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Control of somite patterning by Sonic hedgehog and its downstream signal response genes

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SUMMARY

In the avian embryo, previous work has demonstrated that the notochord provides inductive signals to activate *myoD* and *pax1* regulatory genes, which are expressed in the dorsal and ventral somite cells that give rise to myotomal and sclerotomal lineages. Here, we present bead implantation and antisense inhibition experiments that show that Sonic hedgehog is both a sufficient and essential notochord signal molecule for *myoD* and *pax1* activation in somites. Furthermore, we show that genes of the Sonic hedgehog signal response pathway, specifically *patched*, the Sonic hedgehog receptor, and *gli* and *gli2/4*, zinc-finger transcription factors, are activated in coordination with somite formation, establishing that Sonic hedgehog response genes play a regulatory role in coordinating the response of somites to the constitutive notochord Sonic hedgehog signal. Furthermore, the expression of *patched*, *gli* and *gli2/4* is differentially patterned in the somite, providing mechanisms for differentially transducing the Sonic hedgehog signal to the myotomal and sclerotomal lineages. Finally, we show that the activation of *gli2/4* is controlled by the process of somite formation and signals from the surface ectoderm, whereas upregulation of *patched* and activation of *gli* is controlled by the process of somite formation and a Sonic hedgehog signal. The Sonic hedgehog signal response genes, therefore, have important functions in regulating the initiation of the Sonic hedgehog response in newly forming somites and in regulating the patterned expression of *myoD* and *pax1* in the myotomal and sclerotomal lineages following somite formation.

Key words: Sonic hedgehog, Somite patterning, Quail, *patched*, *gli*, *myoD*, Notochord

INTRODUCTION

Early in embryonic development, multipotential cells of the germ layers become determined to their cell fates and form lineages of progenitor cells that differentiate into cell types later in development. The regulatory mechanisms that control cell lineage determination in the vertebrate embryo are not well understood, but recent genetic and molecular studies are beginning to uncover the regulatory mechanisms that determine multipotential somitic mesoderm cells to lineages that form the epaxial and hypaxial skeletal muscles. Gene targeting studies in the mouse have established that *myoD* and *myf5*, which encode bHLH transcription factors (Davis et al., 1987; Braun et al., 1989b), are essential for the establishment of two lineages of skeletal muscle that arise from somites in the early embryo (Braun et al., 1992; Rudnicki et al., 1992, 1993). In the absence of *myf5* function, lineage marking experiments show that somite cells, which would normally express *myf5,* adopt one of the alternative somitic fates as cartilage and dermal lineages (Tajbakhsh et al., 1996), establishing that *myf5* function is essential for determination of somite cells to the myogenic lineage. Presumably, *myoD* also functions as an essential gene for the proper determination of somitic cells to the *myoD*-dependent muscle lineage. In avian and mouse embryos, the activation of *myoD* and *myf5* is highly patterned within the somite (Buckingham, 1992; Pownall and Emerson, 1992), reflecting the very early determination of myogenic lineages in the medial and lateral domains (Cossu et al., 1996). The spatially patterned expression of regulatory genes such as *pax1* and *scleraxis* in the sclerotome (Deutsch et al., 1988; Cserjesi et al., 1995) and *dermo-1* in the dermatome (Li et al., 1995) also is established early on and is coordinated with myogenic determination. Microsurgical and cell culture experiments with avian embryos have shown that the patterned activation of regulatory genes in these lineage domains of the somite is controlled by a complexity of activating and inhibiting signals produced by adjacent notochord, neural tube, ectoderm and lateral mesoderm (Christ and Ordahl, 1995; Lassar and Munsterberg, 1996).

In avian embryos, the notochord synthesizes a diffusible signal that is essential for activation of *myoD* and *myf5* in the medial myotomal domain, and for activation of *pax1* in the ventral sclerotomal domain (Ebensperger et al., 1995; Pownall et al., 1996). In addition, we showed that signals from the

dorsal neural tube are necessary to maintain *myoD* and *myf5* expression in somites (Pownall et al., 1996), a function likely fulfilled by a member of the *Wnt* gene family (Munsterberg et al., 1995). During early development, notochord signals are sufficient for *pax1* activation, but are insufficient for *myoD* activation and myogenic determination (Borycki et al., 1997). The activation of *myoD* and *pax1* genes in the somite also is tightly coordinated with somite formation, as the cells of the unsegmented paraxial mesoderm are not competent to respond to the notochord signals (Borycki et al., 1997).

Sonic hedgehog (Shh) is a secreted protein, produced in the vertebrate embryo by the notochord and the zone of polarizing activity (ZPA) of the limb bud, that specifies ventral fate in the neural tube and the limb bud (Echelard et al., 1993; Riddle et al., 1993). It also has been suggested to play a role in organogenesis of eye (Ekker et al., 1995; Burke and Basler, 1997; Jensen and Wallace, 1997), tooth (Koyama et al., 1996) and gut (Roberts et al., 1995). Finally, Shh has been implicated as a notochord signal molecule that controls determination of sclerotome and myotome lineages in somites (Bumcrot and McMahon, 1995; Lassar and Munsterberg, 1996). In presomitic mesoderm explant cultures from mouse and chicken embryos, Shh activates the sclerotome gene, *pax1* (Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995), and in combination with Wnts, Shh also induces *myoD* expression (Munsterberg et al., 1995). Retrovirus-mediated ectopic expression of *shh* in chicken somites leads to ectopic expression of both *pax1* and *myoD* (Johnson et al., 1994). Together, these data suggest that Shh has a dual function in somites, controlling both dorsal and ventral patterning and cell lineage determination. However, the biological role of Shh in sclerotome and myotome determination has been questioned by recent *shh* gene targeting studies in the mouse embryo (Chiang et al., 1996). These studies show that *pax1*, *myf5* and *myoD* are activated in somites of *shh* null mice, suggesting that, in vivo, Shh is not essential for somite patterning or determination. Furthermore, in mouse and avian embryos, Shh is expressed constitutively in the notochord along the entire anteroposterior axis of the embryo, even in regions of paraxial mesoderm and at developmental stages when sclerotomal and myotomal genes are not yet expressed (Marti et al., 1995b), indicating that Shh does not have an active role in initiating the precisely regulated and patterned activation of myotomal and sclerotomal genes during somite formation.

In this study, we have tested the in vivo functions of Shh in *myoD* and *pax1* activation during the determination of myotome and sclerotome lineages in the somites of the developing quail embryo. The avian embryo has significant advantages for undertaking these studies. First, the temporal and spatial developmental regulation of *myoD* and *pax1* has been described in detail (Charles de la Brousse and Emerson, 1990; Ebensperger et al., 1995; Borycki et al., 1997); second, the notochord has been shown to be the necessary and sufficient source of inductive signals for both *myoD* (Pownall et al., 1996) and *pax1* (Ebensperger et al., 1995) activation during somite formation; third, the notochord and other tissues of the avian embryo are readily amenable to surgical manipulation, allowing grafting, retrovirus and bead experiments for ectopic expression studies to test the developmental and gene regulatory functions of purified signaling molecules in vivo in the intact embryo (Bronner-Fraser, 1996), and fourth, antisense methods are available to knockout expression of specific genes when they are activated in the embryo to test their essential and specific developmental functions (Angela Nieto et al., 1994; Srivastava et al., 1995).

Here, we report that recombinant Shh protein is sufficient to replace the inductive activity of the notochord in the patterned ventral activation of *pax1* and the dorsal activation of *myoD* and *myf5* in somites. We also show that Shh expression is essential for the activation of both of these genes, thus establishing that Shh is the necessary and sufficient notochord signal for myotome and sclerotome determination in the avian embryo. To examine how the constitutively expressed Shh signal can regulate the activation of *myoD* and *pax1* in coordination with somite formation, we investigated the developmental regulation and functions of genes in the downstream pathway for Shh signaling. These genes include *patched* (*ptc*), the Shh receptor (Marigo et al., 1996a; Stone et al., 1996), and *gli* and *gli2/4*, which are zinc-finger transcription factors that mediate the nuclear transcriptional activity of Shh signaling (Marigo et al., 1996b). Our studies establish that *ptc*, *gli* and *gli2/4* are activated in coordination with somite formation, immediately preceding *myoD* and *pax1* activation. Furthermore, expression of *gli* and *gli2/4* is patterned in the early somite such that *gli* is expressed predominantly in the ventral somite, encompassing both *pax1* and *myoD* domains of activation, whereas *gli2/4*, following expression throughout newly formed somites, becomes restricted to the dorsal domain of myotome formation and *myoD* expression. In addition, analysis of the mechanisms controlling *ptc, gli* and *gli2/4* activation in somites revealed that *ptc* and *gli* expression are induced by Shh, but *gli2/4* expression is induced independently by signals from the surface ectoderm, providing a mechanism to initiate the response of somite cells to the Shh signal. The developmental regulation of the *ptc* and *gli* and *gli2/4* genes during somite formation, therefore, can coordinate the transduction of the Shh signal with somite formation, and mediate the differential activation of *myoD* and *pax1* during myotomal and sclerotomal lineage determination.

MATERIALS AND METHODS

Cloning and sequencing of the quail Shh cDNA

A cDNA library of stage 12 quail embryos was constructed into the ZAPexpress vector (Stratagene). The chicken *shh* cDNA probe (Riddle et al., 1993) was used to screen the library and a full-length quail cDNA was partially sequenced (Accession numbers AF022881, AF022882). Two antisense oligonucleotides were used in experimental studies (5 were tested), and those are OT5 (16 mer) = 5′-GAATTCTTGTCAACAG-3′ and OT7 (15 mer) = 5′-GGTCAGCTTTTTGGG-3′. The two control random oligonucleotides are OR5 (16 mer) = 5′-(C/G) (A/T) N (A/T) (A/T) (C/G) (A/T) (A/T) (C/G) (A/T) (C/G) (A/T) (A/T) (C/G) (A/T) (C/G)-3′ and OR7 (15 mer) = 5′-(C/G) (C/G) (A/T) (C/G) (A/T) (C/G) (C/G) (A/T) (A/T) N (A/T) (A/T) (C/G) (C/G) N-3′. All oligonucleotides were modified by addition of deoxyphosphorothioate groups at all linkages, and oligonucleotides were purified through HPLC columns before use. Oligonucleotides are resuspended and stored in water at the final concentration 400 μM.

