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

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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 trancriptional 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*. Finally, 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.

MATERIALS AND METHODS

Plasmids and in vitro assays

Descriptions of plasmids used in this study are provided in Table 1. Myc-tagged *Msx1* was cloned into the *Kpn*I-*Hin*dIII sites of *pCB6+*. The *Pax3* coding region was amplified from the murine cDNA by PCR with *Bam*HI and *Eco*RI primers and cloned into *pcDNA3* (Invitrogen) or *pGEX2T* (Pharmacia). For some applications, we introduced an in-frame double Myc epitope by PCR amplification at the 5′ end of the *Pax3* coding region followed by subcloning into *pcDNA3*. Avian retroviruses were made by subcloning Myc-tagged murine *Pax3* into the *Eco*RI-*Hin*dIII sites of the *SLAX13* shuttle vector (Hughes et al., 1987) and then partially digested with *Cla*I to subclone into *RCASBP(A)* (Hughes et al., 1987; Fekete and Cepko, 1993). Complete sequences of PCR-amplified constructs were confirmed.

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).

In situ hybridization analysis

Chicken embryos were staged according to Hamburger and Hamilton (1951). For section in situ hybridization, embryos were collected between stages 18-24, fixed in 4% paraformaldehyde, and embedded in Tissue-Tek OCT (Sakura). Adjacent cryosections (12 μm) were hybridized with digoxigenin-labeled antisense riboprobes as described by Sciavolino et al. (1997). For whole-mount in situ hybridization, embryos were collected at stages 25-26, fixed in 4% paraformaldehyde, and hybridized with digoxigenin-labeled antisense riboprobes as described by Catron et al. (1996). Some embryos were embedded in OCT following whole-mount in situ hybridization and 12 μm transverse cryosections were obtained. Orientation was continuously monitored during sectioning to ensure that sections were symmetrical.

Retroviral infection of chicken embryos and phenotypic analysis

Replication-competent avian retroviruses were made in chicken embryonic fibroblasts (CEFs) following transfection of *RCAS* plasmids using standard protocols. Viral supernatants were collected in low serum medium and concentrated by ultracentrifigation as described by Fekete and Cepko (1993). Virus titers (5×10^8-1.5×10^9) were determined by immunohistochemistry with 3C2 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. For muscle histology, stage 36-37 embryos were paraffin-embedded; zeugopods of control and infected wings embedded together for comparison at the same proximodistal level. Sections were stained with a modified Russell-Movat Pentachrome method (McElroy, 1994). For each experiment, expression of exogenous *Msx1* or *Pax3* was verified by in situ hybridization. Co-infection was performed using a 1:1 (equal titer) mixture using viruses encoding alternative envelope proteins [*RCAS(A)-Pax3* and *RCAS(B)-Msx1*].

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 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.

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, *GMyoD* is expressed in the myotome, but 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

