Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite

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We have demonstrated previously that a combination of signals from the neural tube and the floor plate/notochord complex synergistically induce the expression of myogenic bHLH genes and myogenic differentiation markers in unspecified somites. In this study we demonstrate that Sonic hedgehog (Shh), which is expressed in the floor plate/notochord, and a subset of Wnt family members (Wnt-1, Wnt-3, and Wnt-4), which are expressed in dorsal regions of the neural tube, mimic the muscle inducing activity of these tissues. In combination, Shh and either Wnt-1 or Wnt-3 are sufficient to induce myogenesis in somitic tissue in vitro. Therefore, we propose that myotome formation in vivo may be directed by the combinatorial activity of Shh secreted by ventral midline tissues (floor plate and notochord) and Wnt ligands secreted by the dorsal neural tube.

[*Key Words:* Neural tube; floor plate; notochord; Shh; Wnts; myogenic bHLH; somitogenesis]

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In vertebrates, paraxial mesoderm, which lies adjacent to the neural tube and notochord, gives rise to transient cellular aggregates, termed somites. Initially, the somite is a sphere of epithelial cells. Subsequently, cells in the ventral half of the somite develop a mesenchymal morphology, termed the sclerotome, which contains precursor cells for vertebrae and ribs. The epithelial sheet that persists in the dorsal-most somite is termed the dermomyotome. The dermomyotome contains precursor cells for both skeletal muscle and dermis; cells located in the medial dermomyotome, adjacent to the neural tube, give rise to back and intercostal muscles (i.e., epaxial musculature; Ordahl and Douarin 1992); cells located in the lateral dermomyotome give rise to ventral body wall and limb muscles (i.e., hypaxial musculature; Ordahl and Douarin 1992); and an additional population of cells gives rise to some of the dermis within the back. Dermomyotomal cells that lie adjacent to the neural tube give rise to the myotome, a secondary sheet of differentiated skeletal muscle cells that lies between the dermomyotome and sclerotome.

The different somitic cell fates are cued by signals from surrounding tissues (Aoyama and Asamoto 1988;

Christ et al. 1992; Ordahl and Douarin 1992; for review, see Christ and Ordahl 1995). Currently, there is a wealth of evidence indicating that signals from the neural tube and notochord are necessary to promote the proper formation of both sclerotome (Holtzer and Detwiler 1953; Watterson et al. 1954; Brand-Saberi et al. 1993; Dietrich et al. 1993; Goulding et al. 1993; Koseki et al. 1993; Pourquie et al. 1993) and myotome (Avery et al. 1956; Vivarelli and Cossu 1986; Kenny-Mobbs and Thorogood 1987; Christ et al. 1992; Rong et al. 1992; Bober et al. 1994; Buffinger and Stockdale 1994; Goulding et al. 1994; Buffinger and Stockdale 1995; Münsterberg and Lassar 1995; Stern and Hauschka 1995). Explant analysis has indicated that signals from either the notochord or floor plate of the neural tube are sufficient to induce chondrification of somitic cells (Grobstein and Holtzer 1955; Lash et al. 1957). In consonance with these earlier findings, it was demonstrated recently that Sonic hedgehog (Shh), a signaling molecule expressed in both floor plate and notochord (Echelard et al. 1993; Krauss et al. 1993; Riddle et al. 1993; Chang et al. 1994; Johnson et al. 1994; Roelink et al. 1994), can activate expression of a sclerotomal marker, Pax-1, in somitic cells (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995).

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We have found recently that myogenesis of somitic tissue in vitro [presegmental plate and somite stages I–III isolated from Hamburger–Hamilton (Hamburger and Hamilton 1951) stage 10 chick embryos] requires signals from both the notochord/floor plate complex and more dorsal regions of the neural tube (Münsterberg and Lassar 1995; see also Vivarelli and Cossu 1986; Kenny-Mobbs and Thorogood 1987; Rong et al. 1992; Buffinger and Stockdale 1994; Buffinger and Stockdale 1995; Stern and Hauschka 1995). In contrast, myogenesis in more mature somites (somite stages IV–VI) can occur in vitro in the presence of neural tube lacking floor plate/notochord (Münsterberg and Lassar 1995). [Following the numbering system of Ordahl, the most recently formed somite is termed stage I, and successively more rostral somites are termed somite stages II, III, etc. (Ordahl 1993; Christ and Ordahl 1995).] Neural tube adjacent to either presegmental plate mesoderm or somites IV–VI is capable of inducing myogenesis in somites IV–VI but does not induce myogenesis in presegmental plate from stage 10 embryos in the absence of floor plate/notochord (Münsterberg and Lassar 1995). This finding suggested that prior exposure to a ventral midline signal (i.e., emanating from the floor plate and/or notochord) had rendered somites IV–VI competent to respond to a muscle-inducing signal from the neural tube. In contrast, presegmental plate mesoderm from similar stage embryos had not yet received this ventral midline signal in vivo and therefore was incompetent to be induced to become skeletal muscle in vitro by signals from the neural tube. These findings have led us to propose that somite myogenesis is combinatorially induced by signals from both the floor plate and neural tube, and that the signal from the former is sent prior to the latter (Münsterberg and Lassar 1995).

