**Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors**

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Accepted 2 September; published on WWW 21 October 1999

**SUMMARY**

The migration of myogenic precursors to the vertebrate limb exemplifies a common problem in development – namely, how migratory cells that are committed to a specific lineage postpone terminal differentiation until they reach their destination. Here we show that in chicken embryos, expression of the Msx1 homeobox gene overlaps with Pax3 in migrating limb muscle precursors, which are committed myoblasts that do not express myogenic differentiation genes such as MyoD. We find that ectopic expression of Msx1 in the forelimb and somites of chicken embryos inhibits MyoD expression as well as muscle differentiation. Conversely, ectopic expression of Pax3 activates MyoD expression, while co-ectopic expression of Msx1 and Pax3 neutralizes their effects on MyoD. Moreover, we find that Msx1 represses and Pax3 activates MyoD regulatory elements in cell culture, while in combination, Msx1 and Pax3 oppose each other’s transcriptional actions on MyoD. Finally, we show that the Msx1 protein interacts with Pax3 in vitro, thereby inhibiting DNA binding by Pax3. Thus, we propose that Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors via direct protein–protein interaction. Our results implicate functional antagonism through competitive protein–protein interactions as a mechanism for regulating the differentiation state of migrating cells.

Key words: Muscle development, Cell migration, Protein–protein interactions, Msx1, Pax3, MyoD

**INTRODUCTION**

Cell migration is a fundamental process that contributes to many aspects of patterning and morphogenesis during development. In many cases, cells become committed to a particular lineage prior to migration, although they do not undergo terminal differentiation until they have reached their final destination. This implies the need for mechanisms that regulate the timing of differentiation of such migrating cells. This phenomenon of delayed differentiation during migration is exemplified by cells of the myogenic lineage, particularly those that form the muscles of the limb. In vertebrates, limb muscles, like most other skeletal muscles, are derived from the somites (Chevallier et al., 1977; Christ et al., 1977; Christ and Ordahl, 1995). However, unlike muscles of the axial skeleton which form in the vicinity of the somites, formation of limb muscles requires migration of precursors away from the somites. Classical chick-quail chimera studies have shown that limb muscle precursors are committed to the myoblast lineage prior to migration (Chevallier et al., 1977; Christ et al., 1977). However, their differentiation is delayed for about two days relative to that of axial muscle precursors, until they have ceased migrating and have coalesced into the dorsal and ventral pre-muscle masses that subsequently form the limb musculature (Christ and Ordahl, 1995). This delay is evident from the postponed expression of myogenic regulatory factors (MRFs), such as MyoD and Myf5, which are detected following the formation of the limb pre-muscle masses (reviewed by Buckingham, 1994). The molecular mechanisms responsible for restraining the differentiation of myogenic precursors while they are migrating are of great interest, but have not been well defined.

Among the genes that are expressed in migrating limb muscle precursors, the paired-type homeobox gene Pax3 is of particular interest because it is essential for migration of these cells (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994; Daston et al., 1996), and also plays a broader role in skeletal muscle differentiation (Maroto et al., 1997; Tajbakhsh et al., 1997; Tremblay et al., 1998). Notably, Pax3 expression marks early stages of commitment in all skeletal muscle lineages, although it is downregulated once MRFs are expressed (Goulding et al., 1994; Williams and Ordahl, 1994). Its role in myogenesis was first inferred from the absence of limb musculature in Splotch mice (Franz et al., 1993), which harbor a mutation in Pax3. The Splotch mutation results in a failure of migration of limb muscle precursors (Daston et al., 1996), as well as various abnormalities in muscle formation (Tremblay et al., 1998) and, in the context of a Myf5 null
background, a complete absence of body muscles (Tajbakhsh et al., 1997). Furthermore, forced expression of Pax3 is sufficient to initiate myogenic differentiation in explant cultures, since it activates MyoD expression in muscle and non-muscle cells (Maroto et al., 1997). Therefore, Pax3 is required for migration of myogenic precursors, which are undifferentiated, yet also has the capability to initiate muscle cell differentiation. This apparent paradox suggests that the myogenic-promoting activity of Pax3 is selectively and transiently blocked in migrating limb muscle precursors, accounting for their delayed differentiation. Furthermore, since Pax3 is also expressed in certain non-muscle lineages, including neural derivatives (Goulding et al., 1991), its muscle-promoting activity is likely to be negatively regulated, albeit more permanently, in such non-muscle cells.

