ 1		10 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1					
	<i>n</i>	% <sup>(a)</sup>	$\bar{x}$ <sup>(b)</sup>	<i>n</i>	%	$\bar{x}$	<i>n</i>	%	$\bar{x}$			
		MHC + explants	MHC + cells		MHC + explants	MHC + cells		MHC + explants	MHC + cells	MHC + explants	MHC + cells	
somite <sup>(d)</sup>	13	0	—	15	27	4 $\pm$ 1.4	15	40	2.3 $\pm$ 0.4	18	100	49 $\pm$ 12.6
rostral seg. plate	20	0	—	20	30	2 $\pm$ 0.4	20	5	4	24	100	51 $\pm$ 7.3
caudal seg. plate	20	5	2	20	15	2 $\pm$ 0.8	20	25	5.4 $\pm$ 2.2	24	96	43 $\pm$ 7.2

(a) Percentage of explants with MHC-positive cells.

(b) Mean number of MHC-positive cells per positive explant.

(c) Somites I-IV.

(d) Somites I-III from stage 13 and somite I from stage 14. At these stages, more rostral somites often contain some cells committed to myogenesis (Stern and Hauschka, 1995).



**Fig. 1.** Paraxial mesoderm explants from a stage 13 embryo cultured without factors (A, somite I), with 30 ng/ml bFGF (B, somite I), or 10 ng/ml TGF- $\beta$ 1 (C, somite II). Notice that the cell numbers are greater in the bFGF-treated culture than in the TGF- $\beta$ 1 or untreated cultures. Scale bar, 150  $\mu$ m.

if tissues from older embryos would exhibit a more robust response to bFGF, similar experiments were performed with stage 13-14 embryos. As with the stage 10-11 tissues, only a limited myogenic response was observed (Table 1B). A 10-fold decrease or increase in dose (to 1 or 100 ng/ml) did not augment the myogenic effect. Thus, bFGF has a minimal ability to promote paraxial mesoderm myogenesis, but cannot mimic the robust response observed with neural tube cocultures (Stern and Hauschka, 1995; Stern et al., 1995).

#### Myogenic effect of TGF- $\beta$ 1 on paraxial mesoderm

Acid-activated TGF- $\beta$ 1, at doses between 1 and 100 ng/ml, did not affect myogenesis in either somites I-IV or rostral or caudal segmental plate from stage 10-11 embryos (Table 1A). Interestingly, tissues from slightly older (stage 13-14) embryos were responsive to TGF- $\beta$ 1 (Table 1B), but the myogenic effect was quite minimal compared to effects observed with neural tube (Stern and Hauschka, 1995; Stern et al., 1995).

#### Synergistic effects of bFGF and TGF- $\beta$ 1 on paraxial mesoderm myogenesis

Although bFGF and TGF- $\beta$ 1 alone have only limited myogenic activity, it seemed possible that these two factors might act synergistically, as has been shown previously in the induction of mesoderm in *Xenopus* animal cap assays (Kimelman and Kirschner, 1987). To test this hypothesis, paraxial mesoderm from stage 10-11 embryos was cultured for 3 days with 10 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1. 15% of somites I-IV, 53% of rostral segmental plate explants and 98% of caudal segmental plate explants exhibited MHC-positive cells (Table 1A) in the presence of both factors. Furthermore, the average number of MHC-positive cells per responding explant was greater than that obtained with either factor alone, particularly for the caudal segmental plate, which had an average of 60 MHC-positive cells per explant compared to only 3 without factors or 3-6 with bFGF or TGF- $\beta$ 1 tested alone.

It was surprising to observe such a strong myogenic response from 'immature' paraxial mesoderm (caudal segmental plate) and a progressively weaker response from increasingly mature paraxial mesoderm (somites) of stage 10-11 embryos. To determine whether this pattern of myogenic responsiveness also occurs in more mature embryos, stage 13-14 paraxial mesoderm explants were treated with 10 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1. 95-100% of all paraxial mesoderm explants from these older stages exhibited a myogenic response with an average of about 45-50 MHC-

positive cells, regardless of their rostrocaudal position of origin (Table 1B). Examples of myogenic responsiveness to these factors are presented in Fig. 2. Thus, bFGF and TGF- $\beta$ 1 exhibit synergy with respect to promotion of paraxial mesoderm myogenesis in older (stage 13-14) as well as younger (stage 10-11) embryos. However, the pattern of response differs: somites and rostral segmental plate explants from stage 13-14 embryos exhibited 5- to 10-fold greater responses than those of similar tissues from stage 10-11 embryos.

In addition to the dramatic effect of bFGF and TGF- $\beta$ 1 on myogenesis, these factors also affected cell survival/proliferation during the 3 day culture period (Table 2, Fig. 1). Analysis of bFGF and TGF- $\beta$ 1 effects both alone and in combination indicated that bFGF was responsible for enhanced cell survival/proliferation since the mean number of cells in cultures with bFGF alone was equivalent to that in cultures with both factors. Since myogenesis was not promoted by bFGF alone (Table 1) and since TGF- $\beta$ 1 alone did not affect cell survival/proliferation (Table 2), the myogenic effect observed when both factors are present does not appear to be due solely to an effect on cell survival/proliferation.