Table 1. Description of plasmids

Purpose and name	Plasmid and characteristics	Reference or source
Transcription-translation		
Msx1	pGEM7zf(+)-Msx1 (1-297)	Zhang, 1996
Msx1-A	pGEM7zf(+)-Msx1 (1-297): K168A, R170A, F173A	Zhang, 1996
Msx1-B	pGEM7zf(+)-Msx1 (1-297): L181A, F185A	Zhang, 1996
Msx1-C	pGEM7zf(+)-Msx1 (1-297): R183A, K184A, R186A, Q187A	Zhang, 1996
Msx1-D	pGEM7zf(+)-Msx1 (1-297): R196A	Zhang, 1996
Msx1-E	pGEM7zf(+)-Msx1 (1-297): I212A, Q215A, N216A	Zhang, 1996
Pax3	pcDNA3-Pax3 (1-479): ORF* in *Bam*HI-*Eco*RI sites	This study
Pax3δ1	pcDNA3-Pax3 (1-292): pcDNA3-Pax3 linearized at *Tfi*I site	This study
Pax3δ2	pcDNA3-Pax3 (1-187): pcDNA3-Pax3 linearized at *Cla*I site	This study
Pax3δ3	pcDNA3-Pax3 (155-479):ORF(155-479) in *Bam*HI-*Eco*RI sites	This study
Myc-Pax3	pcDNA3-Myc-Pax3 (1-479): 5′ Myc plus ORF in *Eco*RI site	This study
GST fusions		
GST-Msx1	pGEX2T-Msx1 (1-297)	Zhang, 1996
GST-Msx1δ1	pGEX2T-Msx1 (1-165)	Zhang, 1996
GST-Msx1δ2	pGEX2T-Msx1 (1-225)	Zhang, 1996
GST-Msx1δ3	pGEX2T-Msx1 (166-225)	Zhang, 1996
GST-Msx1δ4	pGEX2T-Msx1 (166-297)	Zhang, 1996
GST-Msx1δ5	pGEX2T-Msx1 (226-297)	Zhang, 1996
GST-Pax3	pGEX2T-Pax3 (1-479): ORF in *Bam*HI-*Eco*RI sites	This study
In situ hybridization		
GMsx1	pBS SK(−)-GMsx1: 1.3 kb cDNA	This study
GPax3	pBS KS(−)-GPax3: 0.5 kb partial cDNA	This study
GMyoD	pBS SK(−)-GMyoD: 0.6 kb exon 1	This study
Msx1	pBS SK(−)-Msx1: 0.9 kb ORF	Catron, 1996
Pax3	pBS SK(−)-Pax3: 1.4 kb ORF	This study
Transcription		
Myc-Msx1	pCB6+/BamHI⁻-Myc-Msx1 (1-297) in *Kpn*I-*Hin*dIII sites	This study
MyoD enhancer/promoter	pGL2-MyoD-2.5/F3 Luc‡: in *Sac*I-*Xho*I sites of pGL2-basic	This study; Woloshin, 1995
e5-luciferase	pGL2-e5Luc: single e5 site in *Xho*I site of pGL2-basic	This study
Retroviral vectors		
RCAS-Msx1	RCASBP(A)-Myc-Msx1 or RCASBP(B)-Myc-Msx1	Hu, 1998
RCAS-Msx1A	RCASBP(A)-Myc-Msx1A or RCASBP(B)-Myc-Msx1A	Hu, 1998
RCAS-Pax3	RCASBP(A)-Myc-Pax3 (1-479)	This study

*ORF, open reading frame.
‡Luc, Luciferase gene.

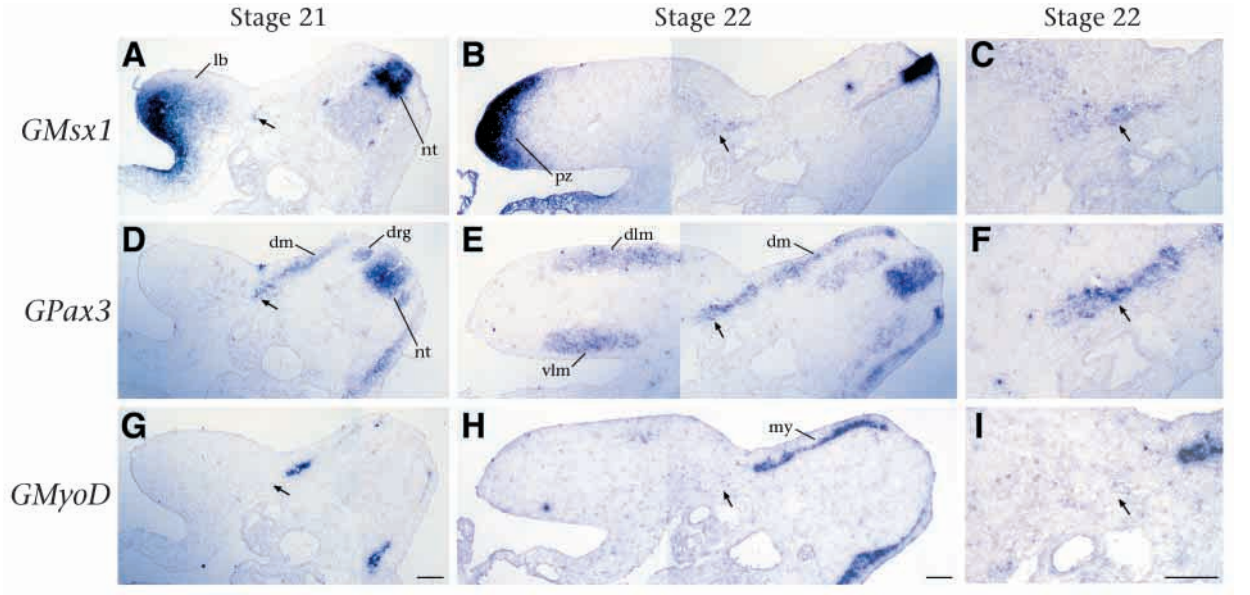


Fig. 1. Overlapping expression of *Msx1* and *Pax3* in migrating limb muscle precursors. In situ hybridization of *GMsx1* (A-C), *GPax3* (D-F) and *GMyoD* (G-I) on adjacent transverse sections at the level of the forelimb of chicken embryos, stages 21 and 22. Note the expression of *GMsx1* at the base 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.