Reassignment of chick Gli3 to the Gli2/Gli4 zinc-finger family

An additional 609 bp of 5′ region of the chicken *gli3* cDNA were sequenced to define the N terminus (Marigo et al., 1996b) (accession

number AF022818). Analysis of the chicken Gli3 N-terminal amino acid sequence and comparison with other members of the Gli3 zinc-finger family proteins show that chicken Gli3 shares only about 60% identity with human (Ruppert et al., 1990), mouse (Thien et al., 1996) and *Xenopus* Gli3 (Marine et al., 1997) (Fig. 4A). In contrast, when compared with mouse Gli2 (Hughes et al., 1997) or *Xenopus* Gli4 (Marine et al., 1997), the overall percent of identity increases to 72-79%, indicating that chicken Gli3 is more closely related to Gli2/Gli4 than to other Gli3 proteins (see Fig. 4A). Examination of the regions of homologies of Gli proteins that have been described previously (Marine et al., 1997) shows high conservation among all members of the family in the zinc-finger region (see Fig. 4B). However, analysis of the sequence in the N-terminal domain, including domain 1 (see Fig. 4B) demonstrates that Gli3 sequences are distinct from mouse Gli2, *Xenopus* Gli4 and chicken Gli3 (Fig. 4C). In contrast, chicken Gli3 shares specific sequence homologies with mouse Gli2 and *Xenopus* Gli4 that are different from other Gli3 proteins (Fig. 4C). These findings lead us to propose the reassignment of chicken Gli3 to the Gli2 /Gli4 family of proteins. Interestingly, only three *gli* genes have been identified in mouse (Hui et al., 1994). A human *gli4*-related gene (Tanimura et al., 1993) was recently shown to be more closely related to the mouse *gli2*, and therefore may be a human *gli2* gene (Hughes et al., 1997). In *Xenopus*, four genes, supposedly different, have been identified by two different groups (Lee et al., 1997; Marine et al., 1997). In the absence of nucleotide sequence data for the *Xenopus gli2* (Lee et al., 1997), it is not possible to resolve whether *Xenopus gli2* and *gli4* are identical, and, therefore, whether the chicken cDNA belongs to the Gli2 family or the Gli4 family. Therefore, in this study, we will refer to chicken *gli3* as *gli2/4*. Finally, supporting this proposition, we report that chicken *gli2/4* is expressed in the somite myotomal region (see below), as described for *Xenopus gli4* (Marine et al., 1997), whereas no member of the Gli3 family has been reported to be expressed in the myotome.

Surgeries and bead implantation experiments

Stage 12 quail embryos were isolated by transfer to paper rings. An oblique ventral-dorsal incision through the neural tube was introduced with an electrolytically sharpened tungsten knife at the level of the segmental plate, leaving dorsal-lateral neural tube in contact with presegmental mesoderm on the operated side and the intact notochord/neural tube complex in contact with the presegmental mesoderm on contralateral side. A bead (Affigel) soaked in PBS (n>100) or in a 100 μg/ml solution of N-Shh (bacterially produced Shh 198) (Marti et al., 1995a) (n>100) was then implanted into the lumen of the neural tube. For axial tissue separation experiments (n=20), a ventral-dorsal incision was performed through the three layers between the segmental plate and the neural tube/notochord complex. When indicated, a control PBS bead was inserted to maintain the tissues separated. For surface ectoderm removal experiments (n=20), embryos were incubated at room temperature for 30 seconds in a Dispase I solution (Boehringer Mannheim) and rinsed in PBS before surface ectoderm was surgically removed over the segmental plate, the neural tube and the lateral plate. For separation of lateral plate from segmental mesoderm (n=15), a dorsal-ventral incision was performed through the three layers between the segmental plate and the lateral plate. Following all surgeries, embryos were cultured for 16 hours, as previously described (Pownall et al., 1996), and then fixed in 4% formaldehyde, 2 mM EGTA in PBS for in situ hybridization analysis.

Explant culture

Segmental plate explants (300 μm) were dissected from stage 12 quail embryos. When indicated, the surface ectoderm was removed over the segmental plate region as described above. Explants were then cultured on a 24-well plate coated with 1% agarose in DMEM- 15% FCS (1:1) for 16 hours at 37°C in a CO_2 incubator. The culture medium was composed of DMEM with 15% FCS and 1% penicillin/streptomycin/fungizone. Explants (n=30) were then transferred to an Eppendorf tube, rinsed with PBS, fixed at 4°C in 4% formaldehyde, 2 mM EGTA in PBS and analyzed by in situ hybridization.

Culture of antisense-treated quail embryos

Quail embryos (stage 11-13, 12-18 somites) were rinsed in the culture medium (Leibovitz's L15 medium, 1% penicillin/streptomycin, Gibco-BRL) and transferred into Eppendorf tubes containing 300 μl of culture medium containing deoxyphosphorothiate oligonucleotides (final concentration 80 μM). Five different antisense oligonucleotides were tested in combinations or alone, and the combination OT5/OT7 was selected because of effective inhibition of Shh protein expression in the posterior embryo. Different oligonucleotide concentrations were tested, and 80 μM gave optimal inhibition with no evident toxicity. After incubation at 37°C for 20 minutes, embryos were transferred into a cell culture insert (pore size 8.0 μ, Falcon) which was suspended inside a 300 μl medium-containing well of a 24-well plate (Falcon). Embryos were cultured at 37°C for 8 hours with continuous presence of oligonucleotides. The medium was replaced after 4 hours with fresh medium and oligonucleotides. After culture, embryos (n>200) were rinsed in PBS and fixed at 4°C for 16 hours in PBS containing 4% formaldehyde, 2 mM EGTA, pH 7.0. Embryos were then processed for in situ hybridization using DIG-labeled RNA probes.

Whole-mount in situ hybridization

Embryos and explants were fixed in 4% formaldehyde, 2 mM EGTA in PBS at 4°C for 16 hours and analyzed by in situ hybridization with DIG-labeled (Boehringer Mannheim) RNA probes as previously described (Pownall et al., 1996) with some modifications. RNA probes were synthesized from quail *myoD* and quail *myf5* cDNAs (Charles de la Brousse and Emerson, 1990; Pownall and Emerson, 1992), quail *pax1* cDNA (a gift of R. Balling), quail *pax3* cDNA (a gift of C. Marcelle), chicken *pax6* cDNA (Li et al., 1994), chicken *HNF3β* cDNA (Ruiz i Altaba et al., 1995), chick *patched* cDNA (Marigo et al., 1996c), chick *gli* and *gli3* (now *gli2/4*) cDNA (Marigo et al., 1996b), chick *Hoxb8* cDNA (a gift of C. Tabin), and chick *Wnt7a* (a gift of R. Riddle). Embryos were photographed using a videocamera (LEICA), embedded in 4% low melting point agarose (Sigma) and 100 μ transverse sections were performed using a Vibrotome 3000. Sections were photographed on a DIC microscope (Leica) at 400× magnification.

Immunoblotting

Single embryos were cut at the level of somite VIII and the anterior and posterior halves were analyzed by immunoblot on a 5% SDS-PAGE (Standiford et al., 1997). Blots were probed with a polyclonal antibody against Shh (Ab80) (Marti et al., 1995a) at 1:1000 dilution using HRP-conjugated anti-rabbit antibodies (Vector) diluted 1:1500 or with a monoclonal antibody against α-tubulin (Sigma) at 1:2000 dilution with HRP-conjugated anti-mouse antibodies (Vector) diluted 1:1500. Immunoblots were quantitated using a Phosphoimager (Molecular Dynamics) and the program IPGel.

RESULTS

Shh is the notochord signal for *myoD* and *pax1* activation in somites

Notochord ablation and grafting studies have established that notochord signals control *myoD* and *pax1* activation in somites of avian embryos (Ebensperger et al., 1995; Pownall et al., 1996). To identify notochord signal molecules responsible for *myoD* activation, we have designed a bead implant assay to test in vivo the ability of candidate proteins to activate *myoD* in

somites formed in the absence of the notochord. In this assay, the notochord and the ventral neural tube are separated from the segmental plate by a ventral-lateral incision in the region of the embryo posterior to newly formed somites (Fig. 1A). This procedure leaves the segmental plate in contact with only the lateral and dorsal neural tube on the operated side, which provides maintenance but not activation signals, and with the notochord and the neural tube on the control, contralateral side. Beads soaked in PBS, with or without test proteins, are then inserted in a ventral position. After culture for 16 hours, 7 somite pairs form around the bead, on both the control and operated sides. In control experiments, with beads soaked in PBS, we observed that *myoD* and *pax1* were activated normally in somites formed in contact with the neural tube/notochord complex. However, in absence of the notochord, *myoD* and *pax1* were not activated (Fig. 1B,E), consistent with our previous results (Pownall et al., 1996) and work by others (Koseki et al., 1993; Fan and Tessier-Lavigne, 1994; Ebensperger et al., 1995), which showed that the notochord is required for both *pax1* expression in the sclerotome lineage and *myoD* expression in the myotome lineage.

This bead implantation assay was then used to test the activity of Shh for *myoD* and *pax1* activation. These studies used recombinant N-terminal Shh, which has been shown to account for all biological activities of Shh (Marti et al., 1995a). 16 hours after implantation of Shh beads, we observed that *myoD* (Fig. 1C) and *pax1* (Fig. 1F) were both induced in somites formed on the operated side of the embryo, in the absence of notochord. Transverse sections through Shh-induced embryos revealed the dorsal myotomal localization of *myoD* expression (Fig. 1D) and ventral sclerotomal localization of *pax1* expression (Fig. 1G). Decreasing the bead concentration of N-Shh by 10-fold resulted in the activation of *pax1* but not *myoD*, indicating that Shh may act on somites in a concentration-dependent manner, as previously demonstrated for Shh signaling of the neural tube (Ericson et al., 1996). However, in the absence of any information on the concentration of Shh protein that diffuses out of the bead, estimation of the activating dose of Shh remains speculative. These data, therefore, show that Shh alone is sufficient to replace the notochord for the activation of both *pax1* and *myoD* in the ventral and dorsal medial compartments of somites.

To determine whether Shh is essential for *pax1* and *myoD* activation during somite formation, we employed an antisense oligonucleotide technique to knockout gene expression in developing avian embryos (Angela Nieto et al., 1994; Srivastava et al., 1995; Isaac et al., 1997). This oligonucleotide-mediated antisense knockout method causes the targeted inhibition of specific gene expression in embryos for short treatment periods (8 hours) at a specific developmental stage, thereby minimizing complications arising from embryonic lethality, activation of compensatory mechanisms and disruption of general processes such as cell proliferation. For these experiments, we cloned and sequenced the quail homologue of *shh* and utilized its sequence to design several sets of antisense oligonucleotides (see Materials and Methods). As controls, random oligonucleotides were generated with a nucleotide composition similar to those of the antisense oligonucleotides. Selected oligonucleotides did not share any sequence homology with other members of the Hedgehog family, Indian hedgehog (Ihh) (Vortkamp et al., 1996) and Desert hedgehog (Dhh) (Echelard et al., 1993) and were modified by incorporation of phosphorothioate groups to prevent their degradation by nucleases (Zon and Stec, 1991).