#### The myogenic response to combinations of bFGF and TGF- $\beta$ 1 is dose dependent

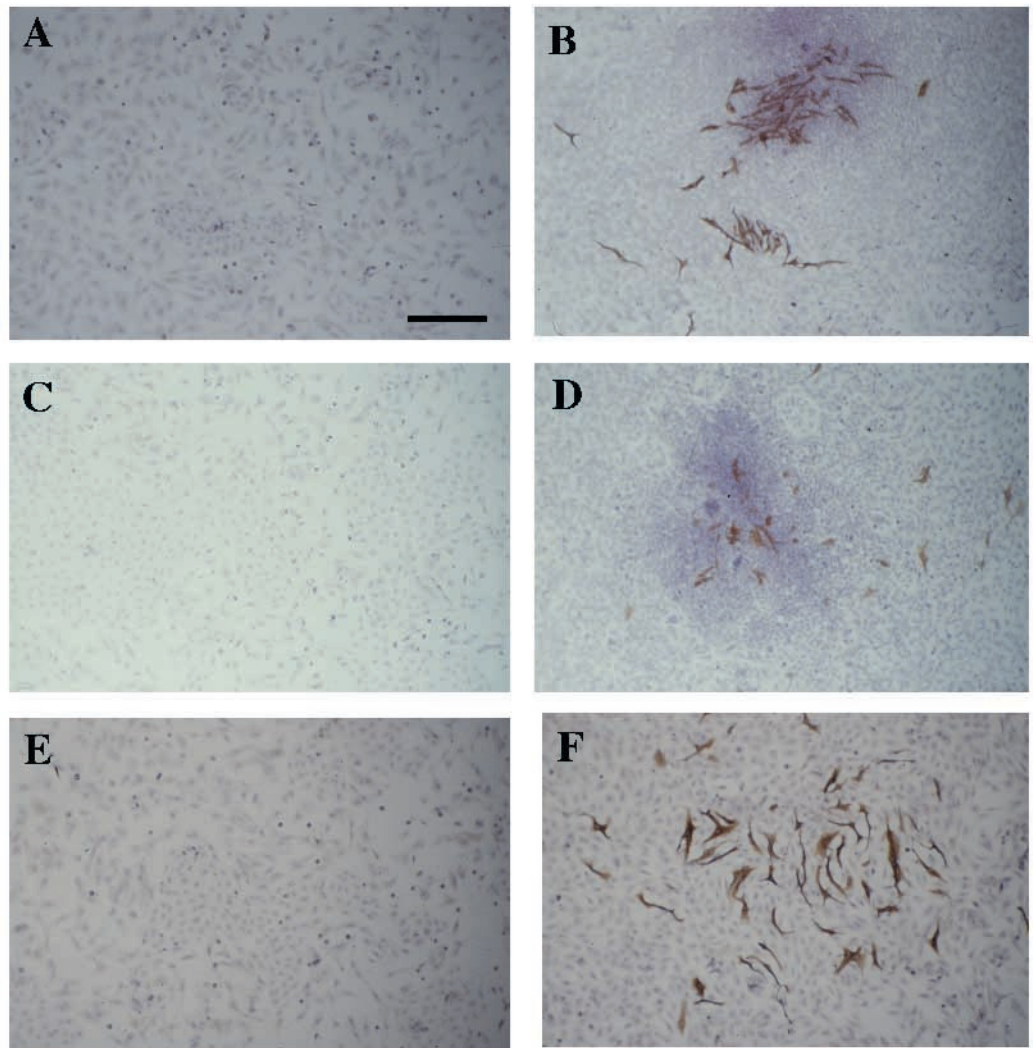
One possible explanation for the lower responsiveness of stage 10-11 somites and rostral segmental plate to bFGF/TGF- $\beta$ 1 is that the dose of either factor may not have

**Table 2. Basic FGF promotes survival and/or proliferation of paraxial mesoderm cells**

Condition <sup>(a)</sup>	<i>n</i>	Avg. number of cells <sup>(b)</sup>
no added factors	16	529 $\pm$ 30
10 ng/ml bFGF	12	1867 $\pm$ 220
10 ng/ml TGF- $\beta$ 1	8	573 $\pm$ 51
10 ng/ml bFGF plus 10 ng/ml TGF- $\beta$ 1	8	2276 $\pm$ 315

(a) The 'no added factor' condition utilized somites that were contralateral to the factor treated somites.

(b) Somites I-IV from stage 10-11 embryos were utilized for cell counts. Somites, rather than segmental plate pieces, were chosen for this study because of their discrete structure, therefore maximizing the likelihood that contralateral tissues had approximately the same number of cells prior to culture. Cell counts were performed on cultures fixed and stained after 3 days. Cell numbers are means  $\pm$  s.e.m. The following are results of *t*-tests performed on some of the conditions above: no factors compared to bFGF,  $P = 0.0001$ ; no factors compared to TGF- $\beta$ 1,  $P = 0.41$ ; bFGF compared to bFGF plus TGF- $\beta$ 1,  $P = 0.10$ .



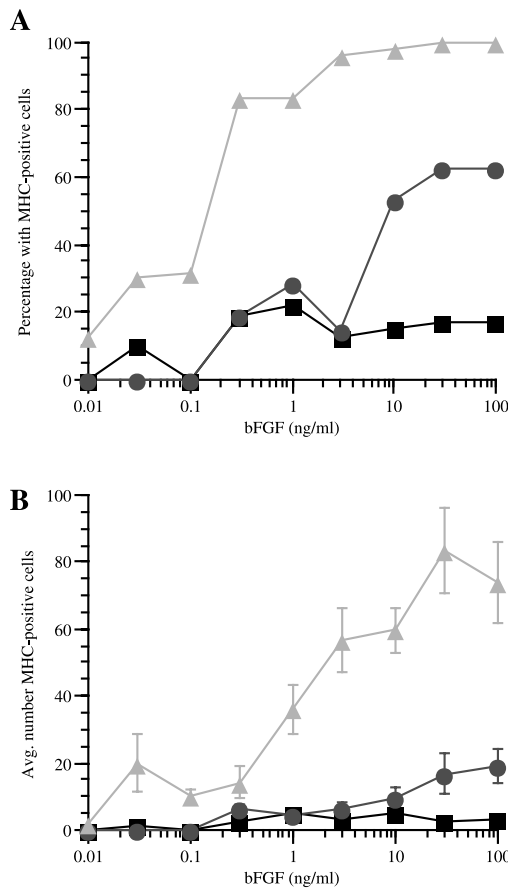
**Fig. 2.** Paraxial mesoderm explants from a stage 13 embryo were cultured for three days with (B, D, F) or without (A, C, E) 10 ng/ml bFGF plus 10 ng/ml TGF- $\beta$ 1 and were immunostained for myosin heavy chain. (A, B) somite II explants; (C, D) rostral segmental plate explants; (E, F) caudal segmental plate explants. Scale bar, 200  $\mu$ m.

been adequate. To address this possibility, a dose-response analysis for bFGF and TGF- $\beta$ 1 was performed on tissue from stage 10-11 embryos, holding one factor at 10 ng/ml and varying the dose of the other. The results are presented as two graphs, one indicating the percentage of paraxial mesoderm explants that responded (Figs 3A, 4A) and the second displaying the average number of MHC-positive cells in the subset of explants that exhibited a response (Figs 3B, 4B). The myogenic response of paraxial mesoderm increased as the dose of bFGF increased with TGF- $\beta$ 1 held constant at 10 ng/ml (Fig. 3). The maximal response was achieved at a bFGF concentration of approximately 30 ng/ml (Fig. 3B). The myogenic response to TGF- $\beta$ 1 (bFGF held constant at 10 ng/ml) also increased with increasing dose (Fig. 4). The maximum response to TGF- $\beta$ 1 occurred at about 10 ng/ml (Fig. 4B). These results demonstrate that the myogenic response of paraxial mesoderm is dose dependent with respect to both bFGF and TGF- $\beta$ 1. However, even at doses as high as 100 ng/ml of either growth factor, the caudal-to-rostral differences in responsiveness were maintained, suggesting that the differential response of stage 10-11 paraxial mesoderm regions are not simply due to differences in dose requirements for bFGF and/or TGF- $\beta$ 1.