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

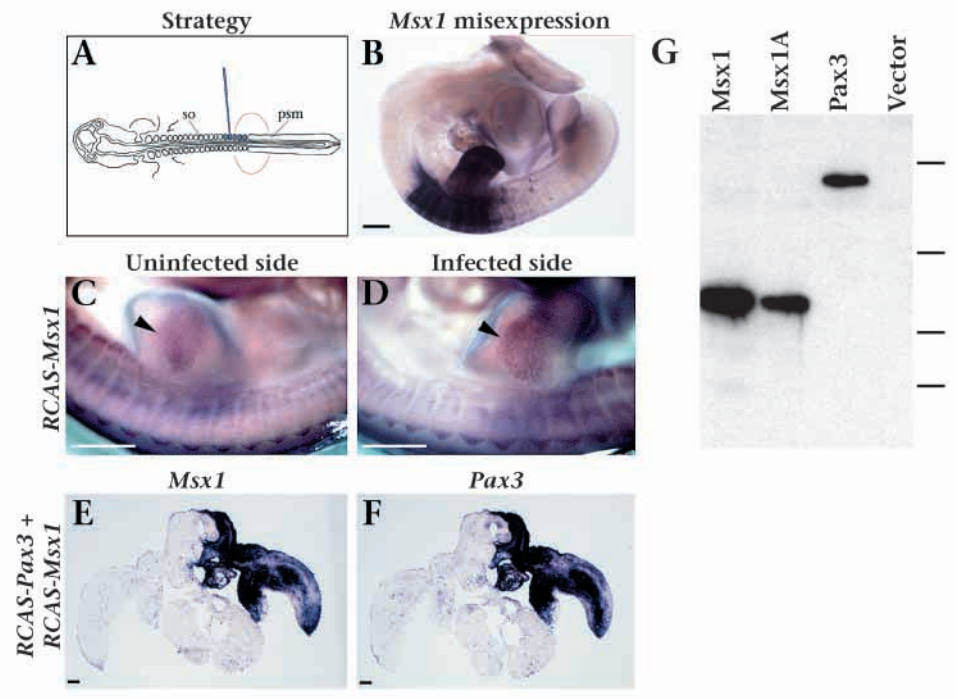


Fig. 2. (A) Retroviral infection of pre-migratory limb muscle precursors. Schematic diagram of a stage 13 chicken embryo (modified from Christ et al., 1977), depicting infection of righthand somites 15-21 (blue), which contribute muscle precursors to the right forelimb; red arcs denote future forelimbs. Abbreviations: so, somite; psm, pre-somitic 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.