Shh expression was knocked out in stage 11 to 13 quail embryos cultured for 8 hours in the presence of 80 μM of individual or combinations of two antisense oligonucleotides. In situ hybridization was used to assay for the activation and expression of *myoD* and *pax1* in somites (Fig. 2). During this short treatment, three somite pairs form, demonstrating the non-toxicity of the oligonucleotide treatment on normal development of quail embryos. Antisense oligonucleotides were tested for their inhibitory effects on *pax1* expression, which is normally activated in somite II-III throughout early quail embryo development (Ebensperger et al., 1995; Borycki et al., 1997). Two antisense oligonucleotides with sequences in the region of the translation start codon were found to inhibit *pax1* expression when oligonucleotides were tested individually, and more effectively when applied together. The

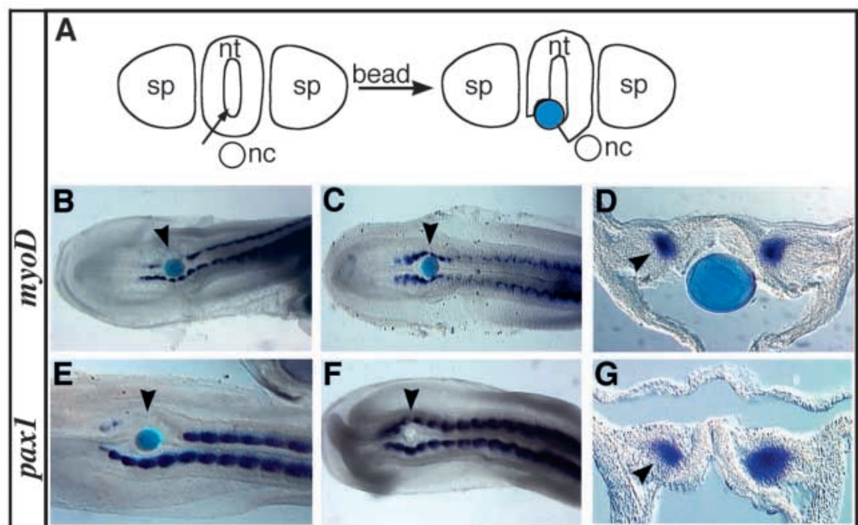

Fig. 1. Shh can replace the notochord for *myoD* and *pax1* activation during somite formation. (A) Representation of bead implantation surgery. The notochord and ventral neural tube were surgically separated from the segmental plate on one side of stage 12-13 quail embryos. The segmental plate on the contralateral side remains in contact with the notochord and ventral neural tube and serves as an internal control. An Affigel bead soaked in PBS (B,E) or in 100 μg/ml Shh (C,D,F,G) was inserted into the incision created in the neural tube, and embryos were cultured for 16 hours. Following fixation, gene expression was assayed by whole-mount in situ hybridization, followed by vibrotome sectioning (D,G). (B-D) *myoD* expression in embryos cultured with a control (B) or a Shh (C,D) bead. (E-G) *pax1* expression in embryos cultured with a control (E) or a Shh (F,G) bead. Occasionally, the bead implant fell out of the embryo during the in situ hybridization procedure (F,G). The arrowhead indicates the operated side lacking notochord/ventral neural tube. nc, notochord; sp, segmental plate; nt, neural tube.

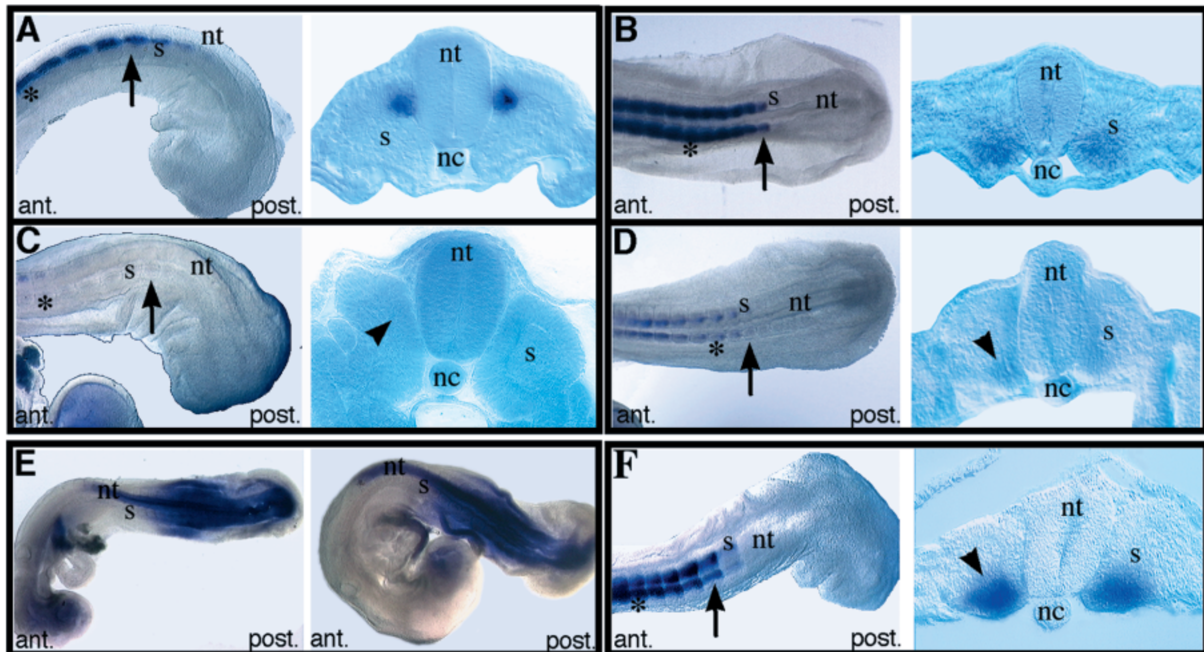

Fig. 2. Antisense-mediated downregulation of Shh inhibits *myoD* and *pax1* activation during somite formation. Intact stage 11-12 quail embryos were cultured for 8 hours with random (A,B,E left panel) or *shh* antisense (C,D,E right panel,F) oligonucleotides, and gene expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. (A,C) *myoD* expression in embryos treated with control (n=40) (A) or *shh* antisense oligonucleotides (n=60) (C). (B,D) *pax1* expression in embryos treated with control (n=40) (B) or *shh* antisense oligonucleotides (n=60)(D). (E) *Hoxb8* expression is unaffected by treatment of embryos with either the random (n=6) (left panel) or the *shh* antisense (n=9) (right panel) oligonucleotides.(F) *pax1* expression is rescued in *shh* antisense-treated embryos by addition of purified N-Shh (10 µg/ml) to the culture medium (n=21). The arrow indicates the site of the transverse section. The arrowhead indicates the loss of *myoD* and *pax1* activation in antisense-treated embryos and the recovery of *pax1* activation in the rescue experiment. The asterisk shows the position along the anteroposterior axis at which embryos were cut in two halves for immunoblot analysis (see Figure 3). nc, notochord; nt, neural tube; s, somite; ant, anterior; post, posterior.

level of Shh protein, as assessed by western blot analysis, was inhibited by 85% in the posterior of the embryo where *pax1* expression was most severely reduced (Fig. 3). Decrease in the level of Shh protein in the anterior of the embryo was not as evident, probably due to the higher accumulation of Shh proteins at this axial level. Control oligonucleotides did not affect *myoD* or *pax1* expression along the entire anterior-posterior axis (Fig. 2A,B). In contrast, *shh* antisense oligonucleotides led to a severe inhibition of *pax1* activation in the most posterior, newly formed somites, up to somite IV, and decreased expression in the more anterior somites that had initiated *pax1* expression prior to addition of *shh* antisense (Fig. 2D). *shh* antisense treatment had an even more dramatic inhibitory effect on *myoD* activation, causing the complete inhibition of *myoD* expression in all but the most anterior somites (Fig. 2C). The expression of the other early myogenic regulatory gene, *myf5*, was also inhibited by *shh* antisense oligonucleotides (data not shown). Therefore, these experiments show that Shh is essential for both *pax1*, *myoD* and *myf5* activation. Our observations also suggest that Shh may be involved in the maintenance of *myoD*, and to a lesser extent *pax1*, expression.

To further demonstrate the specificity of the *shh* antisense oligonucleotide treatment, we assayed the expression of *Hoxb8* (Krumlauf, 1994), an homeobox gene specifically expressed in the posterior region of the embryo where *pax1* and *myoD* gene expression is most effectively blocked by antisense treatment. We found that *Hoxb8* expression was unaffected by treatment with control or *shh* antisense oligonucleotides (Fig. 2E). We also examined the expression of other genes known to be modulated by Shh signals, including *HNF3β* (Sasaki et al., 1997), which is involved in the establishment of the dorsal-ventral patterning in the neural tube and ventral cell fate (floor plate, motor neurons), and *pax3* and *pax6*, which are involved in the dorsal specification of the neural tube (Roelink et al., 1995; Chiang et al., 1996). We observed that *shh* antisense treatment led to a ventral expansion of *pax3* and *pax6* expression in the neural tube (data not shown), as observed in *shh* null mice (Chiang et al., 1996) and in neural tube explants after treatment with Shh-blocking antibodies (Ericson et al., 1996). However, in antisense-treated quail embryos, ventral-specific gene expression was not completely lost, as shown by *HNF3β*, where expression was greatly reduced, but not entirely eliminated in the floor plate region (data not shown). Finally, we showed that *pax1* expression can be rescued by addition of 10 µg/ml N-Shh protein to the culture medium of *shh* antisense-treated embryos (Fig. 2F). These data, therefore, establish that the inhibitory effects of *shh* antisense treatment on *pax1* and *myoD* expression in quail embryos are highly specific for *shh* expression and, together with the results of the Shh bead implantation experiments, these *shh* antisense experiments establish that Shh is an essential and a sufficient

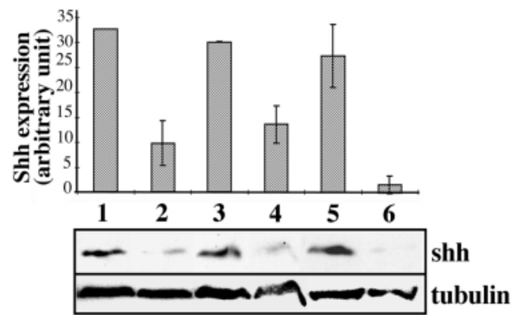

Fig. 3. Treatment with *shh* antisense oligonucleotides downregulates Shh protein in quail embryos. Embryos were cultured for 8 hours in absence of oligonucleotides (1,2) and in presence of random (3,4) and *shh* antisense (5, 6) oligonucleotides. Embryos were cut at the level of somite VII, leaving anterior (1,3,5) and posterior (2,4,6) halves, that were subject to immunoblotting using a monoclonal antibody against the N terminus of Shh (Ab80) or a monoclonal antibody against α-tubulin, as an internal control. Shh expression was quantitated by scanning the Shh band, using a Phosphoimager (Molecular Dynamics) and the program IPGel, and normalizing values to the tubulin band. The graph shows the average levels of Shh protein in embryos for each culture condition (*n*=6). Shh protein level is decreased (7-fold) in the posterior part of *shh* antisense-treated embryos (6) compared to control (2,4) embryos. The error bars are the standard deviation of the values for each culture condition.

notochord signal for the activation of *myoD* and *pax1*, during the early regulatory steps involved in myotomal and sclerotomal lineage determination.