In contrast to Pax3, the Msx1 homeobox gene inhibits muscle cell differentiation and represses MyoD expression in cell culture (Song et al., 1992; Woloshin et al., 1995). Msx1 encodes a transcriptional repressor (Catron et al., 1995), whose activities are mediated through protein-protein interactions with components of the general transcription complex, such as the TATA binding protein (Zhang et al., 1996), as well as other homeoproteins, such as members of the Dlx and Lhx families (Zhang et al., 1997; Bendall et al., 1998). Msx1 is expressed in several embryonic regions, including the limb and craniofacial mesenchyme and the dorsal neural tube (reviewed by Davidson, 1995). Notably, Msx1 expression has been reported in somites, based on expression of a lacZ reporter gene under the control of Msx1 regulatory sequences (MacKenzie et al., 1997), as well as in myogenic precursors, based on expression of a lacZ allele “knocked-in” to the Msx1 locus (Houzelstein et al., 1999).

Here we investigate the roles of Msx1 and Pax3 in muscle cell differentiation in the chick. We show that expression of Msx1 overlaps with Pax3 in migrating limb muscle precursors, which do not express MyoD or other MRFs. We find that misexpression of Msx1 represses MyoD expression in vivo, while Pax3 activates MyoD expression, and that misexpression of both genes negates their effects on MyoD. Although we present evidence that the opposing activities of Msx1 and Pax3 on muscle differentiation are mediated through direct protein-protein interactions, which inhibit Pax3 DNA binding in vitro and repress MyoD regulatory elements in cell culture. We propose that Msx1 negatively regulates the differentiation of myogenic precursors by directly antagonizing the myogenic activity of Pax3.

**RESULTS**

**Overlapping expression of GMsx1 and GPax3 in migrating limb muscle precursors**

As a first step to compare the roles of Msx1 and Pax3 in myogenesis, we examined their expression patterns in somitic derivatives during chicken embryogenesis. The limb musculature is derived from the dermomyotome, which is the source of all myogenic precursors within the somite (Christ and Ordahl, 1995). In chicken embryos, limb muscle precursors detach from the ventrolateral dermomyotome beginning around stage 15 and migrate to the limb to coalesce into dorsal and ventral pre-muscle masses, around stage 15 and migrate to the limb to coalesce into dorsal and ventral pre-muscle masses, around stage 22 (Christ and Ordahl, 1995). These migrating precursors express GPax3, whereas MRF expression (e.g. GMyoD) is not detected until they condense into pre-muscle masses. This contrasts with axial muscle precursors, which arise from the dorsomedial dermomyotome and express MRFs immediately upon entering the myotome.

Gel retardation assays and transient transfections were performed as described by Catron et al. (1993, 1995). Procedures for in vitro transcription/translation and GST interaction assays have been described by Bendall et al. (1998). Note that we were unable to co-immunoprecipitate Msx1 and Pax3 from transfected cells due to technical difficulties encountered in extracting Msx1 from nuclei under conditions that preserve protein complex formation (A.J.B. and C.A.-S., unpublished).

**Retroviral infection of chicken embryos and phenotypic analysis**

Replication-competent avian retroviruses were made in chicken embryonic fibroblasts (CEF) following transfection of RCAS plasmids using standard protocols. Viral supernatants were collected in low serum medium and concentrated by ultracentrifugation as described by Fekete and Cepko (1993). Virus titers (5x10^1-1.5x10^9) were determined by immunohistochemistry with 32C monoclonal antibody (Potts et al., 1987). RCAS-Msx1 retroviruses in the A and B viral coats have similar phenotypes (Hu et al., 1998). Injection of stage 13–14 chicken embryos was performed as described by Goff and Tabin (1997). Embryos at stage 24–26 were fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridization and 12 μm transverse cryosections were obtained. Orientation was continuously monitored during sectioning to ensure that sections were symmetrical.
Section in situ hybridization analysis of stage 21 and 22 chicken embryos revealed low levels of GMsx1 expression in a dispersed population of cells at the base of the forelimb bud (Fig. 1A-C, arrows). In adjacent sections, this GMsx1 expression overlaps with that of GPax3, but not GMyoD (Fig. 1C,F,I, arrows). In particular, at stage 21 GPax3 is continuous from the dermomyotome to the base of the forelimb (Fig. 1D) and, by stage 22, GPax3 is also expressed in the newly forming dorsal and ventral pre-muscle masses (Fig. 1E). In contrast, and, by stage 22, is also expressed in the newly forming GPax3 in the dorsal neural tube (Fig. 1A,B). Indeed, high level expression of GMsx1 in mesenchymal cells of the progress zone precluded detection of expression of GMsx1 in migrating myogenic precursors at earlier stages (J. D., A. J. B., M. M. S and C. A.-S., unpublished). Notably, GMsx1 is expressed in the myotome, and is excluded from cells at the base of the forelimb (Fig. 1G-I, arrows). The relatively low level expression of GMsx1 at the base of the forelimb contrasts with its robust expression in other embryonic regions, such as the progress zone of the limb and the dorsal neural tube (Fig. 1A,B). Indeed, high level expression of GMsx1 in mesenchymal cells of the progress zone precluded detection of GMsx1 expression in migrating limb muscle precursors at earlier stages (J. D., A. J. B., M. M. S and C. A.-S., unpublished). Notably, GMsx1 also coincides with GPax3 in the dorsal neural tube (Fig. 1A,D), which does not express MRFs. We conclude that GMsx1 is expressed in migrating limb muscle precursors, since the expression domain at the base of the forelimb is positive for GPax3 and negative for GMyoD. Our findings are concordant with a recent report showing that an Msx1-lacZ knock-in allele is expressed in somites that contribute myogenic precursors to the forelimb and overlaps with Pax3 in migrating myogenic progenitors (Houzelstein et al., 1999).