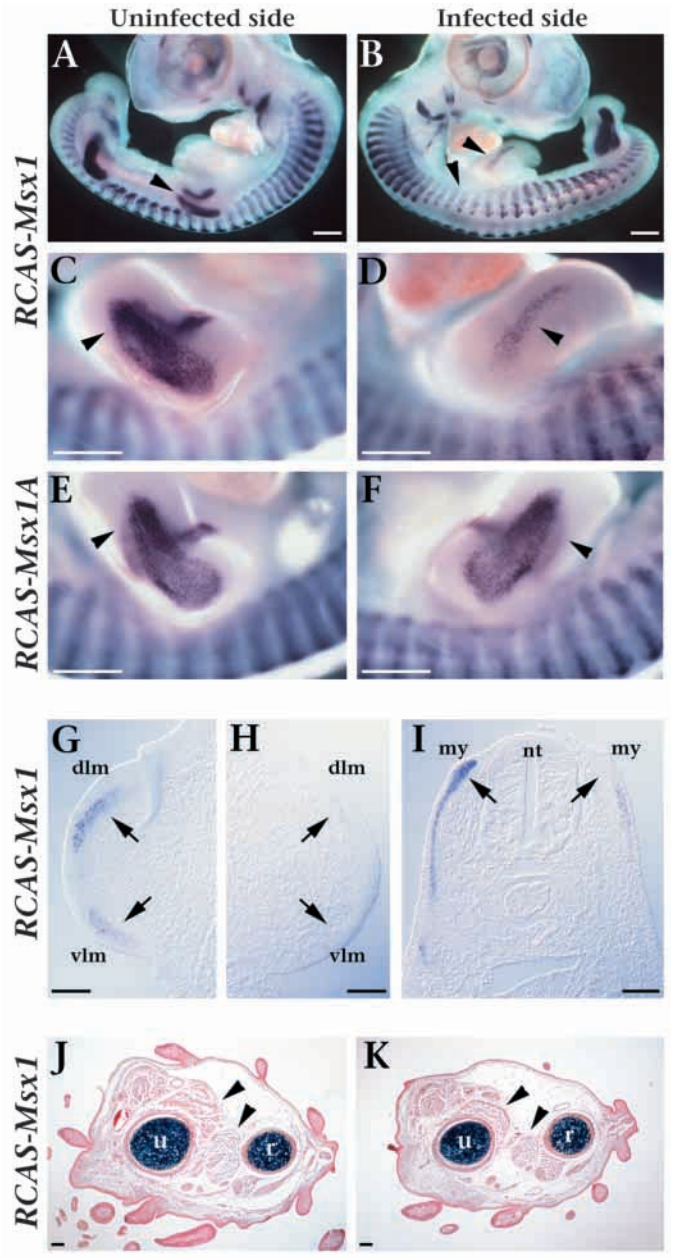


Fig. 3. Ectopic expression of *Msx1* represses *GMyoD* in the forelimb and somites. (A-F) Chicken embryos were injected with *RCAS-Msx1* (A-D) or *RCAS-Msx1A* (E,F) at stage 13-14, and *GMyoD* expression was examined by whole-mount in situ hybridization at stage 25-26. (A,B) Low-power views of an *RCAS-Msx1*-infected 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-Msx1*-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