#### **Paraxial mesoderm myogenesis requires long-term exposure to bFGF but requires only short-term exposure to TGF- $\beta$ 1**

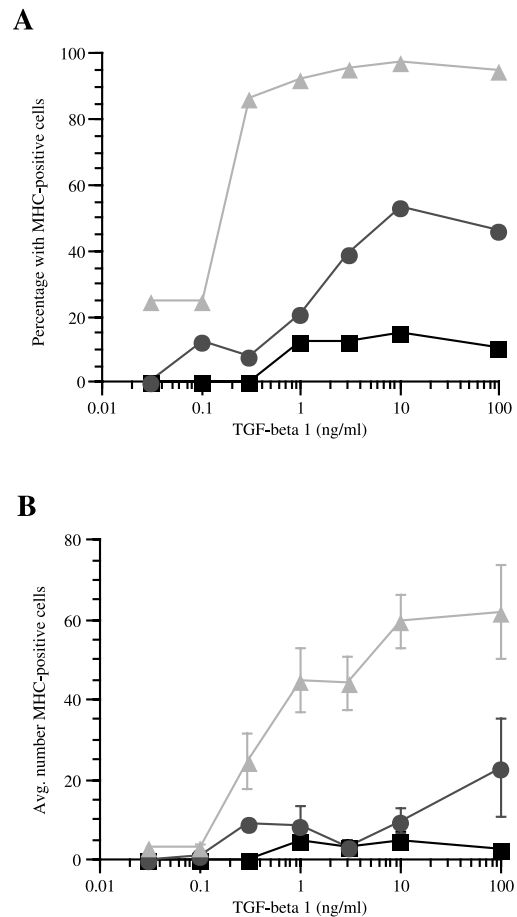
The demonstration that soluble factors can mediate a myogenic signal provides an opportunity to examine the duration of exposure required for promoting myogenesis in paraxial mesoderm. Explants from stage 10-11 caudal segmental plates (the tissue exhibiting the most robust response in the above experiments) were cultured for various lengths of time in the presence of 30 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1 followed by rinsing and incubation without added factors for a total culture time of 72 hours. There was little to no increase in the percentage of explants exhibiting myogenic cells when cultures were exposed to bFGF and TGF- $\beta$ 1 together for 24 hours, nor was there an increase in the mean number of MHC-positive cells in the explants that were exposed to bFGF plus TGF- $\beta$ 1 for up to 36 hours. In contrast, 36-48 hours of exposure was sufficient to trigger a myogenic response as measured by both the percentage of explants responding and by the average number of MHC-positive cells (Fig. 5A, B, squares).

To investigate whether the exposure time required for myogenic commitment differs for the two factors, we performed experiments as above except that only one of the



**Fig. 3.** Rostral-caudal differences in the myogenic responsiveness of stage 10-11 paraxial mesoderm explants to varying doses of bFGF in the presence of 10 ng/ml TGF- $\beta$ 1. (A) Percentage of explants that exhibit MHC-positive cells as a function of bFGF dose. (B) Average number of MHC-positive cells in the responding explants as a function of bFGF dose. Somites I-IV (squares); rostral segmental plate explants (circles); caudal segmental plate explants (triangles). Error bars represent the standard error of the mean. Each point on the graph represents at least 12 explants taken from 3 embryos, except for the 0.01 ng/ml data point which represents 8 explants from 2 embryos. The results for the 0.01 ng/ml data point were equivalent to data from cultures treated with 10 ng/ml TGF- $\beta$ 1 without any bFGF (see Table 1A).

factors was removed at various time points, i.e. by restoring one factor after the rinses. Myogenic commitment in response to TGF- $\beta$ 1 treatment (with continuous exposure to bFGF) occurred rapidly. After only 6 hours, TGF- $\beta$ 1 could be removed and 55% of the explants still exhibited myogenic cells (Fig. 5A, circles), which is significantly greater than the 25% level observed without added factors (Fig. 5A, 0 hours); but, the average number of myogenic cells per MHC-positive explant was only about 6 (Fig. 5B, circles, 6 hours), which is equivalent to the extent of autonomous myogenesis in untreated controls (Fig. 5B, 0 hours). However, after 12 hours of exposure to TGF- $\beta$ 1 plus bFGF, followed by TGF- $\beta$ 1 deprivation for the remaining culture period, the percentage of explants with MHC-positive cells was 95% (Fig. 5A, circles), which is equivalent to the response observed when TGF- $\beta$ 1 was present throughout (Fig. 5A, point X). Moreover, the



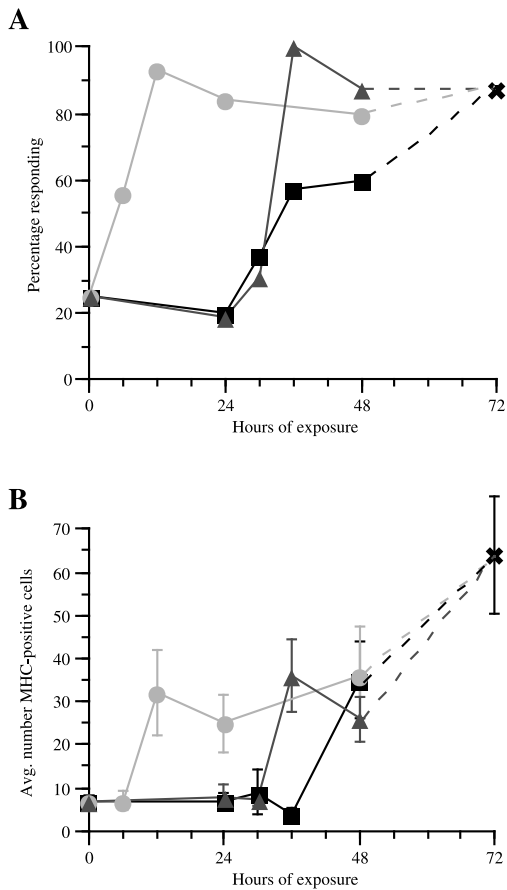
**Fig. 4.** Rostral-caudal differences in the myogenic responsiveness of stage 10-11 paraxial mesoderm explants to varying doses of TGF- $\beta$ 1 in the presence of 10 ng/ml bFGF. (A) Percentage of explants that exhibit MHC-positive cells as a function of TGF- $\beta$ 1 dose. (B) Average number of MHC-positive cells in the responding explants as a function of TGF- $\beta$ 1 dose. Somites I-IV (squares); rostral segmental plate explants (circles); caudal segmental plate explants (triangles). Error bars represent the standard error of the mean. Each point on the graph represents at least 12 explants taken from 3 embryos, except for the 0.1 ng/ml data point which represents 8 explants from 2 embryos. The results for the 0.03 ng/ml data point were equivalent to data from cultures treated with 10 ng/ml bFGF without any TGF- $\beta$ 1 (see Table 1A).

average number of MHC-positive cells per explant increased 5-fold (Fig. 5B, circles).