**Ectopic expression of Msx1 represses GMyoD expression in the forelimb and somites and inhibits muscle development**

To investigate the biological significance of Msx1 expression in myogenic precursors, we examined the consequences of its overexpression in somites from which these precursors arise. We infected chicken embryos with a replication-competent Msx1-expressing retrovirus (RCAS-Msx1), and examined GMyoD expression by whole-mount in situ hybridization. Since muscle precursors of the forelimb originate from somites 15-21 (Chevallier et al., 1977; Christ et al., 1977), we infected these somites at stage 13-14, prior to the onset of migration (Fig. 2A). Three days after injection (stage 25-26), exogenous Msx1 was highly expressed throughout the forelimb and trunk on the infected side, while it was not expressed on the

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<td>RCASBP(A)-Myc-Pax3 (1-479)</td>
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*ORF, open reading frame.
‡Luc, Luciferase gene.
uninfected (control) side (Fig. 2B,E). RCAS-Msx1 infection resulted in high level expression of Msx1 protein as shown by western blot analysis of lysates prepared from infected cells (Fig. 2G).

Ectopic Msx1 repressed GMyoD expression in the forelimb and somites on the infected side, but there was no repression on the uninfected side (Fig. 3A-D). Thus, by stage 25-26 GMyoD is normally expressed at high levels in the dorsal and ventral pre-muscle masses of the fore- and hindlimbs, as well as in the somites (Fig. 3A,C). However, infection with RCAS-Msx1 resulted in a significant reduction in GMyoD expression in the forelimb and surrounding somites in a majority of

Fig. 1. Overlapping expression of Msx1 and Pax3 in migrating limb muscle precursors. In situ hybridization of GMsx1 (A-C), GPax3 (D-F) and GMyoD (G-I) on adjacent transverse sections at the level of the forelimb of chicken embryos, stages 21 and 22. Note the expression of GMsx1 at the base of the forelimb (A-C, arrows), where it overlaps with that of GPax3 (D-F, arrows) but not GMyoD (G-I, arrows). Abbreviations: dlm, dorsal limb pre-muscle mass; dm, dermomyotome; drg, dorsal root ganglion; lb, limb bud; my, myotome; nt, neural tube; pz, progress zone; vlm, ventral limb pre-muscle mass. Scale bars, 0.1 mm.

Fig. 2. (A) Retroviral infection of pre-migratory limb muscle precursors. Schematic diagram of a stage 13 chicken embryo (modified from Christ et al., 1977), depicting infection of righthand somites 15-21 (blue), which contribute muscle precursors to the right forelimb; red arcs denote future forelimbs. Abbreviations: so, somite; psm, pre-somatic mesoderm. (B) Whole-mount in situ hybridization shows ectopic expression of murine Msx1 at stage 26 following injection of RCAS-Msx1 into somites 15-21 of a stage 13-14 chicken embryo. (C,D) High-power views of RCAS-Msx1-infected embryos showing equivalent expression of GPax3 in the pre-muscle masses of infected and uninfected forelimbs (arrowheads). (E,F) In situ hybridization on adjacent transverse sections from stage 25-26 chicken embryos co-infected with RCAS(B)-Msx1 and RCAS(A)-Pax3 showing overlapping expression of exogenous Msx1 (E) and Pax3 (F). The uninfected (left) and infected (right) wings are shown. (G) Western blot of cell lysates prepared from CEFs infected with RCAS-Msx1, RCAS-Msx1A, RCAS-Pax3, or RCAS (Vector) using an anti-Myc antisera. Dashes indicate molecular weight markers: 77, 48, 34, 29 kDa. Scale bars, 0.5 mm in B-D and 0.1 mm in E,F.
Muscle development was examined in RCAS-Msx1-infected chicken embryos by histological staining of transverse paraffin sections through the zeugopod at stage 36-37. Note the general reduction in muscle mass on the infected side (arrowheads). Decreased bone size following RCAS-Msx1 infection has been described previously (Hu et al., 1998). Anterior is right and dorsal is top; K is inverted vertically for comparison with J. Additional abbreviations: r, radius; u, ulna. Scale bars, 0.5 mm in A-F and 0.1 mm in G-K.