In this study we demonstrate that somite myogenesis can be induced in presegmented paraxial mesoderm in vitro by administration of Shh in combination with some but not all Wnt ligands. Whereas presegmental plate mesoderm requires both Shh and Wnt signals to activate MyoD expression, more mature somites (stages IV–VI) require only Wnt signals. Wnt family members that induce somite myogenesis (Wnt-1, Wnt-3, and Wnt-4) are expressed predominantly in the dorsal half of the developing neural tube and noninducing Wnt family members (Wnt-7a and Wnt-7b) are expressed predominantly in the ventral half of the neural tube. Therefore, we propose that myotome formation in vivo may be directed by the combinatorial activity of Shh secreted by ventral midline tissues (floor plate and notochord) plus Wnt ligands secreted by the dorsal neural tube.

Results

Shh acts in combination with signals from the neural tube to induce somite myogenesis

Presegmental plate mesoderm was isolated from a stage 10 chick embryo and cultured in vitro, in a collagen gel, either alone or in the presence of axial tissues. Following a 3-day culture period, RNA was isolated from the ex-

plants and gene expression was assayed by reverse transcriptase–PCR (RT–PCR). Muscle-specific genes (*myoD*, *myogenin*, and *myosin heavy chain*) were highly expressed in presegmental plate mesoderm when cocultivated with neural tube plus the notochord/floor plate complex (Fig. 1A, lane 2). In contrast, muscle-specific genes were expressed either at only trace levels or were undetectable in presegmental plate mesoderm when cocultured with neural tube in the absence of ventral midline tissues (Fig. 1A, lanes 1,3). Notochord and floor plate are known to express Shh and ectopic expression of Shh in the dorsal somite expands the domain of MyoD expression (Johnson et al. 1994). Therefore, we tested whether bacterially produced Shh (peptide N3; Marti et al. 1995), which included the 19-kD amino-terminal processed fragment of Shh plus 19 amino acids of the carboxy-terminal peptide (termed here as N–Shh), was sufficient to induce somite myogenesis in the presence of neural tube lacking floor plate. (In the following text we refer to neural tube lacking floor plate as “neural tube –FP”.) The 19-kD amino-terminal-processed fragment of Shh has been shown previously to be sufficient to induce both Pax-1 expression in somitic tissue (Fan et al. 1995) and floor plate and/or motor neuron markers in intermediate neural tube cultures (Marti et al. 1995; Roelink et al. 1995). Addition of soluble N–Shh (175 ng/ml) to presegmental plate mesoderm/neural tube –FP cocultures led to high-level expression of MyoD and myosin heavy chain plus the sclerotomal marker Pax-1 (Fig. 1A, lane 4). The trace levels of Shh transcript in this culture suggests that an ectopic floor plate was not induced in this explant (Fig. 1A, lane 4). Thus, the amino-terminal fragment of Shh can functionally substitute for the muscle-inducing capacity of floor plate/notochord. Addition of N–Shh to presegmental plate mesoderm cultured in the absence of the adjacent neural tube led to activation of Pax-1; however, no detectable somite myogenesis was observed (Fig. 1A, lane 6). These findings indicate that induction of myogenesis in presegmental plate mesoderm can be elicited by either the amino-terminal fragment of Shh or the floor plate/notochord complex (Münsterberg and Lassar 1995) in combination with signals from the neural tube –FP. N–Shh administration significantly increased glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in presegmental plate mesoderm (Fig. 1A, cf. lanes 5 and 6), consistent with recent findings that Shh is mitogenic for this tissue (Fan et al. 1995). Whereas N–Shh treatment of presegmental plate mesoderm is sufficient to activate Pax-1 expression and increase somitic cell number, N–Shh administration alone is not sufficient to induce myogenesis in the absence of additional signals from the neural tube.

Because the amino-terminal fragment of Shh is capable of inducing both a sclerotomal marker (Pax-1) and myotomal markers in the presence of neural tube, we monitored whether these distinct somitic cell fates were induced by different concentrations of N–Shh (Fig. 1B). Titration of increasing amounts of N–Shh plus presegmental plate mesoderm indicated that concentrations of N–Shh (100 ng/ml) that induced half maximal levels of

Pax-1 induced only trace levels of MyoD (in the presence of neural tube; Fig. 1B, lane 4). A dose-response curve comparing the relative levels of MyoD and Pax-1 induction in presegmental plate/neural tube -FP cocultures treated with increasing amounts of soluble N-Shh indicated that Pax-1 is more readily induced at lower concentrations of soluble N-Shh than is MyoD (Fig. 1C; cf. solid circles with filled squares). Whereas induction of MyoD by soluble N-Shh was absolutely dependent on the presence of adjacent neural tube -FP, Pax-1 was induced to comparable levels in the absence or presence of the neural tube (Fig. 1C; cf. solid and open circles). Thus, robust expression of myotomal markers in presegmental plate mesoderm requires (1) a higher concentration of the amino-terminal fragment of Shh than is necessary for

induction of Pax-1, and (2) the presence of the adjacent neural tube.

Some Wnt family members mimic the muscle-inducing activity of the neural tube

Somitic myogenesis was not observed in presegmented plate mesoderm cocultured with neural tube -FP alone (Fig. 2A, lane 2). In contrast, myogenesis was induced in more rostrally located somites (somite stages IV–VI) after coculture with adjacent neural tube -FP (Fig. 2A, lane 4) but not after coculture with either the notochord/floor plate complex (Münsterberg and Lassar 1995), the notochord alone (Fig. 2A, lane 8), or bacterially produced N-Shh (Fig. 2A, lane 12). However, both notochord and

Shh and Wnt synergistically induce MyoD

Wnt-1, Wnt-3, or Wnt-4 increased somitic GAPDH levels (Fig. 2B, cf. lanes 2 and 3, 5 and 6, 8 and 9), suggesting that these Wnt family members may affect both cell proliferation (see, e.g., Takada et al. 1994) as well as myogenesis in the somite.