Downstream *Shh* signal response genes, *patched*, *gli* and *gli2/4,* are regulated during somite formation

Although Shh is an essential signal for *pax1* and *myoD* activation in newly forming somites, it is clear that the temporal regulation of *pax1* and *myoD* activation cannot be attributed directly to Shh because Shh proteins are synthesized constitutively by the notochord along the entire axis of the early embryo (Marti et al., 1995b), even in posterior regions of segmental mesoderm where *myoD* and *pax1* are not yet activated (Borycki et al., 1997). Furthermore, *pax1* and *myoD* transcription is activated in spatially distinct ventral and dorsal medial domains of the newly formed somite. Therefore, the spatial and temporal control of *myoD* and *pax1* activation must be regulated by mechanisms that operate downstream in the Shh response pathway. These considerations led us to examine the developmental expression of *patched* (*ptc*), the Shh receptor, and *gli* and *gli3* (now designated *gli2/4*) zinc-finger transcription factors. These are downstream genes that mediate the Shh response (Marigo et al., 1996b). In our sequence analysis of the chick *gli3* cDNA (Marigo et al., 1996b), we discovered that its N-terminal sequences are homologous to mouse *gli2* and *Xenopus gli4* and distinct from *gli3*, leading to the designation of this gene as *gli2/4* (see Materials and Methods; Fig. 4).

Using whole-mount in situ hybridization, we examined the expression of these Shh response genes in stage 12 embryos. We observed high level expression of *ptc* transcripts in newly forming somites, but no *ptc* transcript was detected in the unsegmented paraxial mesoderm, showing that *ptc* activation is coordinated with somite formation and precedes the activation of *myoD* and *pax1* (Fig. 5A). In contrast, *ptc* is expressed in the neural tube along the entire anteroposterior axis (Fig. 5A) and, therefore, is regulated differently in somites and the neural tube. In transverse sections, we observed that *ptc* is expressed in a ventral medial domain of newly formed posterior somites (somite I) but, in more anterior somites, *ptc* transcripts are progressively downregulated in the ventral domain and upregulated in the dorsal medial domain, and then expressed exclusively in the myotome-forming region (somite VIII) (Fig. 5A). Since it is generally thought that *ptc* expression marks cells receiving Shh signals (Chen and Struhl, 1996; Goodrich et al., 1996; Marigo et al., 1996c), these observations indicate that Shh is directly signaling both the ventral and the dorsal compartments of the somite. Consistent with this interpretation, treatment of stage 12 quail embryos with *shh* antisense oligonucleotides inhibits *ptc* activation in somites (Fig. 5B), establishing that *ptc* expression in somites is upregulated in response to Shh signals. *ptc* expression in the neural tube is decreased but not eliminated by *shh* antisense treatment (Fig. 5B), suggesting either that the low amount of Shh protein remaining after antisense treatment (see Fig. 2) is sufficient to maintain neural tube expression of *ptc*, or that neural tube *ptc* mRNA is stable and therefore persists during the short time of antisense treatment.

gli and *gli2/4* gene expression also has been examined by in situ hybridization in stage 12 quail embryos (Fig. 6). Our results show that *gli*, like *ptc*, is not expressed in the unsegmented paraxial mesoderm, whereas neural tube expression occurs just posterior to the first somite, coinciding with floor plate formation (Lee et al., 1997) (Fig. 6A). Significantly, *gli* is activated in coordination with somite formation, in the newest formed somite I, and is expressed in a medial domain encompassing the domains of early *myoD* and *pax1* expression. In more anterior somites, *gli* expression remains restricted to the ventral medial domain (Fig. 6A). *gli2/4* also is activated in somite I, in coordination with somite formation (Fig. 6B), and its expression in the neural tube extends along the entire anteroposterior axis. In contrast to the ventral somite expression of *gli*, *gli2/4* is first expressed in the entire newly formed somite, and then its expression becomes restricted to the dorsal medial myotome-forming domain where *myoD*, as well as *ptc,* are co-expressed (Fig. 6B). Examination of sections from 16- and 20-somite-old embryos labeled with *gli2/4*-DIG riboprobe shows that dorsalization of *gli2/4* transcripts occurs immediately prior to dermomyotome formation, at the level of somite VIII and VII, respectively. This observation indicates that dorsalization of *gli2/4* expression does not play a direct role in *pax1* activation, which occurs at the level of somite III (Borycki et al., 1997). However, the activation of *ptc*, *gli* and *gli2/4* in the sclerotome and myotome domains during somite formation and their differential expression in the dorsal and ventral somite compartments indicates these components of the Shh response pathway have important functions in *pax1* and *myoD* expression in the ventral and dorsal somite in response to the constitutively expressed Shh signals.

We also have performed an in situ hybridization analysis of *ptc*, *gli* and *gli2/4* expression in quail in earlier stage embryos when *myoD* is not activated in any somite. We found that *ptc*, *gli* and *gli2/4* are activated in all 4 somites formed in embryos

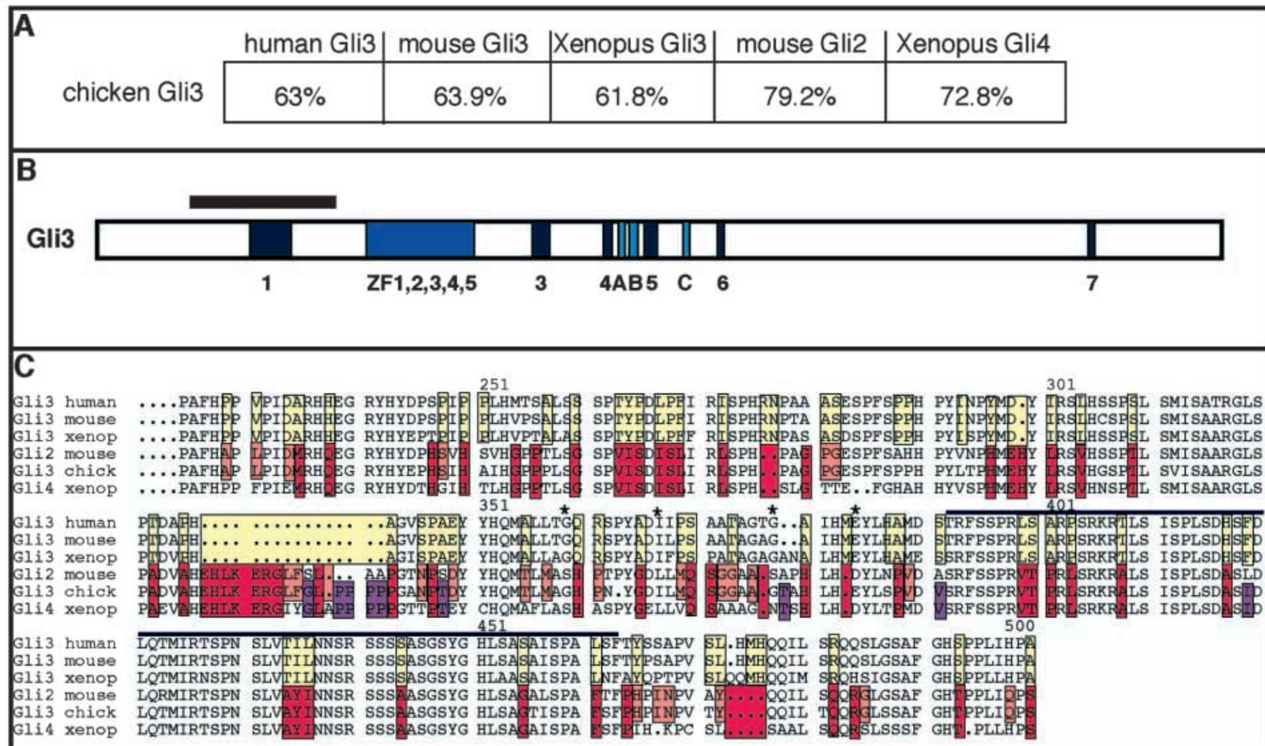

Fig. 4. Chick Gli3 is more homologous to Gli2 and Gli4 than to Gli3. (A) Percent identity of protein sequence of chick Gli3 (Marine et al., 1997) with human Gli3 (Ruppert et al., 1990), mouse Gli3 (Thien et al., 1996), *Xenopus* Gli3 (Marine et al., 1997), mouse Gli2 (Hughes et al., 1997), *Xenopus* Gli4 (Marine et al., 1997). (B) Schematic representation of the different homology domains in the Gli3 protein, as described by Marine et al. (1997). These comprise five zinc-finger domains (ZF1,2,3,4,5), 6 sequence homology domains (1-7, excluding the zinc-finger domain), and 3 protein kinase A phosphorylation sites domains. The black bar above the homology domain 1 indicates the position of the sequences shown in C. (C) The 605 bp of the 5′ region of the chick *gli3* cDNA was sequenced (Genbank accession number AF022818). Its open reading frame translation product was compared to the human, the mouse, and the *Xenopus* Gli3 protein sequences, as well as to the mouse Gli2 and the *Xenopus* Gli4 protein sequences. Comparison of sequences, including homology domain 1, is shown. Dots show gaps in the sequence. Yellow boxes encircle sequences identical only between human, mouse and *Xenopus* Gli3. Fuschia boxes encircle sequences identical only between mouse Gli2, chick Gli3 and *Xenopus* Gli4. Pink boxes encircle sequences identical only between mouse Gli2 and chick Gli3. Purple boxes encircle sequences identical only between chick Gli3 and *Xenopus* Gli4. Stars above the alignment indicates sequences identical only between all Gli3 proteins. The bar above the alignment indicates the position of the homology domain 1 shown in B. Uncircled sequences are either completely identical or highly variable between all Glis. Note that chick Gli3 displays higher homology with mouse Gli2 and *Xenopus* Gli4 than with Gli3. On this basis, we are re-naming chick *gli3* as chick *gli2/4*.

as early as stage 8 (data not shown). However, we observed that, in stage 8 and 9 embryos, *ptc* expression remains restricted to the most ventral domain of somites (Fig. 7A) whereas, in anterior somites of stage 11 embryos, *ptc* transcripts extend more dorsally into the dorsal medial region where *myoD* begins to be expressed (Fig. 7B). These findings indicate that the dorsal medial somite cells of early embryos do not receive Shh signals, which may explain why *myoD* is not activated in any somite until stage 11, whereas *pax1* is activated in ventral somites, even at early stages (Borycki et al., 1997).

gli is regulated by the notochord and Shh, and *gli2/4* by the dorsal surface ectoderm

To investigate the mechanisms responsible for the spatially patterned activation of *gli* and *gli2/4* in newly forming somites, we performed series of surgical experiments. Segmental plate mesoderm was surgically separated from the notochord and neural tube, and *gli* and *gli2/4* expression was examined by in situ hybridization in somites formed after 16 hours of culture. Fig. 8A shows that *gli* is not activated in the newly formed somites in the absence of the axial tissues. On the contralateral control side, *gli* activation occurs normally. We also examined *gli* activation in somites formed in absence of the notochord and the floor plate, and found that *gli* activation in somites, like in the ventral neural tube (Lee et al., 1997), requires ventral midline tissues (Fig. 8B). Finally, we implanted beads soaked in N-Shh in place of the ventral midline tissues and found that N-Shh is sufficient to induce high level of *gli* expression in somites and neural tube on both the operated and the contralateral side (Fig. 8C). These experiments establish that Shh produced by the notochord controls *gli* expression, both in newly forming somites and in the neural tube.