**Fig. 3.** Ectopic expression of Msx1 represses GMyoD in the forelimb and somites. (A-F) Chicken embryos were injected with RCAS-Msx1 (A-D) or RCAS-Msx1A (E,F) at stage 13-14, and GMyoD expression was examined by whole-mount in situ hybridization at stage 25-26. (A,B) Low-power views of an RCAS-Msx1-infected embryo showing reduced expression of GMyoD in the forelimb and somites on the infected versus the uninfected side (arrowheads). (C,D) High-power views of an RCAS-Msx1A-infected embryo showing reduced expression of GMyoD in the pre-muscle masses of the infected forelimb compared with the uninfected forelimb (arrowheads). (E,F) High-power views of an RCAS-Msx1A-infected embryo showing equivalent levels of expression of GMyoD in the pre-muscle masses of the infected and uninfected forelimbs (arrowheads). (G-I) Cryosections of whole-mount embryos (as in A-D) showing inhibition of GMyoD in the forelimb and somites following RCAS-Msx1 infection. G,H show the uninfected and infected forelimbs, respectively, from the same section; note reduced expression of GMyoD in the dorsal and ventral pre-muscle masses on the infected side (H, arrows). (I) A section rostral to the forelimb from the same embryo showing reduced GMyoD expression in the myotome on the infected (right arrow) compared with the uninfected (left arrow) side. (J,K) Ectopic expression of Msx1 inhibits wing muscle development on the infected (K) but not the uninfected (J) side. Muscle formation was examined in RCAS-Msx1-infected chicken embryos by histological staining of transverse paraffin sections through the zeugopod at stage 36-37. Note the general reduction in muscle mass on the infected side (arrowheads). Decreased bone size following RCAS-Msx1 infection has been described previously (Hu et al., 1998). Anterior is right and dorsal is top; K is inverted vertically for comparison with J. Additional abbreviations: r, radius; u, ulna. Scale bars, 0.5 mm in A-F and 0.1 mm in G-K.

Embryos (77% affected, n=91) (Fig. 3B,D). This reduction was not a consequence of Msx1 downregulation of GPax3 expression since RCAS-Msx1-infected embryos had equivalent levels of GPax3 expression on the infected and uninfected sides (n=15) (Fig. 2C,D). Furthermore, repression of GMyoD required a functionally active form of Msx1, since infection with a retrovirus encoding a transcriptionally inactive (mutant) Msx1 protein (RCAS-Msx1A) (Zhang et al., 1996) did not inhibit GMyoD expression (n=22) (Fig. 3E,F). Analysis of sections from RCAS-Msx1-infected embryos revealed reduced GMyoD expression in the dorsal and ventral pre-muscle masses of the forelimb, as well as in the myotome (Fig. 3G-I). These findings indicate that MyoD is a target for transcriptional repression by Msx1 in vivo.

To ask whether ectopic Msx1 affected muscle development as well as GMyoD expression, RCAS-Msx1-infected embryos were allowed to develop to stage 36-37, by which time the limb musculature has assumed its adult pattern (Sullivan, 1962). Histological staining of transverse sections through the wing revealed a general reduction in muscle size on the infected versus the uninfected side (n=5) (Fig. 3J,K; arrowheads). Thus, repression of MyoD by Msx1 reflects its broader role as a negative regulator of muscle development.

**Ectopic expression of Pax3 activates GMyoD expression in the forelimb and somites**

We performed complementary experiments to examine the consequences of ectopic expression of Pax3. As we observed with RCAS-Msx1, infection of somites 15-21 of stage 13-14 embryos with RCAS-Pax3 resulted in exogenous Pax3 expression in the forelimb and trunk on the infected side (Fig. 2F), and RCAS-Pax3 produced high level protein expression (Fig. 2G). However, in contrast to RCAS-Msx1, infection with RCAS-Pax3 resulted in ectopic expression of GMyoD in the forelimb and somites in a majority of stage 25-26 embryos (86% affected, n=37) (Fig. 4A-D, I-K). By whole-mount in situ hybridization, ectopic expression of GMyoD in the somites was evident from the ventral expansion of its expression domain on the infected side (Fig. 4A,B). Analysis of sections rostral to the forelimb from these RCAS-Pax3-infected embryos revealed an apparent increase in GMyoD expression levels in the myotome, as well as ventral expansion of expression on the infected side (Fig. 4K; arrows).