In contrast to TGF- $\beta$ 1, myogenic commitment in response to bFGF (with continuous exposure to TGF- $\beta$ 1) occurred more slowly. More than 30 hours of bFGF plus TGF- $\beta$ 1 signaling was required to achieve myogenic commitment in the subsequent absence of bFGF (Fig. 5, triangles). Thus, the promotion of myogenic commitment in response to TGF- $\beta$ 1 and bFGF appears to have different temporal requirements for the two ligands.

#### **bFGF is not required for myogenic commitment during at least the first 12 hours of culture**

The observation of temporal differences in myogenic commitment following exposure to bFGF and TGF- $\beta$ 1 suggests that



**Fig. 5.** Time required for bFGF and/or TGF- $\beta$ 1 to promote myogenic commitment in stage 10-11 caudal segmental plate explants. (A) Percentage of explants that exhibit MHC-positive cells as a function of time of exposure to growth factor(s). (B) Average number of MHC-positive cells in the responding explants as a function of time of exposure to growth factor(s). All cultures except for 0 hr time points were initially treated with 30 ng/ml bFGF and 10 ng/ml TGF- $\beta$ 1. At various times, both factors were removed by rinsing 3 times with F10C and then 3 times with fresh medium (squares). In some cases, only TGF- $\beta$ 1 was removed by rinsing and then re-adding 30 ng/ml bFGF (circles). In other cases, only bFGF was removed by rinsing and then re-adding 10 ng/ml TGF- $\beta$ 1 (triangles). For comparison, some cultures were treated with both factors for the full 72 hour time period (x). Note that the culture medium in all of these experiments contains 1% chick embryo extract, which has a level of FGF equivalent to about 0.3 ng/ml bFGF (see Results). Error bars represent the standard error of the mean. Each point on the graph represents at least 12 explants taken from 3 embryos, except for the 30 and 36 hour time points (squares) which represent 8 explants from 2 embryos.

these factors may not act simultaneously at a single step in myogenesis, but rather, may act sequentially. If the latter hypothesis is correct, the order of exposure to the two ligands may be critical. To address this issue, caudal segmental plate explants from stage 10-11 embryos were cultured with 10 ng/ml TGF- $\beta$ 1 for 12 hours, rinsed and then cultured with 30 ng/ml bFGF (without TGF- $\beta$ 1) until fixation at 72 hours. 93% of these cultures exhibited MHC-positive cells (Table 3, line A). The average number of MHC-positive cells per responding explant was  $72 \pm 17$  (s.e.m.), which is slightly greater than the

**Table 3. The order of growth factor exposure and the presence of factors in CEE are critical parameters in the myogenic effect of bFGF/TGF- $\beta$ 1 on paraxial mesoderm**

Treatment: factor(exposure time) <sup>(a)</sup>	n <sup>(b)</sup>	% MHC-positive	Avg. no. MHC-positive
A. TGF(0-12), FGF(12-72)	15	93	$72 \pm 17$
B. FGF(0-36), TGF(36-72) <sup>(c)</sup>	8	0	—
C. TGF(0-12), FGF(12-72) (without CEE)	12	33	$6 \pm 2$
D. TGF + anti FGF(0-12), FGF + anti TGF- $\beta$ 1,2,3(12-72)	12	25	$9 \pm 5$
E. TGF + anti FGF(0-12), FGF(12-72)	10	90	$40 \pm 12$

(a) Cultures of stage 10-11 caudal segmental plate were treated with growth factors in a consecutive manner rather than simultaneously. The order of growth factor exposure is indicated by the order listed for each treatment. Most experiments were conducted in 1% CEE except for the one experiment indicated 'without CEE'. As indicated in the text, 1% CEE contains an FGF level equivalent to about 0.3 ng/ml bFGF. TGF = TGF- $\beta$ 1 at 10 ng/ml; FGF = bFGF at 30 ng/ml; anti-FGF = anti-bFGF antibody at 40  $\mu$ g/ml. (This antibody concentration neutralizes at least 0.3 ng/ml bFGF in the MM14 cell mitogenic assay.) anti-TGF- $\beta$ 1,2,3 = anti-TGF- $\beta$ 1,2,3 antibody at 40  $\mu$ g/ml.

(b) Number of explants tested.

(c) 36 hour (as opposed to 12 hour) exposure to bFGF was chosen based on the requirement for 30-36 hours of bFGF exposure indicated in Fig. 5.

response obtained when bFGF was added at the beginning of the culture period (compare with Fig. 5B, circles). The reverse order of factor exposure was examined by treating caudal segmental plate explants first with bFGF for 36 hours (the longer time based on findings in Fig. 5) then with TGF- $\beta$ 1 for 36 hours. None of these cultures exhibited a myogenic response (Table 3, line B), suggesting that the TGF- $\beta$ 1 exposure must come first.