In contrast to the robust myogenesis detected in somites IV–VI when cocultured with Rat B1a cells expressing either Wnt-1, Wnt-3 or Wnt-4, myogenesis was not observed in similar stage somites when cocultured with Rat B1a cells expressing either Wnt-7a or Wnt-7b (Fig. 2B, lanes 11,14). All of the Rat B1a–Wnt-expressing lines produced approximately the same amount of epitope-tagged Wnt protein as detected by Western blot analysis of total cell protein lysates (Fig. 2C). Thus, the absence of muscle gene expression after coculture of somites with cells expressing either Wnt-7a or Wnt-7b cannot be attributed to quantitative differences in the amount of Wnt expression in these cells. In summary, these findings indicate that some Wnt family members (Wnt-1, Wnt-3, and Wnt-4) are capable of inducing myogenesis in somites IV–VI, yet like signals from the neural tube – FP, are incapable of inducing myogenesis in presegmental plate mesoderm. In contrast, other Wnt family members (i.e., Wnt-7a and Wnt-7b) apparently cannot induce myogenesis in either presegmental plate mesoderm or somites IV–VI.

Combinatorial action of Wnt and Shh are sufficient to induce somitic myogenesis

Both neural tube – FP and fibroblasts expressing Wnt-1, Wnt-3 or Wnt-4 share the capacity to induce myogenesis in somites IV–VI but cannot induce myogenesis in presegmental plate mesoderm isolated before stage 11. Because neural tube – FP is capable of inducing myogenesis in presegmental plate mesoderm when cultured in the presence of the soluble amino-terminal fragment of Shh (Fig. 1), we determined whether the combination of Wnt-expressing cells plus administration of soluble N–Shh was sufficient to induce myogenesis in presegmental plate mesoderm. MyoD and myosin heavy chain expression were both induced in presegmental plate mesoderm when this tissue was cocultured with Rat B1a cells expressing either Wnt-1 or Wnt-3 in the presence of soluble N–Shh (Fig. 3, lanes 4,6). In contrast, culture of presegmental plate mesoderm with either N–Shh in the presence of the parental Rat B1a fibroblasts (lane 2) or with the Wnt-producing Rat B1a fibroblasts alone (lanes 3,5) was not sufficient to induce myogenesis. These findings indicate that the combination of N–Shh plus either Wnt-1 or Wnt-3 is sufficient to induce myogenesis in presegmental plate mesoderm.

Shh signaling is continuously required for the maintenance of Pax-1 expression, but only transiently required for the induction of somite myogenesis

The above findings indicate that some Wnt family members together with Shh synergistically activate myogenesis in presegmental plate mesoderm. The ability of

Figure 3. Shh and Wnt combinatorially induce myogenic bHLH gene expression in presegmented paraxial mesoderm (psm). psm was dissected from chick embryos (stages 9+ and 10) and cultured as indicated above each lane. psm was cultured with pellets of Rat B1a fibroblasts infected with the retroviral expression vehicle LNCX, in the absence (lane 1) or the presence of N–Shh (200 ng/ml, lane 2), with pellets of Rat B1a fibroblasts expressing either retroviral encoded Wnt-1 (lanes 3,4) or Wnt-3 (lanes 5,6) in the absence (lanes 3,5) or presence (lanes 4,6) of N–Shh (200 ng/ml). After a 3-day culture period RT–PCR was performed to detect MyoD, myosin heavy chain (MHC), Pax-1, and GAPDH. Similar results were obtained in at least three independent experiments.

more mature somites IV–VI to initiate myogenesis in response to Wnt signals in the absence of Shh (or neural tube in the absence of floor plate) suggests that somites IV–VI had been exposed previously to Shh signaling in the embryo. Interestingly, however, Pax-1 expression, which is induced by Shh (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995, and this study), is either undetectable or expressed at only trace levels in these somites (IV–VI) after coculture with either neural tube in the absence of floor plate (Fig. 2A, lane 4) or with Wnt-expressing cell lines (data not shown). Furthermore, whole-mount in situ hybridization studies have documented Pax-1 expression in somites IV–VI (Johnson et al. 1994; Ebensperger et al. 1995). Thus, the absence of detectable Pax-1 transcripts in somites IV–VI after in vitro culture suggests that continued exposure to Shh is necessary to maintain Pax-1 gene expression in somitic tissue. To test this possibility directly we examined Pax-1 expression in somites IV–VI either immediately following dissection of this tissue (Fig. 4A, lanes 1,3) or following in vitro culture of this tissue for 65 hr in the absence or presence of soluble N–Shh (Fig. 4A, lanes 2 and 4, respectively). We found that whereas somites IV–VI express readily detectable levels of Pax-1 immediately after dissection from a stage 10 embryo, this expression decays following 65 hr in culture in the absence of N–Shh (Fig. 4A, lane 2). In contrast, in vitro culture of this tissue in the presence of soluble N–Shh is sufficient to sustain high level expression of Pax-1 (Fig. 4A, lane 4). These findings, together with those of others (Fan and Tessier-Lavigne 1994; Johnson et al. 1994; Fan et al. 1995), suggest that continued exposure to Shh is necessary to both initiate and maintain expression of Pax-1 in somitic mesoderm.