Notably, infection with RCAS-Pax3 also resulted in ectopic GMyoD expression in scattered cells at the base of the forelimb (Fig. 4; compare C,D), which is likely to correspond to precocious GMyoD expression in migrating limb muscle precursors. Sections from these RCAS-Pax3-infected embryos...
showed that GMyoD was continuous from the dorsomedial myotome to the dorsal limb pre-muscle mass on the infected side (Fig. 4J; arrow), contrasting with its normal expression pattern (uninfected side) where GMyoD is excluded from the base of the forelimb (Fig. 4I; arrow). Interestingly, while ectopic Pax3 altered GMyoD expression, muscle histology was not significantly affected in the RCAS-Pax3-infected wings (A). The specific requirement for Msx1 was evident in co-infection studies performed with RCAS(B)-Msx1 and RCAS(A)-Pax3 wherein we observed Pax3-mediated ectopic GMyoD expression at the base of the forelimb (n=14) (Fig. 4G,H). Thus, Msx1 and Pax3 appear to have mutually opposing actions on MyoD expression in vivo.

**Msx1 and Pax3 form a protein complex in vitro, mediated by the homeodomain of Msx1 and the paired domain of Pax3**

To address the mechanism underlying the opposing actions of Msx1 and Pax3 in muscle cell differentiation, we asked whether their protein products can physically associate. To test their interaction in vitro, we performed glutathione S-transferase (GST) interaction assays using recombinant GST-Pax3 or GST-Msx1 and the complementary 35S-labeled Msx1 or Pax3 proteins obtained by in vitro translation (Fig. 5A). These GST interaction assays showed that 35S-labeled Msx1 interacted specifically with GST-Pax3 and, conversely, that 35S-labeled Pax3 interacted with GST-Msx1.

We next asked whether Msx1 and Pax3 can interact in cell extracts (Fig. 5B). For this purpose, we expressed a plasmid encoding Myc-tagged Pax3, or a control vector, in COS-1 cells. The Pax3-expressing and control cell extracts were mixed with extracts (Fig. 5B). For this purpose, we expressed a plasmid encoding Myc-tagged Pax3, or a control vector, in COS-1 cells. The Pax3-expressing and control cell extracts were mixed with extracts (Fig. 5B).
Msx1 inhibits Pax3 activation of MyoD

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Fig. 5. Msx1 interacts with Pax3 in vitro. (A) GST interaction assays were performed with GST or GST-Pax3 (4 μg) and 35S-labeled Msx1, or with GST or GST-Msx1 (4 μg) and 35S-labeled Pax3. Immobilized proteins (arrows) were resolved by SDS-PAGE and visualized by autoradiography. “Input”, 100% (2 μl) of 35S-labeled protein. (B) GST interaction assays were performed using GST or GST-Msx1 (8 μg) and cell lysates prepared from COS-1 cells transfected with a control plasmid (Vector) or one expressing Myc-tagged Pax3 (Pax3). Immobilized proteins (arrow) were resolved by SDS-PAGE and visualized by western blot analysis using an anti-Myc antibody. “Input”, 10% of the input protein extract. (C) GST interaction assays were performed with GST or GST-Msx1 (4 μg) and 35S-labeled Pax3 (arrow). Some reactions contained 0.4 units of micrococcal nuclease (MCN) without (+) or with (+) 10 mM EGTA, or 50 or 200 μg/ml ethidium bromide (ETBR) (triangle). “Input”, 100% (2 μl) of 35S-labeled protein. In A–C, dashes indicate molecular mass markers: 110, 77, 48, 34, 29, 20 kDa (77 kDa, upper dash in A and C; 110 kDa, upper dash in B).

GST-Msx1 or GST alone, and proteins retained on the GST beads were detected by western blot analysis using an antibody directed against the Myc epitope. We found that Pax3 interacted strongly and specifically with GST-Msx1, while no such interaction was detected in control cell extracts (Fig. 5B). Furthermore, since protein complex formation between Msx1 and Pax3 was not inhibited by micrococcal nuclease or ethidium bromide (Fig. 5C), we conclude that Msx1 and Pax3 can form a protein complex in vitro that does not require the presence of DNA.

To map the regions of Msx1 required for its interaction with Pax3, we performed GST interaction assays using an 35S-labeled Pax3 protein and a series of truncated GST-Msx1 fusion proteins (Fig. 6A,B). We found that 35S-labeled Pax3 interacted with GST-Msx1 fusion proteins containing the homeodomain (GST-Msx1, GST-Msx1Δ2, GST-Msx1Δ3, GST-Msx1Δ4), but not with those lacking the homeodomain (GST-Msx1Δ1, GST-Msx1Δ5). To define residues within the Msx1 homeodomain required for interaction with Pax3, we performed GST-interaction assays using GST-Pax3 and a series of mutated Msx1 polypeptides containing clustered alanine substitutions in the N-terminal arm, or helices I, II or III (Fig. 6C,D). We found that interaction of Msx1 with GST-Pax3 was abrogated by alanine substitutions in the N-terminal arm (Msx1A), reduced by alanine substitutions in helices I and III (Msx1B and Msx1E), and not affected by other alanine substitutions in helices I or II (Msx1C, Msx1D). These findings demonstrate that the Msx1 homeodomain is required for interaction with Pax3, and show that residues in the N-terminal arm are of primary importance.