In contrast to *gli*, *gli2/4* activation during somite formation is not controlled by Shh. *gli2/4* expression is not disrupted by separation of newly forming somites from the axial tissues (Fig. 9A). Results in Fig. 9B show that the lateral plate alone is not essential for *gli2/4* activation in somites. When surface ectoderm is removed over the segmental plate only, *gli2/4* activation is not affected (Fig. 9C). However, removal of the

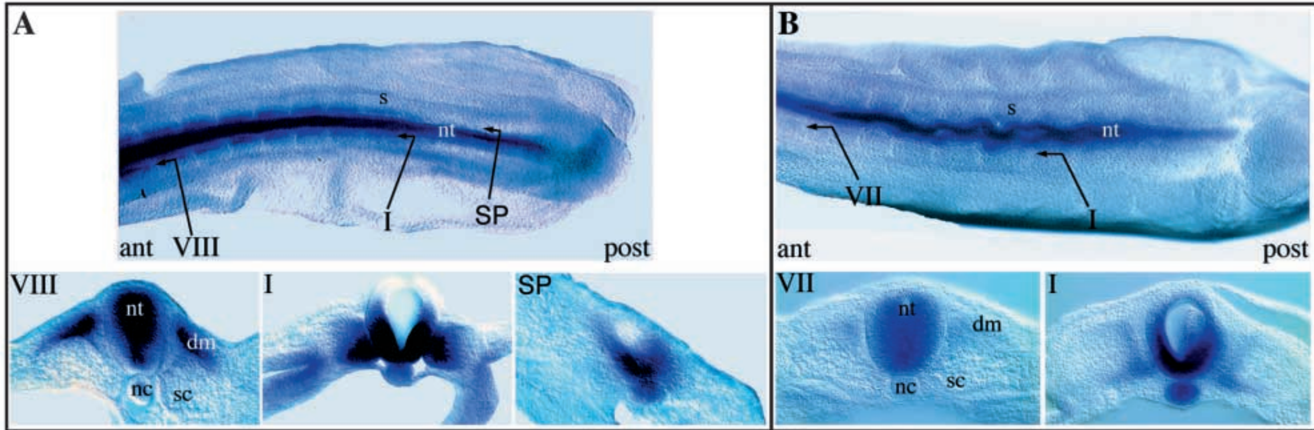

Fig. 5. Shh is required for *patched* (*ptc*) expression in the ventral and dorsal somite. (A) Stage 11-12 quail embryos were cultured for 8 hours with random oligonucleotides (*n*=24). *ptc* expression was assessed by whole-mount in situ hybridization (upper panel) followed by vibrotome sectioning (lower panel) in unsegmented mesoderm, at the level of somite I (epithelial) where *ptc* expression is first activated and at the level of somite VIII (myotome). Note the absence of *ptc* expression in the unsegmented mesoderm, the ventral and dorsal expression of *ptc* in somite I, the subsequent dorsal localization of *ptc* expression to the myotome in somite VIII, and the neural tube expression of *ptc*, extending into the posterior region of the embryo, including expression in the posterior-most notochord. (B) Stage 11-12 quail embryos were cultured for 8 hours with *shh* antisense oligonucleotides (*n*=32). *ptc* expression was assessed by whole-mount in situ hybridization (upper panel) followed by vibrotome sectioning (lower panel) at the level of somite I (epithelial somite) and at the level of somite VII (myotome). Note that *ptc* expression is almost completely absent in somites following Shh down-regulation by *shh* antisense oligonucleotides, but *ptc* continues to be expressed in the neural tube. The arrows indicate the levels of the sections. s, somite; nt, neural tube; nc, notochord; sc, sclerotome; dm,: dermomyotome; ant, anterior; post, posterior.

surface ectoderm over the segmental plate, the neural tube and part of the lateral plate results in a significant reduction in *gli2/4* expression (data not shown). To test whether the axial tissues compensate for the lack of surface ectoderm in *gli2/4* activation, we concomitantly separated the axial tissues and removed the surface ectoderm over the segmental plate, neural tube and lateral plate. Under these conditions, we found that *gli2/4* is not activated on the side lacking surface ectoderm and axial tissues (Fig. 9D, right side), and is reduced significantly on the side lacking the surface ectoderm only (Fig. 9D, left side). To monitor surface ectoderm removal, we assayed *Wnt7a* expression, which has been previously showed to be expressed by the surface ectoderm (Parr and McMahon, 1995). We show that *Wnt7a* transcripts are absent in the region of surface

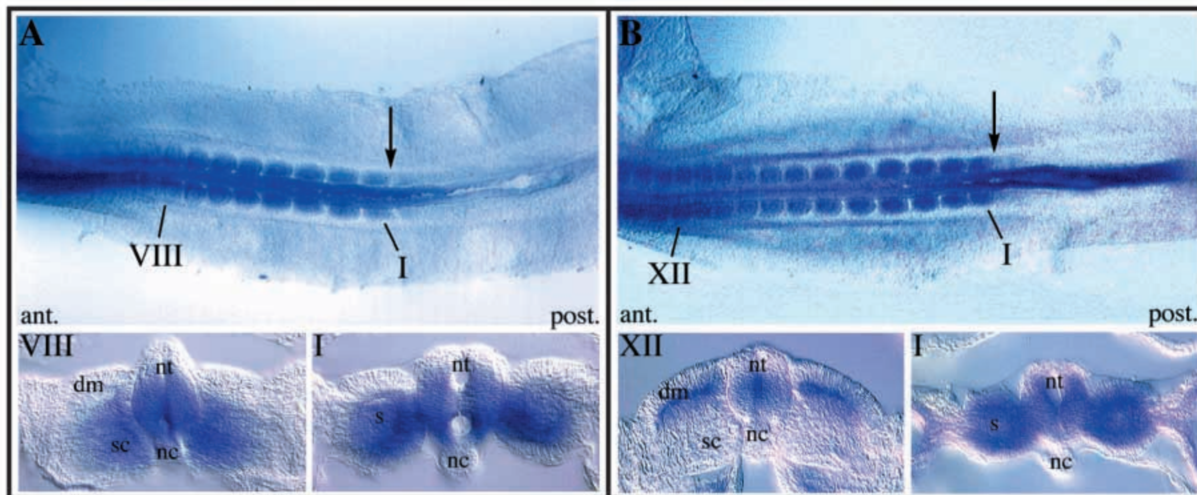

Fig. 6. *gli* and *gli2/4* are differentially activated and patterned in their expression in somites of stage 12 quail embryos. (A) Whole-mount in situ hybridization using *gli* probe (upper panel). Embryos were sectioned at the level of somite I (bottom right panel) and somite VIII (bottom left panel). *gli* expression is activated in somite I and restricted to the ventral and medial somite, including regions of *myoD* and *pax1* activation. (B) Whole-mount in situ hybridization using *gli2/4* RNA probe (upper panel). Embryos were sectioned at the level of somite I (bottom right panel) and somite XII (bottom left panel). *gli2/4* expression is first activated and expressed throughout somite I (bottom right panel), and becomes restricted to expression in the dorsal medial lip, where *myoD* is activated, and the forming myotome (bottom left panel). Note that *gli* and *gli2/4* transcripts are not detected in the paraxial mesoderm posterior to somite I (arrow), while transcripts are detected in the neural tube in regions posterior to somite formation. nc, notochord; nt, neural tube; sc, sclerotome; dm, dermomyotome; ant, anterior; post, posterior.

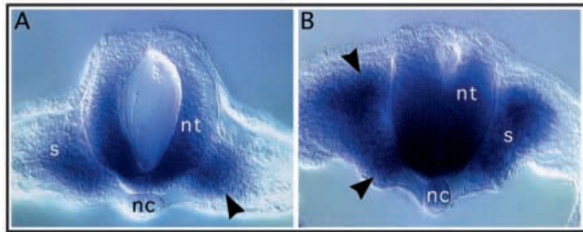

Fig. 7. Dorsal medial *ptc* expression in somites of stage 11, but not in stage 9 embryos. Stage 9 (A) and 11 (B) quail embryos were analyzed by in situ hybridization using a *ptc* probe. Vibrotome sections of the most anterior somite also are shown. Arrowheads indicate the ventral expression of *ptc* in stage 9 embryos and the ventral and dorsal expression of *ptc* in stage 11 embryo. Note that *ptc* transcripts never get completely dorsalized in stage 11 embryos like in stage 12 embryos (see Fig.5A). nc, notochord; nt, neural tube; s, somite.

ectoderm removal (Fig. 9E). Thus, the low level of *gli2/4* expression in absence of surface ectoderm is not the result of incomplete removal or rehealing of the surface ectoderm (Fig. 9E), supporting our conclusion that additional signals from the axial tissues compensate for the lack of surface ectoderm signals in vivo. To completely eliminate influences from adjacent tissues in *gli2/4* activation, we explanted segmental plate mesodermal tissue into culture in the presence or absence of surface ectoderm (Fig. 10A). After 16 hours in culture, we observed that *gli2/4* expression is activated in somites that formed from segmental plate mesoderm in contact with the overlying ectoderm, but not in unsegmented mesoderm (Fig. 10B). In contrast, when segmental plate explants were cultured without surface ectoderm, somite formation did not occur (Fig. 10C), and *gli2/4* was not activated in the paraxial mesoderm of these explants (Fig. 10C), although *Hoxb8* transcripts could be detected demonstrating their viability (Fig. 10C insert). Thus, these data demonstrate that *gli2/4* is activated in coordination with somite formation, and the surface ectoderm is required both for somite formation and for *gli2/4* activation.