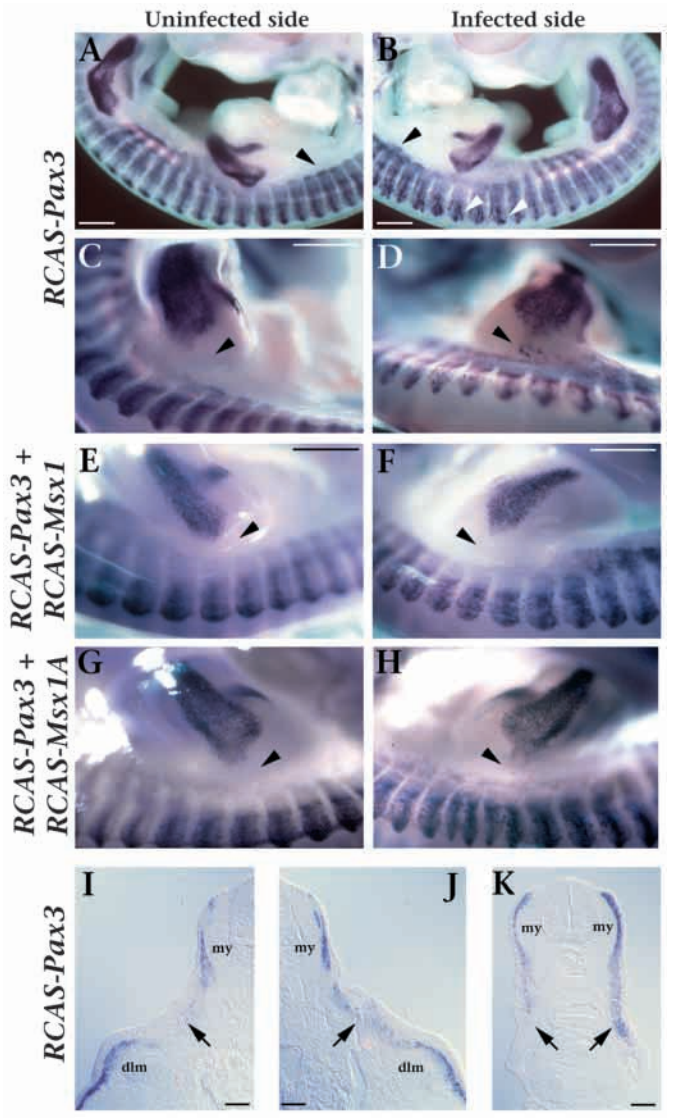


Fig. 4. Ectopic expression of *Pax3* activates *GMyoD*, while co-ectopic expression of *Pax3* and *Msx1* neutralizes their effects on *GmyoD*. (A-H) Chicken embryos were infected with *RCAS-Pax3* alone (A-D) or together with *RCAS-Msx1* (E,F) or *RCAS-Msx1A* (G,H) at stage 13-14, and *GMyoD* expression was examined by whole-mount in situ hybridization at stage 25-26. (A,B) Low-power views of an *RCAS-Pax3*-infected embryo showing punctate expression (white arrowheads) and lateral expansion (black arrowheads) of *GMyoD* in the somites on the infected versus the uninfected side. (C,D) High-power views of an *RCAS-Pax3*-infected embryo showing ectopic *GMyoD* in scattered cells at the base of the forelimb only on the infected side (arrowheads). (E,F) High-power views of an embryo co-infected with *RCAS-Pax3* and *RCAS-Msx1* show equivalent expression of *GMyoD* in the pre-muscle masses of the infected and the uninfected forelimb, and the absence of *GMyoD*-expressing cells at the base of the forelimb (arrowheads). (G,H) High-power views of an embryo co-infected with *RCAS-Pax3* and *RCAS-Msx1A* showing scattered *MyoD*-positive cells at the base of the infected forelimb. (I-K) Cryosections of whole-mount embryos (as in A-D) show ectopic *GMyoD* in the forelimb and somites following *RCAS-Pax3* infection. I and J show continuous expression of *GMyoD* from the somites to the limb on the infected (J, arrow) but not the uninfected (I, arrow) side from the same section at the level of the forelimb. (K) A section rostral to the forelimb from the same embryo with increased expression and ventral expansion of *GMyoD* on the infected (right arrow) but not the uninfected (left arrow) side. Scale bars, 0.5 mm in A-H and 0.1 mm in I-K.

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 G*MyoD* expression, muscle histology was not significantly affected in the *RCAS-Pax3*-infected wings (A. J. B. and C. A.-S., unpublished). Together, these findings suggest that *MyoD* is a target for transcriptional activation by *Pax3* in vivo.

Msx1 and *Pax3* oppose each other's actions on *GMyoD* expression in vivo

To address the combinatorial actions of *Msx1* and *Pax3* on *GMyoD* expression in vivo, we infected somites 15-21 of stage 13-14 embryos with the *RCAS-Msx1* and *RCAS-Pax3* retroviruses in equal amounts (i.e., equal titer). To ensure efficient co-infection of both exogenous genes, we used retroviruses having two different viral coats (e.g., *RCAS(B)-Msx1* and *RCAS(A)-Pax3*). We verified that this co-infection protocol resulted in ectopic expression of both *Msx1* and *Pax3* in the right forelimb and nearby trunk by in situ hybridization (Fig. 2E,F).

Following co-infection of *RCAS(B)-Msx1* and *RCAS(A)-Pax3*, *GMyoD* expression was neither reduced nor significantly enhanced in the forelimb and somites on the infected side compared with the uninfected side (n=22) (Fig. 4E,F). Thus, co-infection of *RCAS(B)-Msx1* and *RCAS(A)-Pax3* did not result in repression of *GMyoD* expression in the dorsal or ventral pre-muscle masses (Fig. 4E,F). Conversely, co-infection did not produce ectopic expression of *GMyoD* at the base of the forelimb (Fig. 4E,F). The specific requirement for *Msx1* was evident in co-infection studies performed with *RCAS(B)-Msx1A* 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 ^{35}S-labeled Msx1 or Pax3 proteins obtained by in vitro translation (Fig. 5A). These GST interaction assays showed that ^{35}S-labeled Msx1 interacted specifically with GST-Pax3 and, conversely, that ^{35}S-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