Although the above result raises the possibility that sequential TGF- $\beta$ 1 followed by bFGF signaling is sufficient to promote myogenesis, it is possible that the chick embryo extract (CEE) in the medium provides essential low levels of FGF during the initial exposure to TGF- $\beta$ 1 and/or low levels of TGF- $\beta$ -like factors during the subsequent exposure to bFGF (i.e., an FGF bioassay using MM14 mouse myoblasts demonstrates that inclusion of 1% CEE in the culture medium results in an FGF concentration equivalent to about 0.3 ng/ml bFGF). In support of these possibilities, treatment of caudal segmental plate with TGF- $\beta$ 1 without CEE followed by bFGF without CEE does not result in a significant increase in myogenesis (Table 3, line C). To further assess whether the CEE requirement reflects a need for low levels of bFGF and/or TGF- $\beta$ 1, stage 10-11 caudal segmental plates were cultured in medium with 1% CEE in addition to 10 ng/ml TGF- $\beta$ 1 and 40  $\mu$ g/ml anti-bFGF antibody for the first 12 hours, followed by 30 ng/ml bFGF with 40  $\mu$ g/ml anti-TGF- $\beta$ 1, anti-TGF- $\beta$ 2 and anti-TGF- $\beta$ 3 antibody for the remaining 60 hours (Table 3, line D). This concentration of bFGF antibody neutralizes at least 0.3 ng/ml bFGF in the MM14 cell mitogenic assay and the concentration of TGF- $\beta$  antibody neutralizes at least 1 ng/ml TGF- $\beta$ 1 in a paraxial mesoderm bioassay (see below). The antibody-treated cultures did not exhibit a myogenic response, demonstrating that either FGF and/or TGF- $\beta$ -like factors in CEE are required. To determine if the CEE requirement for myogenic induction in caudal segmental plate cultures exposed first to TGF- $\beta$ 1 and then to bFGF is due solely to the CEE's provision of bFGF, a

similar experiment was performed except that only the anti-bFGF antibodies were utilized. These cultures exhibited a significant myogenic response (Table 3, line E). Thus, bFGF is not required during the initial 12 hours of TGF- $\beta$ 1 exposure, but a TGF- $\beta$ 1-like factor in CEE is required during the subsequent 60 hour exposure to bFGF. These findings are consistent with a two-step myogenic commitment process for stage 10-11 segmental plate. An initial process requiring 6-12 hours is dependent on TGF- $\beta$ 1 (or a homolog) and a subsequent process requiring about 36 hours is dependent on both bFGF and TGF- $\beta$ 1 (or a homolog).

### The dorsal neural tube myogenic signal is inhibited by anti-bFGF but not by anti-TGF- $\beta$







Because bFGF and TGF- $\beta$ 1 can promote paraxial mesoderm myogenesis when tested in combination or in sequence, they are candidates for the myogenic signals from the neural tube. To examine this possibility, the dorsal neural tube was co-cultured with paraxial mesoderm in the presence or absence of a monoclonal antibody that inactivates bFGF or a monoclonal antibody that inactivates TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. When the dorsal neural tube was co-cultured with paraxial mesoderm, addition of antibodies to bFGF resulted in a marked decrease in the percentage of explants that exhibited a myogenic response (27% versus 89%, Table 4, lines B versus A). Furthermore, explants that responded exhibited significantly fewer myogenic cells (2 versus 38).

Although adding the bFGF antibody to dorsal neural tube-somite co-cultures results in fewer MHC-positive cells, it was possible that the antibody did not inhibit signals from the neural tube, but rather inhibited a subsequent step in the myogenic pathway. To test this possibility, more rostrally located somites, which are known to contain committed yet undifferentiated myogenic cells (Stern and Hauschka, 1995), were cultured with and without bFGF antibody. All of the cultures exhibited MHC-positive cells and the average number of MHC-positive cells was equivalent with and without antibody (Table 4, lines E and D,  $P=0.3$ ). Therefore, the antibody to bFGF does not inhibit muscle differentiation per se, but rather appears to interfere with myogenic signals from the dorsal neural tube.

Experiments similar to those presented above were conducted to determine whether myogenic-inducing signals from the dorsal neural tube involve TGF- $\beta$ 1, TGF- $\beta$ 2 or TGF- $\beta$ 3. Paraxial mesoderm explants were co-cultured with dorsal neural tube plus 40  $\mu$ g/ml of a monoclonal TGF- $\beta$  antibody that binds to and inhibits the activity of TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 in an Mv1Lu mink lung cell growth inhibition assay (Genzyme) (Ogawa and Seyedin, 1991). The set of explants treated with antibody exhibited a myogenic response equivalent to control cultures without antibody (Table 4, lines C and A) and myogenesis was not perturbed by the anti-TGF- $\beta$  antibody in somites containing committed but initially undifferentiated muscle cells (Table 4, lines D and F). To verify that the TGF- $\beta$  antibody was functional, paraxial mesoderm explants from stage 13 embryos were cultured with 10 ng/ml bFGF and 1 ng/ml TGF- $\beta$ 1 plus or minus 40  $\mu$ g/ml anti-TGF- $\beta$  antibody. (According to the manufacturer, 20-30  $\mu$ g/ml antibody neutralizes 0.5 to 1.0 ng/ml TGF- $\beta$ 1, TGF- $\beta$ 2 or TGF- $\beta$ 3.) A 1 ng/ml dose of TGF- $\beta$ 1 was chosen, because a dose of 10 ng/ml, as used in Table 1B, would require an impractical amount of antibody to neutralize activity. 1 ng/ml TGF- $\beta$ 1 was nevertheless capable of promoting myogenesis in combination with bFGF (Fig. 4). Only 3 of 17 (18%) explants with anti-TGF- $\beta$  antibody exhibited MHC-positive cells (in all cases only 1 MHC-positive cell), whereas 88% of the control cultures exhibited MHC-positive cells (average =  $26 \pm 5$ ), demonstrating that the TGF- $\beta$  antibody is functional in the paraxial mesoderm assay system. Consequently, the inability of the TGF- $\beta$  antibody to affect myogenic signaling from the dorsal neural tube suggests that TGF- $\beta$ 1, TGF- $\beta$ 2 or TGF- $\beta$ 3 may not be necessary endogenous components of the dorsal signal, unless the dorsal neural tube secretes greater than 1 ng/ml TGF- $\beta$ 1, TGF- $\beta$ 2 or TGF- $\beta$ 3 under our culture conditions. Rather, a TGF- $\beta$  family member other than these may serve as part of the dorsal neural tube myogenic signal.

The finding that 40  $\mu$ g/ml of an anti-TGF- $\beta$  antibody does not inhibit dorsal neural tube-induced paraxial mesoderm myogenesis also demonstrates that monoclonal antibody addition, per se, is not detrimental. This observation serves as an additional control showing that myogenic inhibition by the bFGF

**Table 4. Effects of bFGF and TGF-beta antibodies on myogenic induction by dorsal neural tube**

Tissues	Antibody	Number of explants tested	Number of explants with MHC-positive cells	Average number of MHC-positive cells <sup>(c)</sup>
A. 	none	46	41 (89%)	37.9 $\pm$ 5.7
B. 	anti-bFGF	22	6 (27%)	2 $\pm$ 0.4
C. 	anti-TGF- $\beta$ <sup>(d)</sup>	24	23 (95%)	36.4 $\pm$ 7.2
D. 	none	33	33 (100%)	47.3 $\pm$ 5.1
E. 	anti-bFGF	14	14 (100%)	38.5 $\pm$ 4.8
F. 	anti-TGF- $\beta$ <sup>(d)</sup>	19	19 (100%)	54.9 $\pm$ 14

(a) Dorsal neural tube co-cultured with somites I-III from stage 12-13 embryos or with explants from the rostral half of the segmental plate.