To map the corresponding regions of Pax3 required for its interaction with Msx1, we generated a series of truncated Pax3 proteins and tested their interaction with GST-Msx1 (Fig. 6E,F). We found that the Pax3 proteins containing the paired domain (Pax3, Pax3Δ1, Pax3Δ2) interacted with GST-Msx1, whereas no such interaction was detected with a protein lacking the paired domain, but containing the homeodomain (Pax3Δ3). Notably, a minimal polypeptide containing the paired domain (Pax3Δ2) interacted as strongly with Msx1 as did full length Pax3 (Fig. 6E,F). Msx1 also interacted in vitro with Pax7 (A. J. B. and C. A.-S., unpublished), which is closely related to Pax3 within the paired domain. Thus, we conclude that the paired domain is necessary and sufficient for interaction with Msx1.

Msx1 inhibits DNA binding activity by Pax3 in vitro

We next asked whether Msx1 and Pax3 interact competitively or cooperatively on DNA using gel retardation assays. Thus, we examined the DNA binding activity of Pax3, in the presence or absence of Msx1, using a Pax3 protein containing the paired domain and homeodomain (Pax3Δ1) (Fig. 7A). We found that Msx1 inhibited DNA binding by Pax3 in a concentration-dependent manner (Fig. 7A, lanes 2-6). We interpret this as inhibition of binding activity, rather than competition for DNA sites, since we used a site that is bound avidly by Pax3 (the e5 site; Treisman et al., 1991) and weakly by full length Msx1 (Fig. 7A, lane 10). Similar results were obtained using other DNA sites and with full length Pax3 (data not shown).

DNA binding by Pax3Δ1 was also inhibited by the Msx1 homeodomain (Msx1Δ3) (Fig. 7A, lanes 7-9), suggesting that this inhibition requires protein regions that mediate the Msx1-Pax3 interaction. Notably, while Msx1Δ3 binds more avidly to the e5 site than does Msx1 (Fig. 7A, compare lanes 10 and 11),
the overall level of Pax3 inhibition is equivalent (compare lanes 3-5 with 7-9). This inhibition was specific for Msx1, since no such inhibition of Pax3 DNA binding activity was observed in the presence of an unrelated homeoprotein, Nkx3.1 (Sciavolino et al., 1997) (Fig. 7A, lanes 12-13), and since the inhibitory effect of Msx1 could be blocked by its prior incubation with an anti-Msx1 antiserum (data not shown). We conclude that Msx1-Pax3 protein complex formation inhibits DNA binding by Pax3.

Pax3 and Msx1 oppose each other’s actions on MyoD regulatory elements in cell culture

Finally, we asked whether the transcriptional activities of Msx1 and Pax3 are competitive or complementary using transient transfection assays in C2C12 cells, a myoblast cell line (Fig. 7B,C). We found that transfection of Pax3 resulted in a modest, but concentration-dependent, activation of an e5-luciferase reporter plasmid (Fig. 7B), consistent with previous studies showing that Pax3 is a weak transcriptional activator (Chalepakis et al., 1994). In contrast, Msx1 was a potent repressor of this reporter, while the Msx1-mediated repression was blocked in a concentration-dependent manner by co-transfection with Pax3 (Fig. 7B). This effect was specific for Pax3, since co-transfection of Msx1 with equivalent levels of a plasmid encoding Nkx3.1 did not affect repression by Msx1 (data not shown). Notably, the ability of Pax3 to block repression by Msx1 is significantly more effective than its ability to activate transcription (Fig. 7B), consistent with the interpretation that Msx1-Pax3 complex formation renders both proteins transcriptionally inactive.