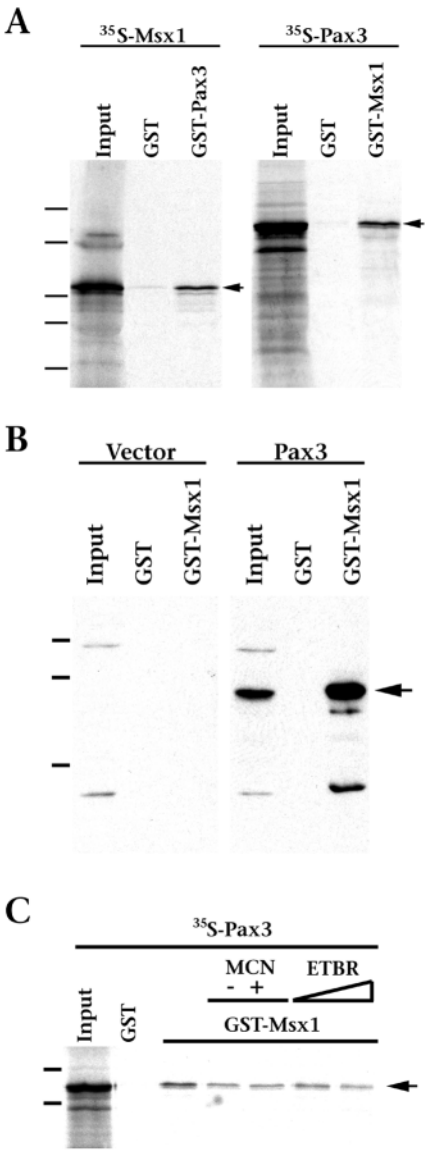


Fig. 5. Msx1 interacts with Pax3 in vitro. (A) GST interaction assays were performed with GST or GST-Pax3 (4 μg) and ^{35}S-labeled Msx1, or with GST or GST-Msx1 (4 μg) and ^{35}S-labeled Pax3. Immobilized proteins (arrows) were resolved by SDS-PAGE and visualized by autoradiography. "Input", 100% (2 μl) of ^{35}S-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 ^{35}S-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 ^{35}S-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 ^{35}S-labeled Pax3 protein and a series of truncated GST-Msx1 fusion proteins (Fig. 6A,B). We found that ^{35}S-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 Pax3δ1 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),