DISCUSSION

In this study, we present antisense inhibition and bead implantation experiments, which establish that Shh is an essential and sufficient notochord signal for the activation of *myoD* and *pax1* during myotomal and sclerotomal lineage determination in somites of quail embryos. In previous studies, we and others have shown that the notochord alone is sufficient to induce both *myoD* and *pax1* in somites (Koseki et al., 1993; Ebensperger et al., 1995; Pownall et al., 1996). Since the notochord is the primary source of Shh signals from the axis, we can now conclude that Shh alone is the source of the notochord inductive signal for both sclerotome and myotome determination in early stage avian embryos. We also provide evidence that genes in the Shh signal response pathway, *ptc*, *gli* and *gli 2/4*, are temporally and spatially regulated in response to Shh and surface ectoderm inductive signals during somite formation. The regulated expression of these Shh response genes provides a framework for understanding the mechanisms by which Shh regulates the differential temporal

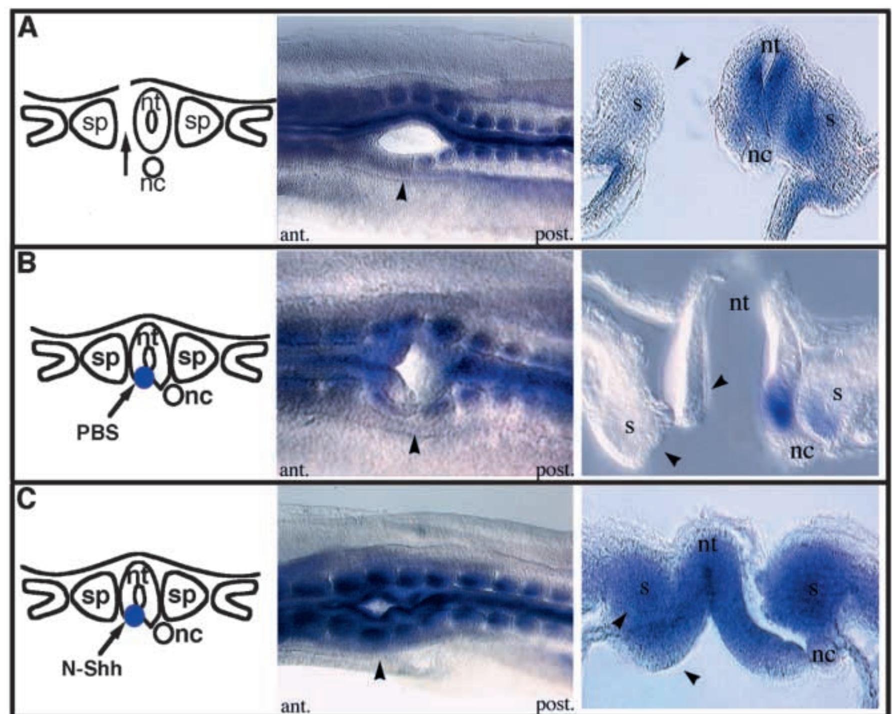

Fig. 8. *gli* activation in somites is controlled by notochord and Shh. (A) Axial tissues are essential for *gli* activation in somites. Notochord and neural tube were surgically separated from the segmental plate of stage 12 quail embryos (left panel). Embryos were incubated for 16 hours and *gli* expression was assessed by whole-mount in situ hybridization (middle panel), followed by vibrotome sectioning (right panel). (B) Notochord and ventral neural tube are essential for *gli* activation in somites and neural tube. Notochord and ventral neural tube were surgically separated from the segmental plate and a control bead soaked in PBS was inserted into the incision (left panel). Embryos were cultured for 16 hours, and *gli* expression was assessed by whole-mount in situ hybridization (middle panel), followed by vibrotome sectioning (right panel). Note that, in addition to a lack of *gli* activation in somites, *gli* is not expressed in the ventral neural tube on the operated side. (C) Shh induces *gli* expression in somites and neural tube in the absence of notochord and neural tube. Notochord and ventral neural tube were surgically separated from the segmental plate and a bead soaked in Shh was inserted into the incision (left panel). Embryos were cultured for 16 hours and *gli* expression was assessed by whole-mount in situ hybridization (middle panel), followed by vibrotome sectioning (right panel). Occasionally, the bead fell out of the embryo during the in situ hybridization procedure (B,C), and tissues were slightly displaced following sectioning and mounting (B,C). The arrow indicates the surgical incision performed in the embryo. The arrowheads indicate the operated side. nc, notochord; nt, neural tube; s, somite; sp, segmental plate; ant, anterior; post, posterior.

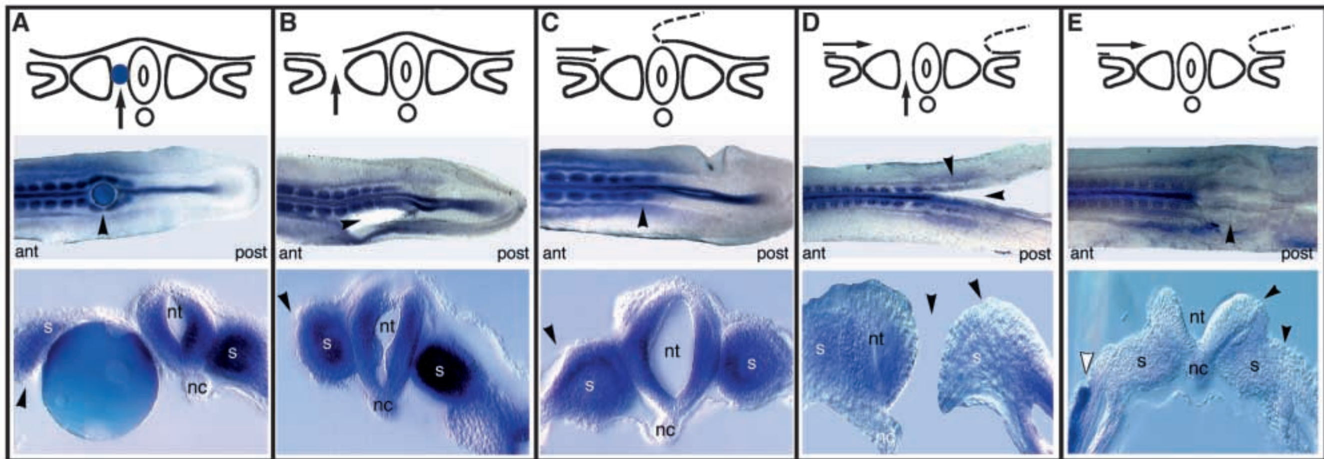

Fig. 9. Axial tissues and lateral plate mesoderm are not required for *gli2/4* activation in somites. (A) Notochord and neural tube are not essential for *gli2/4* activation in somites. Notochord and neural tube were surgically separated from the segmental plate of stage 12 quail embryos. Embryos were cultured for 16 hours and *gli2/4* expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. (B) Lateral plate is not essential for *gli2/4* activation in somites. Lateral plate mesoderm was surgically separated from the segmental plate of stage 12 quail embryos. Embryos were cultured for 16 hours and *gli2/4* expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. (C) Surface ectoderm removal does not affect *gli2/4* activation in somites. Surface ectoderm was surgically removed from the segmental plate of stage 12 quail embryos. Embryos were cultured for 16 hours and *gli2/4* expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. (D) Surface ectoderm and axial tissues provide signals for *gli2/4* activation. Surface ectoderm was surgically removed from the segmental plate, the neural tube and the lateral plate of stage 12 quail embryos, and segmental plate was separated from axial tissues on one side. Embryos were cultured for 16 hours and *gli2/4* expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. Note partial down-regulation of *gli2/4* expression on the side lacking only surface ectoderm, but more dramatic effect following surface ectoderm removal and axial separation. (E) Ectodermal expression of *Wnt7a* is lost following surface ectoderm removal. Surface ectoderm was removed from the segmental plate, the neural tube and the lateral plate of stage 12 quail embryos. Embryos were cultured for 16 hours and *Wnt7a* expression was assessed by whole-mount in situ hybridization, followed by vibrotome sectioning. Note ectodermal *Wnt7a* expression in areas where the surface ectoderm was preserved (white arrowhead) but absence of *Wnt7a* expression over somites and neural tube where the surface ectoderm was removed (black arrowhead). The arrows indicate the surgical incision. Arrowheads in middle and lower panels indicate the operated side. nc, notochord; nt, neural tube; s, somite; ant, anterior; post, posterior.

and spatial activation of genes in the myotomal and sclerotomal lineages during somite formation.

Shh is an essential and sufficient notochord signal for *myoD* and *pax1* activation in avian somites

Our findings extend previous work on the role of Shh in somite induction. Earlier studies showed that retroviral expression of Shh can induce ectopic *myoD* and *pax1* expression in developing avian embryos (Johnson et al., 1994). Cultures of segmental plate explants also induce *pax1* in response to Shh (Fan and Tessier-Lavigne, 1994; Munsterberg et al., 1995) and induce *myoD* in response to Shh and either Wnt1, Wnt3 or Wnt4 (Munsterberg et al., 1995). Our Shh bead implantation experiments now demonstrate the in vivo inductive role of Shh and provide new information that Shh alone is sufficient to replace the notochord as an inductive signal for the patterned activation of both *myoD* and *pax1* during the early determination of myotomal and sclerotomal lineages. Finally, our *shh* antisense knockout experiments establish that Shh is an essential signal for the activation of these genes in the avian embryo. These findings do not exclude a role for additional signal molecules, such as Wnts, which are produced by the neural tube and surface ectoderm and have been implicated as working together with Shh in *myoD* regulation (Munsterberg et al., 1995).

Shh also has an essential role for early myogenesis in zebrafish embryos. Misexpression of *shh* in zebrafish embryos induces ectopic expression of *myoD* (Weinberg et al., 1996) and ectopic slow muscles (Blagden et al., 1997). In addition, two *hh*-related genes, *echidna hh* and *tiggy winkle hh*, are expressed in the notochord and the floor plate of zebrafish embryos; misexpression of *ehh* induces *myoD* and, in combination with *shh*, the formation of supernumerary pioneer cells (Currie and Ingham, 1996). Furthermore, a zebrafish mutant of *shh* has been identified and shown to be essential for the formation of muscle pioneer cells and for *myoD* activation (P. Haffter, personal communication).

In the mouse, *shh* is the only *shh*-related gene yet identified to be expressed in the notochord and floor plate (Bitgood and McMahon, 1995) and, therefore, is the only known *shh* that could initiate somite gene expression. Knockout of *shh* in mouse does not affect *myoD* expression and reduces, but does not eliminate *myf5* and *pax1* expression (Chiang et al., 1996), a result that contrasts with the ability of Shh protein to induce *pax1* in explant cultures of mouse presomitic mesoderm (Fan and Tessier-Lavigne, 1994). A possible explanation for the discrepancy in the findings in mouse with those in avian and fish embryos is that the role of Shh has redundant function in mammalian embryos. There is already evidence for a redundant pathway for myogenic determination in the mouse, and this redundant pathway may compensate for myogenesis in Shh null mice. Specifically, two separate pathways have been identified for *myoD* activation in mouse (Cossu et al., 1996). One pathway uses a signal from the neural

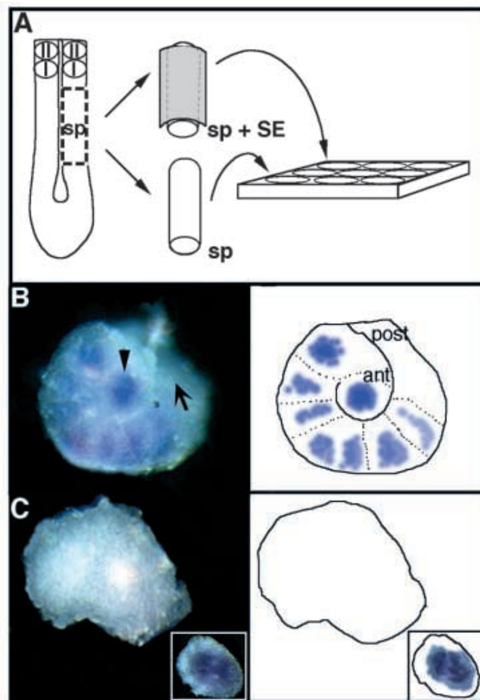

Fig. 10. *gli2/4* activation in paraxial mesoderm explants is regulated by surface ectoderm (SE) and by somite formation. (A) Segmental plate (sp) from stage 12 quail embryos was explanted and cultured for 16 hours with or without associated SE, in agarose-coated 24 well plates. (B) Segmental plate explant with SE following in situ hybridization with *gli2/4* probe. Note that *gli2/4* is activated only in condensed somites (arrowhead), and not in unsegmented mesoderm (arrow). ant: anterior; post: posterior. (C) Segmental plate explant without SE, following in situ hybridization with *gli2/4* RNA probe. Note that, in absence of SE, somites do not form and *gli2/4* is not activated. (insert) Segmental plate explant without SE, following in situ hybridization with *Hoxb8* RNA probe. Note expression of *Hoxb8*, demonstrating the viability of the explants.