(b) Somites VI to XI from stage 13 embryos. These somites undergo myogenesis in vitro without neural tube/notochord (Stern and Hauschka, 1995).

(c) Mean  $\pm$  standard error of the mean for explants which exhibit MHC-positive cells.

(d) The anti-TGF- $\beta$ 1 antibody is capable of neutralizing 1 ng/ml TGF- $\beta$ 1 activity in this culture system as indicated in the text. The anti-bFGF antibody is capable of neutralizing at least 0.3 ng/ml bFGF activity as indicated in the text.



antibody is antibody type-specific, and thus indicative of a true involvement of bFGF in neural tube-mediated myogenesis.

### Dsl-1 does not substitute for TGF- $\beta$ 1 in promoting myogenesis

Because TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 are not likely to mediate myogenic signals from the dorsal neural tube, it was useful to explore another TGF- $\beta$  family member. Dsl-1 is a BMP-like protein expressed in the dorsal neural tube (Basler et al., 1993). Transfected COS cells expressing myc-tagged dsl-1 or mock-transfected COS cells were used to make dsl-1-conditioned medium (CM) and mock CM, respectively. The dsl-1 CM at a 4% (v/v) dilution was found to have BMP-like activity as indicated by a two-fold increase in alkaline-phosphatase-specific activity after 3 days in an MC3T3-E1 osteoblast cell assay (Asahina et al., 1996) compared to the same dilution of mock CM. (BMP-like activity assays of dsl-1 CM were kindly performed by Dr P. Hauschka and E. Skazkina, Children's Hospital, Boston.) These conditioned media were utilized in tests of paraxial mesoderm myogenic activity at concentrations ranging from 1% to 25% CM diluted in fresh medium or in 100% CM. Under no conditions did mock or dsl-1-containing CM promote myogenesis in stage 10-11 caudal segmental plate. Affinity-purified myc-tagged dsl-1 (purified from CM) and a similar immunoprecipitate from mock CM tested at dilutions of 1:10,000 to 1:100 also were devoid of myogenic activity (data for crude CM and affinity-purified material are pooled together, Table 5, lines A and B versus E).

Dsl-1 CM and purified dsl-1 were tested also in combination with bFGF to assess synergistic effects on stage 10-11 caudal segmental plate myogenesis. Myogenesis occurred at only the 'background' rate expected for stage 10-11 caudal segmental plate in the presence of bFGF (see Table 1) when cultured with 1 to 100% dsl-1 CM plus 30 ng/ml bFGF or with 1:10,000 to 1:100 affinity-purified *dsl-1* plus 30 ng/ml bFGF (Table 5, lines C and D versus E). The mock immunoprecipitate with bFGF may have had a mild positive myogenic effect with 35% of explants responding, but only about 8 MHC-

positive cells were detected per responding explant. Contralateral control caudal segmental plate explants from all the embryos tested exhibited a significant myogenic response to bFGF plus TGF- $\beta$ 1 (Table 5, line E). These data demonstrate that *dsl-1* is not a substitute for TGF- $\beta$ 1 in this assay system.

## DISCUSSION

This study demonstrates that combinations of bFGF and TGF- $\beta$ 1 promote paraxial mesoderm myogenesis in a dose-dependent manner. The magnitude of the response varies depending upon the rostrocaudal position of the paraxial mesoderm and the age of the embryo. Only 6-12 hours of exposure to TGF- $\beta$ 1 is required to promote myogenesis, but bFGF must be present for at least 36 hours. Although bFGF is unnecessary during the first 12 hours of culture, a TGF- $\beta$ -like factor must be present during the subsequent exposure to bFGF. The potential biological relevance of these results, especially those obtained with bFGF, is underscored by the finding that antibodies to bFGF inhibit myogenic signals from the dorsal neural tube. Interestingly, although *dsl-1* is a TGF- $\beta$  family member expressed in the right place and time to be a candidate in vivo paraxial mesoderm myogenic-inducing factor, it does not exhibit myogenic activity in our assay system either alone or in combination with bFGF.

### FGF and possibly TGF- $\beta$ family members are myogenic signals from the neural tube

The finding that bFGF and TGF- $\beta$ 1 can promote paraxial mesoderm myogenesis in vitro suggests that these factors, or other members of these families, could be components of the myogenic signal from the neural tube. Previous studies have indicated that bFGF mRNA (Murphy et al., 1994) and protein (Ford et al., 1994) are expressed in the mouse neural tube. Furthermore, bFGF protein is expressed by cultured embryonic day-2 quail neural tubes, and it is localized to the basement membrane surrounding the dorsal neural tube of day-4 quail embryos (Kalcheim and Neufeld, 1990). Also, bFGF mRNA has been detected in the dorsal neural tube of 2-day chick embryos (Savage and Fallon, 1995). Basic FGF is thus present in an appropriate place to serve as a myogenic signal. Furthermore, there is evidence that FGF receptors are expressed in the segmental plate and epithelial somites. For example, as determined by in situ hybridization, FGFR1 mRNA is expressed at high levels in the rostral segmental plate and in the rostral half of somites I-II of 10-15 somite mouse embryos (Yamaguchi et al., 1992). Message levels of FGFR1 in the rostral half of somites were most intense on the medial side. This region corresponds with the area of the somite that will give rise to myotomal cells (Kaehn et al., 1988). In addition, FGFR2 mRNA is expressed in the most recently formed mouse somites (Orr Urtreger et al., 1991) and FREK mRNA (a newly discovered chick FGF receptor, Marcelle et al., 1994) is expressed in undifferentiated myotome cells at later stages of myotome development (Marcelle et al., 1995). In further support of the hypothesis that neural-tube-derived FGF signals are important for myogenesis, our data demonstrate that an antibody to bFGF inhibits myogenic signaling from the dorsal neural tube (Table 4). The neutralization of bFGF by this antibody could block either inductive signals (e.g., instructive)

**Table 5. Can dsl-1 (with or without bFGF) promote myogenesis in stage 10-11 caudal segmental plate explants?**

Condition	No. explants tested	% MHC-positive	Avg. no. of MHC-positive cells <sup>(d)</sup>
A. Mock <sup>(a)</sup>	11	0	—
B. dsl-1 <sup>(b)</sup>	12	0	—
C. Mock + bFGF <sup>(a)(c)</sup>	26	35	8 ± 3
D. dsl-1 + bFGF <sup>(b)(c)</sup>	53	9	7 ± 5
E. TGF- $\beta$ 1 + bFGF <sup>(c)</sup>	51	92	74 ± 10

(a) Data from cultures treated with 0.1% immunoprecipitate of mock CM with anti-myc antibody are pooled with data from cultures treated with 5 to 25% mock CM. There are no significant differences between the data sets.