We also examined the transcriptional activities of Msx1 and Pax3 using a luciferase reporter plasmid containing the MyoD enhancer/promoter (Woloshin et al., 1995) (Fig. 7C). We used this region of MyoD since the upstream control elements contained within this enhancer/promoter are sufficient to recapitulate the endogenous MyoD expression pattern in transgenic mice (Goldhamer et al., 1992, 1995). Transfection of Pax3 resulted in a modest, but concentration-
dependent, activation of the MyoD-luciferase reporter (Fig. 7C), suggesting that MyoD is a direct target for transcriptional activation by Pax3, although its modest activation indicates a requirement for additional regulatory factors. Notably, transgenic analysis of the MyoD enhancer in Splotch mutant mice is also consistent with a direct role for Pax3 in MyoD activation (Kucharczuk et al., 1999). In contrast to Pax3, Msx1 was a potent repressor of the MyoD-luciferase reporter, although this Msx1-mediated repression was significantly reduced when co-transfected with Pax3 (Fig. 7C). Taken together with the observation that Msx1 inhibits Pax3 DNA binding activity, the antagonistic actions of Msx1 and Pax3 on MyoD regulatory elements suggests a biochemical mechanism to account for their opposing effects on myogenesis in vivo.

**DISCUSSION**

The findings presented herein provide insight into the mechanisms regulating myogenic commitment during cell migration. Thus, migrating limb muscle precursors, which are committed to the myogenic lineage but are not differentiated, express Msx1 as well as Pax3, but not MyoD. We show that Msx1 and Pax3 have opposing effects on MyoD expression in vivo and on its transcription in cell culture, and that Msx1 forms a protein complex with Pax3 and inhibits its DNA binding activity. Based on these observations, we propose that Msx1 antagonizes the myogenic activity of Pax3 through direct protein-protein interactions, and suggest that the differentiation status of migrating limb muscle precursors can be regulated by antagonistic protein-protein interactions.

**Negative regulation of muscle development by Msx1 in vivo**

While previous studies have implicated Msx1 as an inhibitor of muscle differentiation in cell culture (Song et al., 1992; Woloshin et al., 1995), the biological relevance of these observations has been unresolved since Msx1 expression is generally not associated with myogenesis (Davidson, 1995). As a putative negative regulator of muscle differentiation, one might expect Msx1 expression to be low and/or transient in myogenic cells and, therefore, difficult to detect. Indeed, we found that Msx1 is expressed at low levels in limb muscle precursors, contrasting with its robust expression in other domains. Recently, Msx1 expression has been described in somitic derivatives during murine embryogenesis using lacZ reporter genes, which overcome difficulties in detecting low-level endogenous gene expression (MacKenzie et al., 1997; Houzelstein et al., 1999). Msx1 expression is also found during limb regeneration in amphibians where it is accompanied by de-differentiation of muscle cells and down-regulation of MRFs (Simon et al., 1995). Intriguingly, the Drosophila homologue msh is expressed in muscle progenitors, some of which are migratory, and its ectopic expression perturbs muscle differentiation in such cells (Nose et al., 1998).

Although these studies support its role in muscle development, targeted gene disruption of Msx1 does not...
produce any gross defects in skeletal muscles (Satokata and Maas, 1994; Houzelstein et al., 1997). One possibility is that other Msx genes functionally compensate for loss of Msx1 in the knockout mice. Indeed, defects in tooth development are more severe in compound Msx1-Msx2 mutants than in Msx1 mutant mice (Bei and Maas, 1998). Although we have found that Msx2 can repress MyoD expression and inhibit muscle differentiation, we have not detected Msx2 expression in migrating limb muscle precursors (G. H., J. D., A. J. B., M. M. S. and C. A.-S., unpublished). Alternatively, a requirement for Msx1 may be functionally compensated for by the actions of other regulatory genes expressed in myogenic precursors, examples of which include the Lbx and Mox homeobox genes (Dietrich et al., 1998; Mennerich et al., 1998; Mankoo et al., 1999).

**Differentiation of migrating limb muscle precursors reflects a balance between levels of activators and repressors**

In contrast to Msx1, Pax3 activates muscle differentiation and is a marker of myogenic commitment (Goulding et al., 1994; Williams and Ordahl, 1994; Maroto et al., 1997). However, Pax3 expression is not always coincident with myogenesis, suggesting that its myogenic activity may be spatially and/or temporally regulated in certain cell populations. Our findings implicate Msx1 as a negative regulator of the myogenic activity of Pax3. Thus, we propose that in migrating limb muscle precursors, where expression of Msx1 and Pax3 overlap, Msx1 blocks the ability of Pax3 to activate muscle cell differentiation, thereby delaying differentiation of these cells during their migration (Fig. 8A).

Implicit in this model is that the onset of muscle differentiation is determined by the balance of myogenic activators and myogenic repressors. As a consequence, overexpression of the repressor Msx1 inhibits muscle cell differentiation (e.g., MyoD expression), whereas overexpression of the activator Pax3 promotes such differentiation (Fig. 8B). Moreover, the expression levels of activator relative to repressor may determine how effectively or permanently myogenesis is inhibited. For example, in the dermomyotome and pre-muscle masses of the limb, where Pax3 expression is not accompanied by Msx1, muscle differentiation is ongoing; in the migrating limb muscle precursors, where Pax3 expression is accompanied by low levels of Msx1, muscle differentiation is delayed; and in the dorsal neural tube, where Pax3 expression is accompanied by high levels of Msx1, myogenesis does not occur under normal conditions. Notably, forced expression of Pax3 in neural tube explants induces myogenic gene expression, which was interpreted as overriding a balance between Pax3 myogenic activity and negative regulators (Maroto et al., 1997).