Figure 4. Shh signaling is continuously required for the maintenance of Pax-1 expression, however, only transiently required for induction of somite myogenesis. (A) Somites IV–VI were dissected from a stage 10 chick embryo and analyzed for the expression of GAPDH and Pax-1 either immediately following dissection (lanes 1,3), or after in vitro culture for 65 hr in the absence (lane 2) or the presence (lane 4) of N–Shh (200 ng/ml). (B) Presegmented paraxial mesoderm (psm) was dissected from chick embryos (stages 10+ and 11–) and cultured as indicated above each lane. psm was cultured with pellets of Rat B1a fibroblasts expressing Wnt-3 in the presence of N–Shh (200 ng/ml) for an initial period of 40 hr. After 40 hr the culture was washed twice with medium to remove all of the soluble N–Shh and then cultured in the absence of N–Shh for another 20 hr (lane 1). psm was cultured in the presence of Rat B1a fibroblasts expressing Wnt-3 and soluble N–Shh for a period of 60 hr (lane 2). After a total culture period of 60 hr RT–PCR was performed to detect MyoD, myosin heavy chain (MHC), Pax-1, and GAPDH. Lane C displays cDNA amplified from 4-day embryo RNA as described in Fig. 1A. Similar results were obtained in two independent experiments.

We were interested to discern whether continued exposure to Shh was also necessary to maintain somitic myogenesis. Presegmental plate mesoderm cultured for a 60-hr period with both Wnt-3-producing fibroblasts and soluble N–Shh expressed high levels of both Pax-1 and MyoD (Fig. 4B, lane 2). In contrast, presegmental plate mesoderm cultured with Wnt-producing cells and soluble N–Shh for an initial period of 40 hr and only Wnt-producing cells for the subsequent 20 hr expressed robust levels of MyoD and myosin heavy chain, but only trace levels of Pax-1 (Fig. 4B, lane 1). The extremely low levels of Pax-1 gene expression following N–Shh withdrawal indicated that high-level expression of MyoD in this explant could not be attributed to residual amounts of Shh contaminating the culture medium following N–Shh removal. These findings indicate that a temporary exposure (40 hr treatment) of presegmental plate mesoderm

to N–Shh is sufficient to induce stable high-level expression of MyoD (for at least 20 hr) when this tissue is cultured in the presence of a Wnt-3 signal.

Discussion

Previous studies have demonstrated that cell fate determination in the somite is cued by signals from adjacent tissues. Sclerotome can be induced by signals emanating from either the floor plate or notochord (Holtzer and Dewitler 1953; Watterson et al. 1954; Brand-Saberi et al. 1993; Pourquie et al. 1993), and loss of these ventral midline tissues leads to absence of sclerotome formation in vivo (Dietrich et al. 1993; Koseki et al. 1993). In contrast, myogenic differentiation in somitic precursor tissue (presegmental plate taken from stage 10 or younger embryos) in vitro is dependent on signals from both the floor plate/notochord complex in addition to signals present in more dorsal regions of the neural tube (Münsterberg and Lassar 1995). These findings are consistent with the observation that excision of either the neural tube (Christ et al. 1992; Rong et al. 1992; Bober et al. 1994) or notochord (Goulding et al. 1994) at early stages of development leads to the absence of myotomal muscle in the somite and indicate that signals from both the neural tube and ventral midline tissues are required for the differentiation of the myotome proper. Here we demonstrate that the muscle-promoting signal from the notochord/floor plate complex can be mimicked by a bacterially produced amino-terminal fragment of the signaling molecule Shh and that the muscle-promoting signal from the neural tube can be mimicked by cell lines programmed to express Wnt-1, Wnt-3 or Wnt-4. Because these signaling molecules are sufficient to induce somitic myogenesis in vitro and are expressed in the inducing tissues (Shh in the floor plate/notochord and Wnt-1, Wnt-3, and Wnt-4 in the dorsal neural tube), we propose that combinatorial signaling by these two classes of signaling molecules is required for axial myogenesis in vivo.

We cannot distinguish at present whether the robust muscle differentiation observed in somites cultured in the presence of Shh and Wnt ligands is attributable to the induction of myogenic precursors or to a clonal expansion of an already existing population of myogenic precursor cells. In situ hybridization studies have demonstrated that low-level expression of MyoD is first detectable in somite III (Ott et al. 1991; Pownall and Emerson 1992; A.E. Münsterberg and A.B. Lassar, unpubl.). However, more sensitive RT–PCR analyses have revealed that Myf-5 and MyoD transcripts are detectable in mouse and chick presegmented mesoderm (Kopan et al. 1994; A.E. Münsterberg and A.B. Lassar, unpubl.). Myogenic basic helix–loop–helix (bHLH) gene expression is no longer detectable after in vitro culture of presegmented mesoderm in the absence of inducing tissues or factors (data not shown). Somitic MyoD and myogenin expression in vivo is similarly initiated but not maintained in chick embryos lacking the neural tube (Bober et al. 1994). Together, these observations suggest that low-

level myogenic bHLH gene expression is unstable in the absence of axial signals.

Although somitic cells require additional cues to assume a myogenic cell fate when explanted as a tissue block (Avery et al. 1956; Vivarelli and Cossu 1986; Kenny-Mobbs and Thorogood 1987; Rong et al. 1992; Buffinger and Stockdale 1994; Buffinger and Stockdale 1995; Münsterberg and Lassar 1995; Stern and Hauschka 1995; Stern et al. 1995), apparently such is not the case when somitic cells are plated as single cells (George-Weinstein et al. 1994). Both somitic and presegmental plate cells have been found to differentiate into either chondrocytes or myocytes following trypsinization and in vitro culture (George-Weinstein et al. 1994). Together, these findings suggest that cell–cell interactions within the paraxial mesoderm act to restrain both myogenesis and chondrogenesis in this tissue in the absence of positive influences. Therefore, signals from the axial tissues that induce the formation of these somitic cell types may act by overcoming an intrinsic inhibitory signal in the intact somite.