(b) Data from cultures treated with 0.1% myc-tagged dsl-1 affinity purified from dsl-1 CM using anti-myc antibody are pooled with data from cultures treated with 5 to 25% dsl-1 CM. There are no significant differences between the data sets. A 4% (v/v) concentration of dsl-1 CM was capable of inducing a two-fold increase in alkaline-phosphatase-specific activity in MC3T3-E1 osteoblast cells when compared to similar dilutions of mock CM, demonstrating BMP-like activity in the dsl-1 CM.

(c) bFGF at 30 ng/ml. TGF- $\beta$ 1 at 10 ng/ml.

(d) Mean ± s.e.m. of explants with MHC-positive cells.

or permissive signals (e.g. proliferative and/or survival). This experiment along with the previously discussed expression studies of bFGF and its receptors suggest that FGF production by the dorsal neural tube may be important for promoting paraxial mesoderm myogenesis. However, it is unclear whether this is a direct effect on myogenesis or rather an indirect effect in which bFGF is necessary for the expression of an as yet unknown myogenic-inducing factor within the paraxial mesoderm.

The hypothesis that bFGF signals from the neural tube promote paraxial mesoderm myogenesis may seem contradictory to a previous experiment in which a dominant negative FGF receptor was introduced into chick somites using a retroviral vector (Itoh et al., 1996). Cells that expressed this dominant negative receptor (as assessed by expression of the dicistronic  $\beta$ -gal) still differentiated into myotome cells; however, the dominant negative FGF receptor was placed into somites which we and others have shown already contain cells committed to myotome development (Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995). Consequently, the dominant negative study demonstrates that FGF signaling is not required once myotomal cells have begun commitment to myogenesis, but the experiment does not address an *in vivo* role for FGFs in promoting paraxial mesoderm myogenic commitment.

Previous experiments have demonstrated that the paraxial mesoderm myogenic response to the neural tube is proximity dependent (Fan and Tessier-Lavigne, 1994; Stern and Hauschka, 1995; Spence et al., 1996). However, this observation does not exclude a role for soluble signaling factors like the FGFs. Although FGFs are soluble, they are readily bound by heparin sulfate proteoglycans in the extracellular matrix and are thought to diffuse not more than a few cell diameters away from the source cell (Fernig and Gallagher, 1994).

TGF- $\beta$  family members are also potentially involved in the neural tube myogenic signal as suggested by our observations (Table 1A,B). Although both TGF- $\beta$ 2 and TGF- $\beta$ 3 are expressed in fetal human and mouse neural tube (Gatherer et al., 1990; Pelton et al., 1991), an antibody that neutralizes TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 does not affect myogenic signaling from the chick dorsal neural tube (Table 4). The chick TGF- $\beta$  family member *dsl-1* is also expressed in the dorsal neural tube at stage 10 (Basler et al., 1993), but it does not have myogenic activity in our system, despite the fact that it had BMP-like activity in an osteoblast differentiation assay. BMP-4 is another TGF- $\beta$  family member expressed in the neural tube. However, prior experiments have suggested that BMP-4 inhibits myotome development (Pourquié et al., 1996). Thus, an *in vivo* role for TGF- $\beta$  family members in paraxial mesoderm myogenesis remains to be determined.

Although the present study focuses on myogenic signals from the dorsal neural tube, it should be noted that other structures have been found to affect paraxial mesoderm myogenesis. For example, some experiments suggest that the ventral neural tube and/or notochord promote myogenesis (Rong et al., 1992; Buffinger and Stockdale, 1994; Stern and Hauschka, 1995; Münsterberg and Lassar, 1995; Buffinger and Stockdale, 1995; Stern et al., 1995; Pownall et al., 1996). Other potentially important tissues include the dorsal ectoderm and lateral plate mesoderm. Some studies have suggested that dorsal ectoderm can promote somite myogenesis (Kenny-Mobbs and

Thorogood, 1987; Fan and Tessier-Lavigne, 1994; Cossu et al., 1996; Maroto et al., 1997), whereas the lateral plate mesoderm inhibits myogenesis (Pourquié et al., 1995; Gamel et al., 1996; Cossu et al., 1996). Thus, although signals from the dorsal neural tube may be important for proper myotome formation, such signals are likely to be modulated by factors from other tissues surrounding the paraxial mesoderm.

### Possible mechanisms for the bFGF/TGF- $\beta$ 1 effect on paraxial mesoderm myogenesis

One possible role for bFGF and TGF- $\beta$ 1 in paraxial mesoderm myogenesis is enhancement of proliferation or maintenance (possibly survival) of myoblast or pre-myoblast cells. In support of this hypothesis, exposure to bFGF resulted in increased cell numbers (Table 2). Our observation that bFGF promotes cell survival/proliferation is in agreement with a similar finding reported by Fan and Tessier-Lavigne (1994). These results support the possibility that cell proliferation and/or survival play a role in myotome development, in agreement with *in vivo* studies which demonstrate that removal of the neural tube/notochord results in somitic cell death (Teillet and Le Douarin, 1983; Rong et al., 1992). However, the absence of any increase in MHC-positive cells in explants treated only with bFGF suggests that survival/proliferation alone is not sufficient to explain the promotion of paraxial mesoderm myogenesis in explants treated with both growth factors. Further experimentation will be necessary to determine if the proliferation/survival status of paraxial mesoderm in general can be extrapolated specifically to the myogenic lineage.

It is important to note that, although combinations of bFGF and TGF- $\beta$ 1 promote myogenesis, only about 2% of the cells in an explant become myogenic. This observation is also true with neural tube co-cultures (Stern and Hauschka, 1995; Stern et al., 1995). Since the response to neural tube was proximity dependent, it was possible that only the closest paraxial mesoderm cells could have responded to the neural tube. However, the *in vitro* response of somites to bFGF plus TGF- $\beta$ 1 is not subject to proximity considerations and, therefore, would be consistent with the hypothesis that only a distinct subpopulation of somite cells is capable of responding to these myogenic signals.