tube/notochord complex, likely Shh, to induce *myf5* expression, which in turn transactivates *myoD* expression (Braun et al., 1989a). A second pathway utilizes different, unknown signal from the surface ectoderm for *myoD* activation (Cossu et al., 1996). Embryos with individual knockout mutations of *myf5* or *myoD* form muscles normally, whereas embryos with double knockouts of *myf5* and *myoD* (Rudnicki et al., 1993), and *myf5* and *pax3*, (essential for *myoD*) (Tajbakhsh et al., 1997), show a complete absence of muscle progenitor cells. Therefore, *shh* mutant embryos likely produce muscle through the surface ectoderm pathway, which induces *myoD* independently of the Shh-induced *myf5* pathway (Tajbakhsh et al., 1997). The specific role of the surface ectoderm signal may be to induce upstream regulators of *myoD* such as *pax3*, which is an essential gene for *myoD* activation (Tajbakhsh et al., 1997) and is controlled by surface ectoderm signals (Fan and Tessier-Lavigne, 1994). In avian embryos, recent studies indicate that surface ectoderm also can promote *myoD* activation, independent of axial tissues that produce Shh (Maroto et al., 1997), suggesting that redundant, Shh-independent pathways may exist in avian embryos for *myoD* activation, as exist in the mouse (Cossu et al., 1996).

Patterned somite expression of genes in the Shh response pathway

To investigate the specific mechanisms by which Shh signals induce the patterned activation of myotomal and sclerotomal genes during somite formation, we have examined the developmental expression of genes known to be in the Shh signaling pathway. These studies establish a number of specific roles for downstream signal response genes in the regulation of Shh signaling in somites.

First, our finding that somites express high levels of Shh response genes, *ptc*, *gli* and *gli2/4*, establishes that somite cells can directly receive Shh signals from the notochord and the floor plate. High level expression of *ptc* is an indicator that cells are receiving Shh signals (Chen and Struhl, 1996; Goodrich et al., 1996; Marigo et al., 1996a), as confirmed by our finding that antisense Shh oligonucleotide treatment knocks out *ptc* expression in newly formed somites. Once newly formed somites can receive Shh signals, the Shh response pathway becomes active, leading to the activation of *ptc*, as well as *gli* and other downstream genes (see below).

Second, our findings show that downstream Shh response genes are activated when somites condense from presomitic mesoderm, thereby providing a mechanism to coordinate the activation of *myoD* and *pax1* with somite formation independent of Shh production, which is synthesized constitutively by the notochord along the anteroposterior axis of embryos throughout development. It is noteworthy that floor plate formation, which is also controlled by Shh, occurs in the neural tube in regions posterior to somite formation (Marti et al., 1995b), showing that somite and neural tube patterning are regulated differently. The patterning of gene expression in somites is regulated by somite formation, which regulates *gli2/4* activation in response to surface ectoderm signals, followed by activation of *gli* and *ptc* under the control of Shh. How somite formation controls somite responsiveness to ectodermal signals and Shh remains to be investigated.

Third, we have observed that, once activated, *ptc* and *gli* and *gli2/4* are patterned in their expression in the somite. Their patterns of expression suggest mechanisms by which Shh could induce lineage-specific responses in somite cells, which are multipotential at the time when somites form and when the downstream Shh response genes are activated (Marti et al., 1995b). In very early embryos (stage 8-9) when *myoD* is not yet activated, *ptc* expression is restricted to a ventral domain, where *pax1* is activated; *ptc* expression does not extend into the dorsal somite region where *myoD* becomes activated at later stages (Borycki et al., 1997). Later in development (stage 11), *ptc* expression extends into the dorsal somite and *myoD* activation becomes coordinated with somite formation. Since high level *ptc* expression is an index that cells are receiving Shh signals, the absence of *ptc* in the dorsal medial region of somites in early embryos likely reflects a failure of Shh to diffuse to that dorsal region from its ventral notochord source. In support of this finding, it has been observed that the level of Shh protein produced by the notochord is lower early in development and increases during the developmental period when Shh becomes expressed in the floor plate of the neural tube (Marti et al., 1995b), providing a higher level of Shh for the more dorsal cells that activate *myoD* in later stage somites (Borycki et al., 1997). We also show in the Shh bead implantation experiments that Shh induction of *myoD* is concentration dependent.

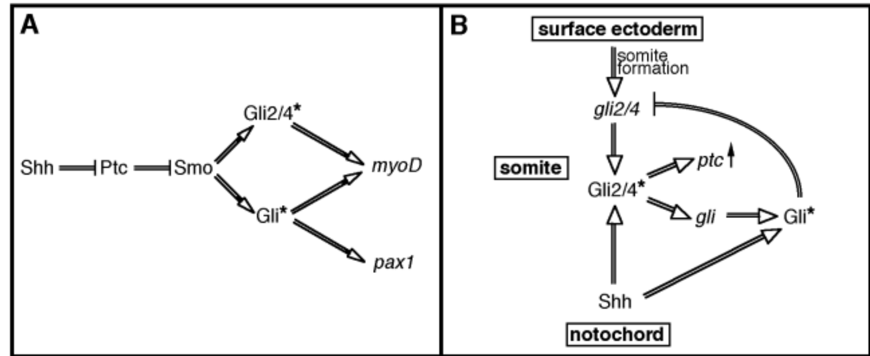

Fig. 11. Models for Shh regulation of *pax1* and *myoD* activation and the initiation of Shh signaling in somites. (A) Model for *myoD* and *pax1* activation by Shh. Shh produced by the notochord controls the induction of *pax1* and *myoD* expression in somites by binding to and inhibiting Ptc, thereby relieving Ptc repression of Smoothened (Smo) and leading to the localized activation of Gli (Gli*) and Gli2/4 (Gli2/4*) in the ventral/dorsal somite. Gli* and Gli2/4* then work in combination to activate *myoD* in the dorsal somite, whereas Gli* works to activate *pax1* in the ventral somite. (B) Model for the initiation of Shh signaling in somites. The surface ectoderm (SE) provides an initial dorsal signal for *gli2/4*, which initially is activated throughout the newly formed somite following somite formation, which makes somite cells responsive to the SE signal. Shh signals from the ventral notochord allow Gli2/4 protein to be activated and translocated into the nucleus in transcriptionally active form (Gli2/4*). Gli2/4* then induces the upregulation of *ptc* and activation of *gli* in the ventral somite. In response to Shh, Gli is converted to an activated form (Gli*), which we propose represses *gli2/4* transcription in the ventral somite, leading to the dorsalization of *gli2/4* and *ptc* expression.

The expression of *gli* and *gli2/4* is precisely patterned in somites. *gli2/4* is initially expressed throughout the somite, and then, concomitant to dermomyotome formation, becomes localized precisely to the dorsal *myoD*-expressing region of the somite, indicating that Gli2/4 initially may have a more general somitic function in the activation of downstream target genes of the Shh signaling pathway, but once its expression becomes localized to the myotomal lineage, it may have a more specific function in the regulation of *myoD* and other myotome-specific genes. Similarly, *gli* is differentially expressed in the ventral medial somite, indicating that Gli may contribute to the regulation of *pax1* and sclerotome-specific genes, as well as *myoD* and myotome-specific genes. These patterns of expression of *gli* and *gli2/4* in the somite suggests a simple combinatorial transcriptional model for *myoD* and *pax1* regulation by Gli2/4 and Gli, in response to Shh (Fig. 11A).

Antisense experiments are in progress to directly test the essential roles of *gli* and *gli2/4* in *myoD* and *pax1* activation and myotomal and sclerotomal lineage determination in somites of the quail embryo. Mutations in the human and mouse *gli* genes result in deficiencies and malformations in rib and vertebral bone formation (*gli2* mutants), limb bones (*gli3* mutants), and in neural development, consistent with their overlapping patterns of expression in the sclerotomal lineages of somites and in limb mesenchyme and the neural tube (Mo et al., 1997). Muscle phenotypes have not been reported in mammalian embryos, but in *Xenopus* embryos, where *gli4* and *myoD* are coexpressed in somitic mesoderm, misexpression of a fusion protein of the Gli zinc-finger DNA-binding domain with an E1a transcriptional activation domain causes ectopic *myoD* expression, consistent with a direct role for Gli proteins in myotome formation (Marine et al., 1997). We have examined the quail and human *myoD* gene transcriptional enhancers (Goldhamer et al., 1992; Pinney et al., 1995) for the presence of Gli-binding sites (GACCACCCA) (Vortkamp et al., 1995; Sasaki et al., 1997) and have not discovered any closely related sites, suggesting that Gli proteins may function indirectly via the activation of intermediate transcription factors. *pax1* regulatory sequences have not yet been defined for analysis of Gli-binding sites and function. Finally, it will be of interest to examine the pattern of somite expression of additional genes in the Shh signal response pathway, specifically, *smoothened* (*smo*), a transmembrane protein that is thought to interact with and to be regulated by Ptc protein (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) and the homologous *gli3*, a member of the Gli transcription factor family (Hui et al., 1994). The expression of these Shh response pathway genes may provide additional insight into the mechanisms by which Shh controls somite patterning.

Fourth, we noted that *pax1* and *myoD* expression is correctly patterned in the dorsal and ventral aspects of the somite in Shh bead implantation experiments, consistent with our working model that the patterned expression of *pax1* and *myoD* is controlled by the dorsal/ventral patterning of the downstream Shh response genes and not by Shh itself (Fig. 11A). However, additional signals, produced by surrounding tissues, also must function to restrict the dorsal and ventral patterns of expression of genes in the dorsal and ventral somite. For instance, a balance between the BMP inhibitory activity of Noggin, produced in the dorsal medial compartment of somites, and BMP4, produced by the lateral plate, may restrict *myoD* expression to the medial somite (Pourquie et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997). Ventral neural tube signals also produces an inhibitory signal that limits the ventral expression of *myoD* in somites (Pownall et al., 1996).