Msx1 is an attractive candidate for a negative regulator of Pax3 in migrating myogenic precursors, since its expression is generally associated with regions of cellular proliferation (Davidson, 1995). While these migrating limb muscle precursors are committed myoblasts, they are not post-mitotic (Sasse et al., 1984). Msx1 may therefore serve a dual function of regulating the muscle-promoting activity of Pax3, while helping to maintain the proliferative capacity of these precursor cells. It is noteworthy that the potent repressor activity of Msx1 contrasts with the weak activator function of Pax3 (Fig. 7B,C); thus, low-level expression of Msx1 in migrating myogenic precursors may be sufficient to block Pax3 myogenic activity. However, Msx1 is unlikely to be the only regulator of Pax3 activity, since its expression does not overlap with Pax3 in all spatial domains that do not express MRFs, such as the dorsal root ganglia (see Fig. 1D).

It is intriguing that BMP signaling, which inhibits myogenic differentiation in vivo (Reshef et al., 1998, and references therein), upregulates Msx gene expression in other tissues (Chen et al., 1996; Phippard et al., 1996). Expression of BMPs is associated with myoblast proliferation, while expression of
**BMP** antagonists, such as **noggin**, is associated with myoblast differentiation (Amthor et al., 1998; Reshef et al., 1998). Furthermore, altered levels of BMP signaling disrupt the normal balance between myoblast proliferation and differentiation, thereby perturbing muscle growth (Amthor et al., 1999). We envision a scenario in which BMP signaling promotes **Msx1** expression, thereby blocking differentiation and promoting proliferation. In support of this idea, **BMP-4** expression in murine embryos is highest at the level of the forelimb, corresponding to the region of **Msx1** expression (Houzelstein et al., 1999). Thus, the consequences of disrupting the balance between proliferation and differentiation of muscle precursors by ectopic **BMP** expression (Amthor et al., 1998, 1999) can be interpreted in terms of altered levels of proteins that repress and activate myogenesis.

**Negative regulation through antagonistic protein interactions: a mechanism for delaying differentiation during cell migration**

Our findings implicate functional antagonism through direct protein-protein interactions as a mechanism for regulating the differentiation status of migratory precursors. Thus, we propose that **Msx1** sequesters **Pax3** in a protein complex, thereby rendering it incapable of activating **MyoD** and, presumably, other myogenic regulatory genes. We propose that **Msx1** negatively regulates **Pax3**, rather than vice versa, since **Msx1** antagonizes other transcriptional activators through direct protein-protein interactions (Zhang et al., 1996, 1997; Bendall et al., 1998). Thus, the present study provides a mechanistic link between the repressive action of **Msx1** as an antagonist of transcriptional activators, and its biological role as a negative regulator of differentiation.

The ability to regulate the differentiation state of precursor cells through protein-protein interactions offers certain advantages over other potential modes of regulation. For instance, negative regulation through protein-protein interactions allows for the continuous expression of activators such as **Pax3**, which is likely to be important for maintaining cell commitment. Furthermore, protein-protein interactions may provide a means of fine-tuning the functions of activators by selectively blocking some functions, but not others. Thus, while **Pax3** is required for migration and differentiation of myogenic precursors, **Msx1** appears to selectively block differentiation, since its co-expression with **Pax3** is compatible with migration, but not differentiation. The need to coordinate differentiation with cell movements is critical for patterning and morphogenesis. Given the versatility of this mode of regulation, we envision that this molecular mechanism for delaying differentiation of committed cells during their migration is not unique to muscle, but may be encountered in other developmental paradigms.

J. D. and G. H. made equal contributions to the present study. We acknowledge the invaluable contribution of Hailan Zhang at the initial stages of this work, and expert technical assistance from Nishita Desai for histology. We thank Frank Rauscher III for the murine **Pax3** cDNA and anti-Pax3 antiserum and Cliff Tabin for the **G Pax3** in situ probe. We thank Tom Curran, Céline Gélinas, Charles Ordahl, Ruth Stewart, Mengqing Xiang and all members of the Abate-Shen, Shen and Xiang laboratories for stimulating discussion and comments on the manuscript. This work is supported by an NIH grant (HD33362) to C. A.-S., by grants from the NIH (HL60212) and American Heart Association (96008379) to M. M. S., and a pre-doctoral fellowship from the American Heart Association, NJ Affiliate, to G. H.

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