Shh serves multiple roles in somite patterning

Shh is autocatalytically processed to yield a secreted amino-terminal 19-kD fragment that is tightly associated with the producing cell and a carboxy-terminal 27- to 28-kD fragment that is released into the medium (Lee et al. 1994; Bumcrot et al. 1995). With regard to somitic cells, the amino-terminal 19-kD fragment of Shh has been demonstrated to be a mitogen (Fan et al. 1995), to induce the expression of a sclerotomal marker (Pax-1) in presegmental plate mesoderm (Fan et al. 1995; this report), to inhibit the expression of the dermomyotomal marker Pax-3 (Fan et al. 1995) and, finally, to promote myogenic differentiation in the presence of neural tube – FP or Wnt family members (this report). Although the amino-terminal 19-kD fragment of Shh is predominantly cell associated, both notochord or COS cells programmed to express N-Shh can apparently activate the expression of Pax-1 at a distance of up to 200 μ m (Fan et al. 1995). However, because conjugation of Pax-1-expressing chick somites VI–X with mouse presegmented mesoderm did not induce expression of mouse Pax-1, it has been argued that Shh directly activates Pax-1 expression as opposed to inducing a cascade of signaling molecules that activate this sclerotomal marker (Fan and Tessier-Lavigne 1994). The amino-terminal fragment of Shh also plays a positive role in the differentiation of myogenic cells. In contrast, the carboxy-terminal fragment of Shh, which is readily secreted into the medium (Lee et al. 1994; Bumcrot et al. 1995), can neither mediate the induction of Pax-1 (Fan et al. 1995) nor activate myogenesis in presegmental plate mesoderm cocultured with Wnt-3-expressing fibroblasts (data not shown). Whereas the amino-terminal fragment of Shh is sufficient to induce somitic expression of Pax-1 (Fan et al. 1995; this report), Shh-mediated myogenesis requires an additional signal from either neural tube – FP or Wnt expressing fibroblasts.

It is not clear whether myogenic differentiation mediated by Shh is a direct effect of Shh on prospective myotomal cells (Fig. 5, left) or whether an intermediate signaling molecule(s) is induced (depicted X in Fig. 5, right) which then promotes myogenesis. We have found that levels of soluble N-Shh sufficient to induce half maximal levels of Pax-1 induce only trace levels of MyoD (Fig. 1B) in vitro. This finding argues that Shh is not simply acting in a concentration-dependent manner to alter somitic cell fate in vivo, because this would be more consistent with sclerotome induction at high concentrations of N-Shh close to the notochord/floor plate and myotome induction at lower concentrations of N-Shh further away from the notochord/floor plate. Rather, the paradoxical requirement for higher levels of Shh for induction of distal myotome versus proximal sclerotome are instead consistent with a dependency of myotome formation on (1) Shh-mediated induction of an intermediate signaling molecule, (2) the combinatorial activity of multiple axial signals, or (3) both of the above. In contrast to this complex scenario in the somite the situation seems to be different in the ventral neural tube where the induction of floor plate and/or motor neurons is thought to be directly determined by the local concentration of N-Shh; high levels inducing floor plate and lower levels inducing motor neurons (Marti et al. 1995; Roelink et al. 1995).

Whereas maintenance of somitic Pax-1 expression requires the continued presence of Shh, somite myogenesis in response to Wnt-expressing Rat B1a cells requires only temporary exposure to Shh. These findings indicate that Shh can induce both unstable and stable genetic programs in somitic cells and further highlight differences in Shh-mediated induction of sclerotome versus myotome. The stability of the myogenic program following Shh withdrawal in vitro suggests that Shh activates a

Figure 5. Shh and Wnt signals combinatorially pattern the somite. Shh secreted by both the floor plate and the notochord induces Pax-1 expression in the ventral somite and mediates myotome formation more dorsally. Myotome differentiation requires both Shh and a Wnt signal from the neural tube. It is unclear whether Shh is acting directly on myogenic cells in combination with a Wnt signal (*left*) or whether Shh indirectly affects myogenic differentiation via an intermediate signaling molecule, depicted as "X" (*right*).

positive regulatory loop that maintains MyoD expression. At present, it is unclear whether this positive loop entails the activation of an intermediate signaling molecule (Signal X in Fig. 5, right) or activation of the myogenic bHLH factors themselves. The ability of myogenic bHLH family members to activate their own synthesis (for review, see Weintraub 1993; Olson and Klein 1994) could explain the stability of myotomal gene expression following Shh withdrawal.