A network of inductive signals controls the initiation of Shh signaling in somites

The discovery that the somite response pathway genes, the Shh receptor, *ptc,* and the *gli* and *gli2/4* transcription factor effectors, are activated in response to different inductive signals provides a mechanism to regulate the initiation of Shh signaling in newly formed somites. This mechanism, depicted in Fig. 11B, is based on the discovery that *gli2/4* is activated in newly formed somites by surface ectoderm signals, independent of Shh signaling, thereby providing the first transcription factor effector required to initiate a Shh signal response in somites. *Gli 2/4* initially is expressed throughout the newly formed somite but Gli2/4 proteins will become activated (*) preferentially in the ventral medial somite, where Shh is present at highest concentrations. As a result of Shh-binding to the low constitutive level of Ptc receptor and Gli2/4

activation, the Shh pathway is stimulated (Marigo et al., 1996a), and we hypothesize that the activated Gli2/4* protein induces the transcription of *gli* and upregulates *ptc* expression. Shh-activated Gli* protein in turn autoregulates its own expression and represses *gli2/4* expression in the ventral somite, as demonstrated in limb mesenchyme (Marine et al., 1997), thereby localizing *gli2/4* and *ptc* expression to the dorsal *myoD*-expressing cells and newly forming myotome. The surface ectoderm signals that activate *gli2/4*, independent of Shh, therefore, provide Gli2/4 to initiate the cascade of activation of downstream genes that lead to the expression of other Shh response genes and the activation of downstream target genes. We are currently testing this model by experimental manipulation of the temporal and spatial expression of *gli2/4* and *gli* in somites.

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REFERENCES

Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E. (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232.

Angela Nieto, M., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264**, 835-839.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**, 126-138.

Blagden, C. S., Currie, P. D., Ingham, P. W. and Hughes, S. M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. *Genes Dev.* **11**, 2163-2175.

Borycki, A. G., Strunk, K., Savary, R. and Emerson, C. P., Jr. (1997). Distinct signal/response mechanisms regulate pax1 and QmyoD activation in sclerotomal and myotomal lineages of quail somites. *Dev. Biol.* **185**, 185-200.

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K. H., Arnold, H. H. and Kohtz, S. (1989a). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. *EMBO J.* **8**, 3617-3625.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E. and Arnold, H. H. (1989b). A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**, 701-709.

Braun, T., Rudnicki, M. A., Arnold, H. H. and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* **71**, 369-382.

Bronner-Fraser, M. (1996). *Methods in Avian Embryology.* San Diego: Academic Press.

Buckingham, M. (1992). Making muscle in mammals. *Trends Genet.* **8**, 144-148.

Bumcrot, D. A. and McMahon, A. P. (1995). Somite differentiation. Sonic signals somites. *Curr. Biol.* **5**, 612-614.

Burke, R. and Basler, K. (1997). Hedgehog signaling in Drosophila eye and limb development – conserved machinery, divergent roles? *Curr. Op. Neurobiol.* **7**, 55-61.

Charles de la Brousse, F. and Emerson, C. P., Jr. (1990). Localized expression of a myogenic regulatory gene, qmf1, in the somite dermatome of avian embryos. *Genes Dev.* **4**, 567-581.

Chen, Y. and Struhl, G. (1996). Dual roles for patched in sequestring and transducing hedgehog. *Cell* **87**, 553-563.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking sonic hedgehog gene function. *Nature* **383**, 407-413.

Christ, B. and Ordahl, C. P. (1995). Early stages of chick somite development. *Anat. Embryol.* **191**, 381-396.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E. and Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. *Development* **122**, 429-437.

Cserjesi, P., Brown, D., Ligon, K. L., Lyons, G. E., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Olson, E. N. (1995). Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. *Development* **121**, 1099-1110.

Currie, P. D. and Ingham, P. W. (1996). Induction of a specific muscle cell type by a hedgehog-like protein in zebrafish. *Nature* **382**, 452-455.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Deutsch, U., Dressler, G. R. and Gruss, P. (1988). Pax 1, a member of a paired box homologous murine gene family, is expressed in segmented structures during development. *Cell* **53**, 617-625.

Ebensperger, C., Wilting, J., Brand-Saberi, B., Mizutani, Y., Christ, B., Balling, R. and Koseki, H. (1995). Pax-1, a regulator of sclerotome development is induced by notochord and floor plate signals in avian embryos. *Anat. Embryol.* **191**, 297-310.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5**, 944-955.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Fan, C. M. and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175-1186.

Goldhamer, D. J., Faerman, A., Shani, M. and Emerson, C. P., Jr. (1992). Regulatory elements that control the lineage-specific expression of myoD. *Science* **256**, 538-542.

Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. and Scott, M. (1996). Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by hedgehog. *Genes Dev.* **10**, 301-312.

Hirsinger, E., Duprez, D., Jouve, C., Malapert, P., Cooke, J. and Pourquie, O. (1997). Noggin acts downstream of Wnt and Sonic Hedgehog to antagonize BMP4 in avian somite patterning. *Development* **124**, 4605-4614.

Hughes, D. C., Allen, J., et al. (1997). Cloning and sequencing of the mouse Gli2 gene: localization to the Dominant hemimelia critical region. *Genomics* **39**, 205-215.

Hui, C. C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol.* **162**, 402-413.

Isaac, A., Sargent, M. G. and Cooke, J. (1997). Control of vertebrate left-right asymmetry by a snail-related zinc finger gene. *Science* **275**, 1301-1304.

Jensen, A. M. and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124**, 363-371.

Johnson, R. L., Laufer, E., Riddle, R. D. and Tabin, C. (1994). Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. *Cell* **79**, 1165-1173.

Koseki, H., Wallin, J., Wilting, J., Mizutani, Y., Kispert, A., Ebensperger, C., Herrmann, B. G., Christ, B. and Balling, R. (1993). A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. *Development* **119**, 649-660.

Koyama, E., Yamaai, T., et al. (1996). Polarizing activity, Sonic hedgehog, and tooth development in embryonic and postnatal mouse. *Dev. Dyn.* **206**, 59-72.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Lassar, A. B. and Munsterberg, A. E. (1996). The role of positive and negative signals in somite patterning. *Curr. Op. Neurobiol.* **6**, 57-63.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). Gli1 is a

target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.

Li, H. S., Yang, J. M., Jacobson, R. D., Pasko, D. and Sundin, O. (1994). Pax-6 is first expressed in a region of ectoderm anterior to the early neural plate: implications for stepwise determination of the lens. *Dev. Biol.* **162**, 181-194.

Li, L., Cserjesi, P. and Olson, E. N. (1995). Dermo-1: a novel twist-related bHLH protein expressed in the developing dermis. *Dev. Biol.* **172**, 280-292.

Marcelle, C., Stark, M. R. and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Shh and Noggin mediate patterning of the dorsal somite. *Development* **124**, 3955-3963.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996a). Biochemical evidence that patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996b). Sonic hedgehog differentially regulates expression of gli and gli3 during limb development. *Dev. Biol.* **180**, 273-283.

Marigo, V., Scott, M. P., Johnson, R. L., Goodrich, L. V. and Tabin, C. J. (1996c). Conservation in hedgehog signaling: induction of a chicken patched homolog by Sonic hedgehog in the developing limb. *Development* **122**, 1225-1233.

Marine, J.-C., Bellefoid, E. J., Pendeville, H., Martial, J. A. and Pieler, T. (1997). A role for Xenopus Gli-type zinc-finger proteins in the early embbryonic paterning of the mesoderm and neuroectoderm. *Mech. Dev.* **63**, 211-225.

Maroto, M., Reshef, R., Munsterberg, A. E., Koester, S., Goulding, M. and Lassar, A. B. (1997). Ectopic pax-3 activates myoD and myf-5 expression in embryonic mesoderm and neural tissue. *Cell* **89**, 139-148.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995a). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants [see comments]. *Nature* **375**, 322-325.

Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995b). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.

Mo, R., Freer, A. M., et al. (1997). Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**, 113-123.

Munsterberg, A. E., Kitajewski, J., Bumcrot, D. A., McMahon, A. P. and Lassar, A. B. (1995). Combinatorial signaling by sonic hedgehog and wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev.* **9**, 2911-2922.

Parr, B. A. and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* **374**, 350-353.

Pinney, D. F., de la Brousse, F. C., Faerman, A., Shani, M., Maruyama, K. and Emerson, C. P., Jr. (1995). Quail myoD is regulated by a complex array of cis-acting control sequences. *Dev. Biol.* **170**, 21-38.

Pourquie, O., Fan, C. M., et al. (1996). Lateral and axial signals involved in avian somite patterning: a role for BMP4. *Cell* **84**, 461-471.

Pownall, M. E. and Emerson, C. P., Jr. (1992). Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. *Dev. Biol.* **151**, 67-79.

Pownall, M. E., Strunk, K. E. and Emerson, C. P., Jr. (1996). Notochord signals control the transcriptional cascade of myogenic bHLH genes in somites of quail embryos. *Development* **122**, 1475-1488.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Roberts, D. J., Johnson, R. L., Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C. (1995). Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development* **121**, 3163-3174.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rudnicki, M. A., Braun, T., Hinuma, S. and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71**, 383-390.

Rudnicki, M. A., Schnegelsberg, P. N., Stead, R. H., Braun, T., Arnold, H. H. and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* **75**, 1351-1359.

Ruiz i Altaba, A., Placzek, M., Baldassare, M., Dodd, J. and Jessell, T. M. (1995). Early stages of notochord and floor plate development in the chick embryo defined by normal and induced expression of HNF-3 beta. *Dev. Biol.* **170**, 299-313.

Ruppert, J. M., Vogelstein, B., Arheden, K. and Kinzler, K. W. (1990). GLI3 encodes a 190-kilodalton protein with multiple regions of GLI similarity. *Mol. Cell. Biol.* **10**, 5408-5415.

Sasaki, H., Hui, C., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.

Srivastava, D., Cserjesi, P. and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. *Science* **270**, 1995-1999.

Standiford, D. M., Davis, M. B., Miedema, K., Franzini-Armstrong, C. and Emerson, C. P., Jr. (1997). Myosin rod protein: a novel thick filament component of *Drosophila* muscle. *J. Mol. Biol.* **265**, 40-55.

Stone, D. M., Hynes, M., et al. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.

Tajbakhsh, S., Rocancourt, D. and Buckingham, M. (1996). Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf5 null mice. *Nature* **384**, 266-270.

Tajbakhsh, S., Rocancourt, D., Cossu, G. and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: pax-3 and myf-5 act upstream of myoD. *Cell* **89**, 127-138.

Tanimura, A., Teshima, H., Fujisawa, J. and Yoshida, M. (1993). A new regulatory element that augments the Tax-dependent enhancer of human T-cell leukemia virus type 1 and cloning of cDNAs encoding its binding proteins. *J. Virol.* **67**, 5375-5382.

Thien, H., Buscher, D. and Ruther, U. (1996). Cloning and sequence analysis of the murine Gli3 cDNA. *Bioch. Bioph. Act.* **1307**, 267-269.

van den Heuvel, M. and Ingham, P. W. (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551.

Vortkamp, A., Gessler, M. and Grzeschik, K. H. (1995). Identification of optimized target sequences for the GLI3 zinc finger protein. *DNA & Cell Biology* **14**, 629-634.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Weinberg, E. S., Allende, M. L., Kelly, C. S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O. G., Grunwald, D. J. and Riggleman, B. (1996). Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos. *Development* **122**, 271-280.

Zon, G. and Stec, W. J. (1991). *Oligonucleotides and Analogues*. (ed. F. Eckstein), pp. 87-108. Oxford University Press.