### Position and age dependence of myogenic responsiveness

An interesting aspect of this study is the observation that the myogenic response of stage 10-11 paraxial mesoderm to bFGF and TGF- $\beta$ 1 varies depending on the rostrocaudal position of the tissue prior to explantation (Table 1A). One hypothesis to explain these rostrocaudal differences relates to potential differences in cell density between rostral and caudal portions of the segmental plate. A careful study of chick somitomeres indicated that they become more condensed as they progress from caudal to rostral in the segmental plate (Meier, 1979). Furthermore, cell-cell adhesiveness increases in progressively more rostral somitomeres (Bellairs, 1979; Cheney and Lash, 1984). A second hypothesis to explain the rostrocaudal differences is that the axial position (rather than cell density) could be critical. In potential contrast to the latter hypothesis, quail-chick heterotopic transplantation experiments in which a somite or presumptive somite is moved from a non-wing to a

wing level, demonstrate that grafted somites adopt the appropriate characteristics for the position in the host (i.e. myogenic cells migrate to the limb bud) (Chevallier et al., 1977). This experiment suggested either that there are no intrinsic differences in somites from various axial positions or that any intrinsic differences are susceptible to modification from new signals in vivo. If the latter is true, it remains possible that any putative intrinsic differences would be observed in an in vitro environment because the tissue would not receive new positional signals as would have been the case in the in vivo experiments.

The fact that bFGF and TGF- $\beta$ 1 are not very efficient at promoting myogenesis in stage 10-11 somites and rostral segmental plate suggests that additional factors may be required to completely mimic the neural tube myogenic signal. Perhaps *Wnt-1*, *Wnt-3* or *Wnt-4* (Stern et al., 1995; Münsterberg et al., 1995) or *Sonic hedgehog* (Münsterberg et al., 1995), may be required before bFGF and TGF- $\beta$ 1 can have a significant effect on somites or rostral segmental plate at stage 10-11. Although we examined the possibility of cooperative effects between *Wnt-1*-expressing fibroblasts and bFGF/TGF- $\beta$ 1, the results were not informative because the soluble growth factors stimulated growth of the *Wnt-1* fibroblasts, thus potentiating the previously reported myogenic inhibitory effects of fibroblast monolayers (Stern et al., 1995).

Another interesting finding was the observation of neural tube/notochord-independent myogenesis in the caudal segmental plate (Table 1). Interestingly, 'cryptic' myogenesis has been previously reported in somite and segmental plate tissue that has undergone dissociation (George-Weinstein et al., 1994, 1996). Perhaps these findings suggest a type of myogenic default pathway that is normally suppressed by cell-cell contacts or signals in vivo.

### bFGF and TGF- $\beta$ 1 play dichotomous roles in myogenesis

The finding that bFGF and TGF- $\beta$ 1 promote paraxial mesoderm myogenesis reinforces the notion that the same growth factors may play positive as well as negative roles in the regulation of myogenesis. The more commonly observed effects of FGFs are the inhibition of myogenic differentiation (Linkhart et al., 1981; Kardami et al., 1985; Spizz et al., 1986) and a recent study suggests that overexpression of FGF or the FGF receptor FGFR1 in chick somites inhibits myotome development (Itoh et al., 1996). TGF- $\beta$ s have also been shown to inhibit differentiation under low serum conditions (Olson et al., 1986; Florini et al., 1986; Massagué et al., 1986) and the TGF- $\beta$  family member, BMP-4, can inhibit chick myotome development (Pourquié et al., 1996). However, in addition to the present study, there have been reports that FGFs and TGF- $\beta$ s have positive myogenic activity. For example, some chick forelimb colony-forming cells require bFGF in order to form differentiated muscle clones (Seed and Hauschka, 1988). Furthermore, bFGF promotes the expression of myogenic markers in *Xenopus* animal caps and addition of TGF- $\beta$ 1 potentiates the response to bFGF (Kimelman and Kirschner, 1987). Recent experiments have shown that expression of a dominant negative type II TGF- $\beta$  receptor delays differentiation of C2C12 mouse myoblasts, suggesting a positive role for TGF- $\beta$ s in myogenesis (Filvaroff et al., 1994). The latter finding is

supported by evidence that TGF- $\beta$ s can promote myoblast differentiation under high serum conditions (Zentella and Massagué, 1992) and can stimulate myogenic differentiation of embryonic stem cells (Slager et al., 1993).

One explanation for such disparate effects of growth factors on myogenesis is the possibility that other factors in the environment might affect how a cell responds to a given factor. For example, it is clear that serum concentrations affect how myoblasts respond to growth factors (for review see Hauschka, 1994). Yet, culture medium effects are clearly not the whole story because bFGF both delays the onset of terminal differentiation and enhances the percentage of muscle clones obtained in chick limb bud cultures (Seed and Hauschka, 1988), demonstrating a diversity among myoblasts with respect to how they respond to a given signal. Thus, the myogenic response of a cell to specific growth factors probably depends upon the entire spectrum of environmental signals to which the cell is being exposed as well as upon the status of the cell's intrinsic receptor and signal transduction pathways for the myogenic factors being tested.

In summary, our study indicates that bFGF and TGF- $\beta$  family members should be considered as candidates for mediating myogenic signaling from the neural tube. Further studies will be necessary to explore the potential involvement of such factors in vivo and to determine how these factors affect expression of earlier myogenic genes such as the MyoD and MEF-2 families.

The authors are grateful to T. Jessell for providing expression plasmids for dorsalin, to Bristol-Myers Squibb for kindly providing the TGF- $\beta$ 1 and to P. Hauschka and E. Skazkina for the bioassays of BMP-like activity in dsl-1-conditioned medium. J. Angello, J. Buskin, C. Fabre-Suver, D. Kimelman and M. Wenderoth are thanked for critical reading of the manuscript, and A. Münsterberg, A. Lassar, M. Spence, J. Yip, C. Erickson, A. Borycki, L. Hart and C. Emerson are appreciated for sharing unpublished observations. This work was supported by a grant from the NIH to S. D. H. (AR18860) and by a predoctoral fellowship from Interdisciplinary Training in Developmental Biology to H. M. S. (NIH T32HD07183)

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(Accepted 11 July 1997)