Ventral midline tissues play both a positive and negative role in the induction of myotome

The ability of the amino-terminal fragment of Shh to influence cell fates in both the ventral regions of the neural tube and in the somite is consistent with the requirement for notochord signals in the induction of floor plate (van Straaten et al. 1988; Smith and Schoenwolf 1989; Placzek et al. 1990; Yamada et al. 1991; Placzek et al. 1993), motor neurons (Yamada et al. 1993), sclerotome (Holtzer and Detwiler 1953; Watterson et al. 1954; Brand-Saberi et al. 1993; Dietrich et al. 1993; Koseki et al. 1993; Pourquie et al. 1993) and myotome (Goulding et al. 1994) and suggests that Shh plays a crucial role in polarization of both the axial and paraxial tissues. Surprisingly, however, implantation of ventral midline tissues between the neural tube and somite (Pourquie et al. 1993), or lateral to the somite (Brand-Saberi et al. 1993), has been demonstrated to induce sclerotome formation at the expense of myotome. However, in contrast to these results, direct infection of somites with a retrovirus encoding Shh led to an expansion of the myotome (Johnson et al. 1994). We think inhibition of myotome formation by implantation of ventral midline tissues adjacent to the somite could be attributable to (1) an alteration of the signaling properties of the neural tube, (2) the suppression of myogenesis by extremely high local concentrations of Shh, or (3) to the presence of a factor in notochord/floor plate (other than Shh) that represses myotome formation at close range.

Combinatorial activation of myogenesis

Three Wnt genes that are predominantly expressed in the dorsal regions of the neural tube (Wnt-1, Wnt-3, and Wnt-4) were each demonstrated to activate myogenesis in somites IV–VI but not in presegmental plate mesoderm. Presegmental plate mesoderm can form muscle in response to signals from both neural tube –FP or cells expressing either Wnt-1 or Wnt-3 by simultaneously exposing this tissue to N–Shh. It is formally possible that induction of somite myogenesis by Wnt-producing cell lines was attributable to a Wnt-mediated induction of a secondary signaling molecule in the Wnt-producing cell lines themselves. Although we cannot rule out this possibility, we feel that this is unlikely given the ability of three different Wnt ligands to elicit this effect while two of the Wnt-ligands were unable to promote myogenesis. In addition, Rat B1a fibroblast have shown no phenotypic changes attributable to Wnt gene expression. Various Wnt family members differ in their capacity to

transform mammary epithelial cells (Wong et al. 1994), induce axis duplication in *Xenopus* embryos (for review, see Du et al. 1995), or induce somitic myogenesis (this report). It is unclear what properties of these signaling molecules account for their differential activities in these assays. Wnt family members that induce somitic myogenesis include those that can transform mammary epithelial cells (Wnt-1 and Wnt-3) and at least one member that is nontransforming in this assay system (i.e., Wnt-4; Wong et al. 1994).

Currently, the localization of the muscle-inducing activity in the neural tube is somewhat controversial. Whereas Stockdale and colleagues have reported that dorsal neural tube contains an inhibitor of myogenesis and that muscle inducing activity is preferentially located in the ventral neural tube (Buffinger and Stockdale 1995), Hauschka and colleagues have found that muscle-inducing activity is preferentially localized to the dorsal neural tube and have found that coculture of somites with Wnt-1-expressing cells can induce somitic myogenesis (Stern et al. 1995). In concert with these latter findings, we have found that Wnt-1, Wnt-3, and Wnt-4, which are expressed predominantly in the dorsal half of the neural tube (Parr et al. 1993), all induce somitic myogenesis. In contrast, Wnt-7a and Wnt-7b, which do not induce somitic myogenesis in vitro, are expressed mainly in the ventral half of the neural tube (Parr et al. 1993). Thus, myotome formation, which is known to be dependent on both neural tube (Christ et al. 1992; Rong et al. 1992; Bober et al. 1994) and notochord (Goulding et al. 1994) may be cued specifically by the combined action of Wnt family members in the dorsal half of the neural tube and Shh expressed by the ventral midline tissues (as illustrated in Fig. 5). Ultimately, functional tests in vivo will be necessary to clarify the role that specific Wnt family members play in the induction of myotomal muscle.

It has been demonstrated recently that signals from the dorsal half of the neural tube can inhibit muscle differentiation in specified somites (i.e., somites VII–XII from stage 12–14 embryos, which are capable of myogenic differentiation in the absence of further signaling from axial tissues; Buffinger and Stockdale 1995). This finding, together with the results of the present study, suggests that dorsal regions of the neural tube contain both positive and negative influences for myogenesis that together may act to define the final position of the myotome in the dorsomedial part of the somite. In addition to the axial tissues (neural tube and notochord) the ectoderm overlying the somite can also induce somite myogenesis in vitro (Kenny-Mobbs and Thorogood 1987; R. Reshef and A.B. Lassar, unpubl.). Wnt family members are expressed in the ectoderm overlying the somite (i.e., Wnt-4 and Wnt-6; Parr et al. 1993) and therefore may mediate the ability of this tissue to induce myogenic differentiation. It has been demonstrated recently that surface ectoderm is either necessary to maintain or initiate the expression of several dermomyotomal markers (Fan and Tessier-Lavigne 1994; Kuratani et al. 1994), although it is unclear in this case which signaling molecules are responsible for these effects.

The synergistic induction of somite myogenesis by Wnt family members and Shh resembles the synergistic induction of dorsal mesoderm in *Xenopus* by Wnt genes plus either fibroblast growth factor (FGF) (Christian et al. 1992) or transformation growth factor- β (TGF- β) family members (Sokol et al. 1991). In *Xenopus*, myogenic bHLH gene expression is activated prior to somitogenesis, whereas in amniotes this regulatory family is activated after somites have formed. It is our speculation that similar signaling molecules activate the myogenic program in these different organisms and that the temporal and spatial differences that mark the onset of myogenesis in avians and amphibia reflect underlying differences in the patterns of expression of the relevant signaling molecules. The above findings in vertebrate systems, together with recent findings that induction of a subset of the somatic musculature in *Drosophila* requires the *wingless* gene product (A. Martinez-Arias and M. Bate, pers. comm.), raise the possibility that induction of skeletal muscle shares a common signaling pathway in metazoa.