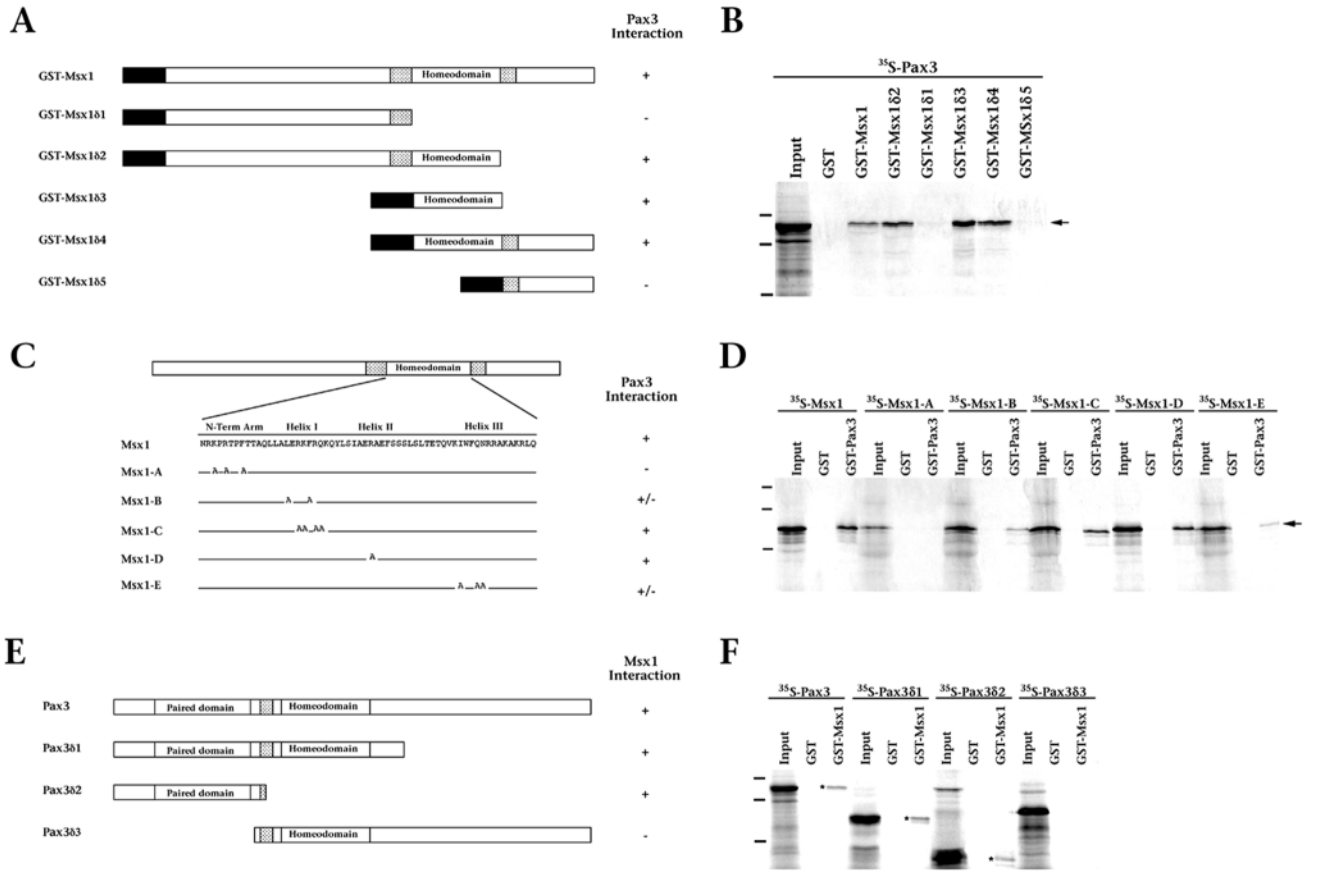


Fig. 6. Msx1 and Pax3 interact through residues in the N-terminal arm of the Msx1 homeodomain and the paired domain of Pax3. (A,C,E) Diagrams of the Msx1 and Pax3 proteins used for in vitro interaction assays. (A) GST-Msx1 fusion proteins (GST-Msx1δ1-5), with the homeodomain, conserved flanking region (stippled boxes), and GST domain (black boxes) indicated. (C) Mutated Msx1 polypeptides (Msx1A-E), with clustered alanine substitutions (A) at indicated positions in the homeodomain. The amino acid sequence of the homeodomain and positions of the N-terminal arm, and helices I, II and III are shown. (E) Pax3 and truncated polypeptides (Pax3δ1-3), with positions of the paired domain, homeodomain, and octapeptide (stippled box) shown. Table 1 provides details of protein sequences. Summaries of protein interactions were compiled from multiple experiments (representative assays shown); (+) indicates a strong interaction, (+/–), a weak interaction, and (–), no interaction. (B,D,F) GST interaction assays were performed using GST or the indicated GST-fusion proteins (4 μg) and the indicated ^{35}S-labeled Pax3 or Msx1 proteins. Immobilized proteins (arrows in B and D, asterisks in F) were resolved by SDS-PAGE and visualized by autoradiography. Dashes indicate molecular mass markers: 77, 48, 34 kDa.

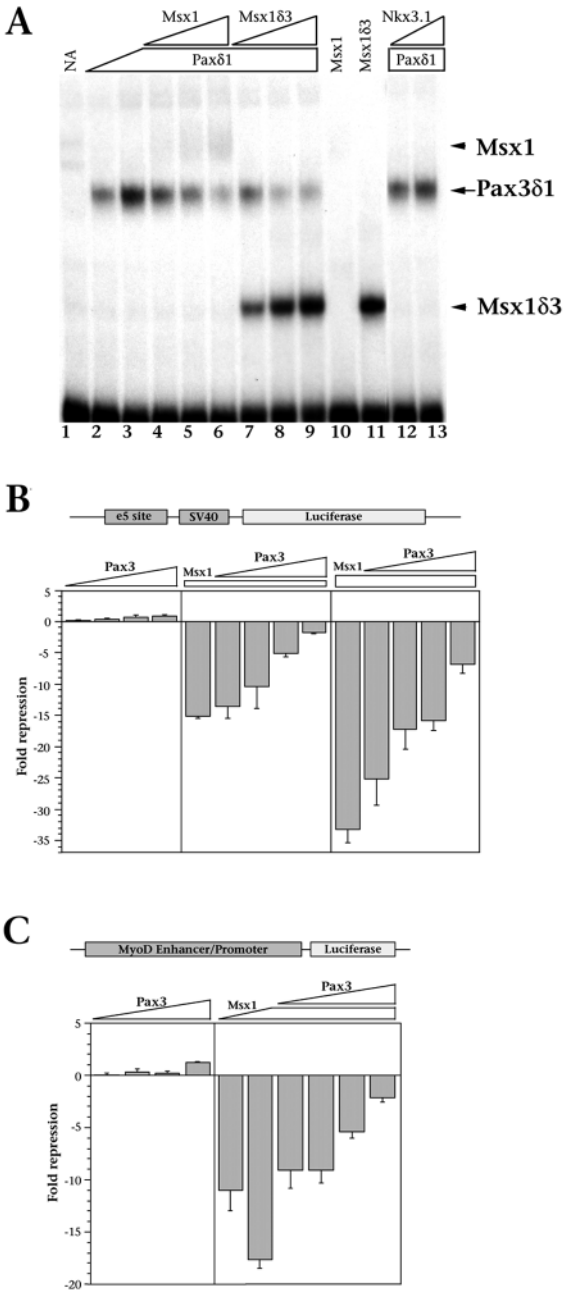
the overall level of Pax3 inhibition is equivalent (compare lanes 3-5 with 7-9). This inhibition was specific for Msx1, since no such inhibition of Pax3 DNA binding activity was observed in the presence of an unrelated homeoprotein, Nkx3.1 (Sciavolino et al., 1997) (Fig. 7A, lanes 12-13), and since the 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 intepretation 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