Materials and methods

Chick embryo dissections and culture of tissues

Fertilized chick eggs (Spafas) were incubated at 38.5°C in a humidified incubator (Petersime). Embryos were staged according to Hamburger and Hamilton (1951), collected in silicon (Dow Corning)-coated petri dishes containing Tyrode's buffer (10 $\times$ stock: 80 grams/liter of NaCl, 2 grams/liter of KCl, 2 grams/liter of CaCl₂, 0.5 gram/liter of NaH₂PO₄, 2 grams/liter of MgCl₂, and 10 grams/liter of glucose, and pinned down with their ventral side facing up using insect pins. To facilitate dissection, three drops of Tyrode's buffer containing dispase (Boehringer Mannheim, 1 mg/ml) were added to each embryo using a glass Pasteur pipette. The endodermal epithelium was removed from the region to be dissected, and the following tissues were explanted using micro feather scalpels (Oasis, CA): paraxial mesoderm (1) alone, (2) with the adjacent neural tube, floor plate and notochord, (3) with the adjacent neural tube excluding floor plate, (4) with floor plate and notochord, (5) or with notochord (see figure legends for details and stages of embryos). After washing in α -minimal essential medium (α -MEM) (GIBCO) supplemented with 10% heat-inactivated horse serum (GIBCO), 2% chick embryo extract and 1% penicillin/streptomycin solution, the tissues were cultured in collagen gels in four-well dishes (Nunc). The cultures were maintained in α -MEM (GIBCO) supplemented with 10% heat-inactivated horse serum (GIBCO), 2% chick embryo extract, 1 ng/ml of FGF (to improve the survival of presegmented paraxial mesoderm; data not shown) and 1% Penn./Strep. solution, for 3 days in 5% CO₂ at 37°C. When indicated, the medium was supplemented with bacterially produced Shh (fragment N3, Marti et al. 1995). Chick embryo extract was prepared as described elsewhere (Münsterberg and Lassar 1995). Preparation of Rat Bl1 fibroblast pellets was essentially as described by (Riddle et al. 1993). The pellets were cut in small pieces approximately the same size as the paraxial mesoderm, transferred to the culture dish, juxtaposed to the chick tissues, and embedded in collagen.

Preparation of rat tail collagen and embedding of tissues

Collagen was prepared from ~10 adult rat tails. The tendons were isolated and dissolved for 24 hr in 150 ml of 3% glacial

acetic acid at 4°C. The undissolved debris was pelleted by centrifugation at 20,000g for 60 min. The supernatant was dialyzed over 3 days against 3 liters of sterile 0.1 $\times$ DME at pH 4.0. The recovered collagen was stored in aliquots at 4°C. For embedding of tissue explants we used a 65% solution of the collagen stock. The collagen was buffered by adding 1/10 volume of a 10 $\times$ DME at pH 4.0 solution and ~1/100 volume of a 0.8 M NaHCO₃ solution. To prevent gelling, the collagen solution was then kept on ice until use. A bed of collagen (6 μ l volume) was prepared in the culture well, and the collagen gelled within 15 min at room temperature. The tissues and cell pellets were then placed on top of the collagen bed, excess medium was removed, and the explants were covered with 3 μ l of collagen. The tissues and cell pellets were positioned before the top collagen gelled; after 15 min at room temperature, the culture was covered with medium.

RNA isolation and reverse transcription

At the end of the culture period medium was aspirated and RNA was prepared from the explants using the method described by Chomczynski and Sacchi (1987). Explants in collagen gels were lysed in 200 μ l of lysis buffer (25 grams of guanidinium thiocyanate, 1.76 ml of 0.75 M sodium citrate at pH 7, 2.64 ml of 10% Sarkosyl, 38 μ l β -mercaptoethanol, and 29.3 ml of H₂O) and transferred to a 1.5-ml centrifuge tube; 20 μ l of 2 M sodium acetate at pH 4 and 200 μ l of water-saturated phenol were added and mixed, and 40 μ l of chloroform/isoamyl alcohol (49:1) was added, mixed, and incubated on ice for 15 min. Following centrifugation at 4°C the supernatant was removed, glycogen was included as a carrier (1.5 μ l of RNase free glycogen, 20 mg/ml, Boehringer Mannheim), and the RNA was precipitated with 500 μ l of ethanol in a new tube. After precipitation at -80°C for at least 1 hr, the RNA was pelleted by centrifugation at 4°C for 20 min, washed with 80% ethanol, air-dried, and resuspended in 50 μ l of buffer (40 mM Tris at pH 8.0, 10 mM NaCl, 6 mM MgCl₂) containing 0.5 μ l of DNase (RNase-free DNase, Boehringer Mannheim, 40 U/ μ l) and 0.1 μ l RNase inhibitor (U.S. Biochemical 120 U/ μ l). This solution was incubated for 1 hr at 37°C, then extracted with Tris-buffered phenol/chloroform and precipitated at -80°C by adding 10 μ l of 2 M NaAcetate at pH 4.5, and 150 μ l of ethanol. After centrifugation the RNA pellet was washed twice with 80% ethanol, air-dried, dissolved in 10 μ l of DEPC-treated H₂O, and kept at -80°C.

Before cDNA synthesis the RNA was incubated at 65°C for 3 min then immediately cooled on ice, 5 μ l were added to a 25- μ l reverse transcription cocktail, containing 1 $\times$ transcription buffer (as provided by Bethesda Research Laboratories [BRL]), 0.5 mM of each dNTP, 200 ng of a random hexamer primer, 0.1 μ l of RNase inhibitor, 3.3 mM DTT, and 200 units of MoMuLV-reverse transcriptase (BRL) and incubated at 42°C for 1 hr. The cDNA was kept at -20°C until used for PCR analysis.

PCR amplification

cDNA (1 μ l) was used as template for each PCR amplification. Each 25- μ l reaction contained 50 mM KCl, 10 mM Tris/HCl at pH 7, 1.5 mM MgCl₂, 0.01% gelatin, 200 mM of each dNTP, 0.1 mCi [³²P]dCTP (3000 Ci/mM, 500 ng of the appropriate primers, and 1 unit of *Taq* polymerase (Boehringer Mannheim). Shh and Pax-1 cDNAs were amplified in the presence of 5% formamide. After an initial denaturation step of 93°C for 3 min, the reactions were cycled between 93°C (30 sec), 60°C (30 sec, for MyoD, MHC, GAPDH) or 49°C (30 sec, for Pax-1 and Shh) and

72°C (1 min) in a MJ Research thermocycler. The reaction products for MyoD, myosin heavy chain, Pax-1, and Shh were amplified for 30 cycles while the product of GAPDH was amplified for 23 cycles. It was determined that after 23 cycles of amplification the GAPDH transcript was in the linear range. GAPDH is ubiquitously expressed and served as a control for the amount of cDNA present in each amplification reaction. We chose 30 cycles of amplification for the other cDNAs to detect trace amounts of gene products that were induced during in vitro culture. Depending on the amount of template in the culture, 30 cycles of amplification of the myogenic bHLH gene products were usually within the linear range. PCR reaction (5 µl of each) was analyzed on a 6% polyacrylamide gel, which was dried and exposed to X-ray film at -20°C for 16 hr. The following primers were used: MyoD [Lin et al. 1989, nucleotides 620–639] 5'-CGTGAGCAGGAGGATGCATA-3', (nucleotides 864–883) 5'-GGGACATGTGGAGTTGTCTG-3'; myosin heavy chain [Kavinsky et al. 1983, nucleotides 392–411] 5'-GATCCAGCTGAGCCATGCCA-3', (nucleotides 1008–989) 5'-GCTTCTGCTCAGCATCAACC-3'; Pax-1 [kindly provided by Rudi Balling (Institut für Sangetiergenetik, Oberschliessheim, Germany) and Haruhiko Koseki (Center for Biomedical Science, Chiba, Japan)] 5'-GCTGGGTGGTGTCTTCTCGTGA-3', (nucleotides 52–71), 5'-GAATACAAGCAGGGCGACCC-3', (nucleotides 287–306); Shh [Riddle et al. 1993, nucleotides 186–205] 5'-TGGAAGATATGAAGGAAGA-3', (nucleotides 561–580) 5'-CTGAGTTTCTGCTTTGACG-3'; GAPDH [Dugaiczek et al. 1983, nucleotides 680–699], 5'-AGTCATCCCTGAGCTGAATG-3', (nucleotides 990–1009) 5'-AGGATCAAGTCCACAACACG-3'. The expected product sizes are as follows: MyoD, 280 bp; MHC, 616 bp; Pax-1, 255 bp; Shh, 395 bp; GAPDH, 330 bp. In all cases, PCR amplification was shown to be dependent on reverse transcription of RNA templates and on the presence of both forward and reverse primers in the PCR reaction. The identity of each of the PCR products was confirmed by restriction enzyme digestion, and in each case the expected size fragments were obtained. The GAPDH primers amplified only the chick GAPDH cDNA from cocultures of Rat B1a fibroblasts and chick paraxial mesoderm (data not shown). PCR products were quantitated on a PhosphorImager (Molecular Dynamics).

Immunoblotting

A 80% confluent 6-cm dish of rat B1a fibroblasts was lysed in 250 µl of 2× SDS sample buffer [Ausubel et al. 1993]. The whole cell lysate was boiled for 2 min and sonicated in a Branson-Sonifier 450-Sonicator. Extract volumes containing equivalent amounts of DNA were determined by fluorimetry using a Hoefer TKO-100 minifluorometer and volumes of extract normalized for equivalent DNA content were resolved on a 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane [Schleicher & Schuell] using a Bio-Rad trans-blot semidry transfer cell. The membranes were blocked over night at 4°C with 5% milk powder in PBT (PBS, 0.1% Tween 20, Fisher Biotech). The membranes were incubated for 3 hr at room temperature with a 1:50 dilution of the primary anti-HA monoclonal 12CA5 in the blocking solution. The membranes were washed three times for 15 min in PBT and then incubated for 1.5 hr at room temperature with the secondary antibody, HRP-donkey-anti-mouse [Jackson Immuno Research], at a dilution of 1:5000 in blocking solution. The membranes were washed as described previously and then detected using chemiluminescence according to the manufacturer's protocol (ECL, Amersham).

Production of soluble Shh

Bacterial production and purification of the 19-kD amino-terminal fragment N3 and the 27-kD carboxy-terminal fragments of Shh are described in Marti et al. (1995).

Rat B1a fibroblast cell lines

The Rat B1a fibroblast cell lines were generated as described in Z. Zheng and J. Kitajewski (in prep.). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO) containing 10% FBS, 1% Penn/Strep. solution, 1% glutamine, and 250 µg/ml of G418. Fresh aliquots of cells were thawed every 2–3 weeks.

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