Fig. 7. Opposing biochemical activities of Msx1 and Pax3. (A) Gel retardation assays were performed using the e5 DNA site (Treisman et al., 1991). Reactions contained in vitro translated Pax3δ1 [1-2 μl (triangle) or 2 μl (rectangle)] plus recombinant Msx1 (140, 280, 560 ng, lanes 4-6), Msx1δ3 (70, 140, 280 ng, lanes 7-9) or Nkx3.1 (280 and 560 ng, lanes 12, 13). Lanes 10 and 11, Msx1 (140 ng) or Msx1δ3 (70 ng) alone. Lane 1, "NA", unprogrammed lysate. The position of the protein-DNA complexes are indicated.
(B,C) Transient transfection assays were performed in C2C12 cells using expression plasmids encoding Pax3 or Msx1. The luciferase reporter plasmid in B contained a single copy of the e5 DNA binding site (Treisman et al., 1991) and a minimal SV40 promoter, and in C the MyoD enhancer/promoter (Woloshin et al., 1995). Amounts of each expression plasmid were 125, 250, 500 or 1000 ng of Pax3 (triangles) and 62.5 or 125 ng of Msx1 (rectangles or triangle). Data are represented as fold luciferase activity; actual values were approximately 10,000 cpms for basal, 22,000 cpms for activated, and 500 cpms for repressed. Assays were repeated three times; a representative assay is shown with error bars indicating difference between duplicates.

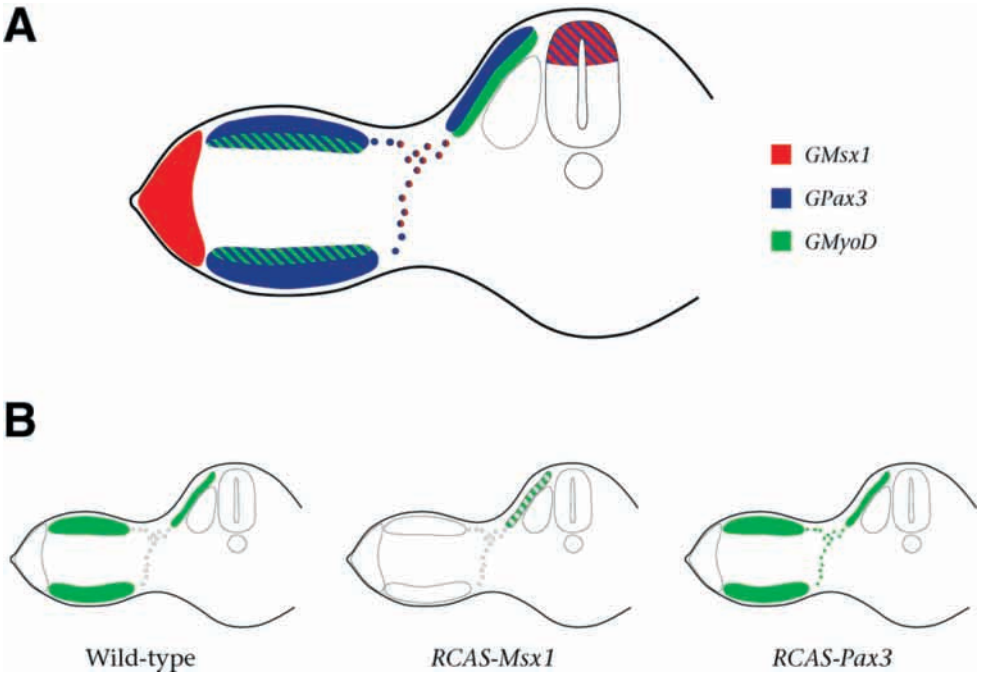


Fig. 8. Model showing the relationship of *Msx1* and *Pax3* expression to myogenesis. The model is described in the text. (A) expression patterns of *GMsx1*, *GPax3* and *GMyoD* at the forelimb level of a stage 22 chicken embryo; overlapping expression indicated by the striped pattern. (B) Diagram of *GMyoD* expression at the forelimb level of a stage 26 chicken embryo (wild-type) or following infection with *RCAS-Msx1* or *RCAS-Pax3*. Reduced *GMyoD* expression shown by the striped pattern.

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, presumbably, 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.

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