The homeobox gene \textit{Msx1} is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb

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SUMMARY

In myoblast cell cultures, the Msx1 protein is able to repress myogenesis and maintain cells in an undifferentiated and proliferative state. However, there has been no evidence that \textit{Msx1} is expressed in muscle or its precursors in vivo. Using mice with the \textit{lacZ} gene integrated into the \textit{Msx1} locus, we show that the reporter gene is expressed in the lateral dermomyotome of brachial and thoracic somites. Cells from this region will subsequently contribute to forelimb and intercostal muscles. Using \textit{Pax} 3 gene transcripts as a marker of limb muscle progenitor cells as they migrate from the somites, we have defined precisely the somitic origin and timing of cell migration from somites to limb buds in the mouse. Differences in the timing of migration between chick and mouse are discussed. Somites that label for \textit{Msx1}\textit{lacZ} transgene expression in the forelimb region partially overlap with those that contribute \textit{Pax3}-expressing cells to the forelimb. In order to see whether \textit{Msx1} is expressed in this migrating population, we have grafted somites from the forelimb level of \textit{Msx1}\textit{lacZ} mouse embryos into a chick host embryo. We show that most cells migrating into the wing field express the \textit{Msx1}\textit{lacZ} transgene, together with \textit{Pax3}. In these experiments, \textit{Msx1} expression in the somite depends on the axial position of the graft. Wing mesenchyme is capable of inducing \textit{Msx1} transcription in somites that normally would not express the gene; chick hindlimb mesenchyme, while permissive for this expression, does not induce it. In the mouse limb bud, the \textit{Msx1}\textit{lacZ} transgene is downregulated prior to the activation of the \textit{Myf5} gene, an early marker of myogenic differentiation. These observations are consistent with the proposal that \textit{Msx1} is involved in the repression of muscle differentiation in the lateral half of the somite and in limb muscle progenitor cells during their migration.

Key words: Mouse, Homeodomain protein, Msx genes, Homologous recombination, Heterospecific grafts, \(\beta\)-galactosidase, Differentiation, In situ hybridisation, Muscle precursor cells, Pax3, Pax-3, Msx-1, Msx2, Msx-2

INTRODUCTION

All muscles of the vertebrate body derive from the segmented paraxial mesoderm of the somites (reviewed in Hauschka, 1994; Christ and Ordahl, 1995). Epaxial muscles, such as those of the back, derive from progenitor cells in their dorsomedial quarter. These initially form the myotome, under the influence of signal(s) from axial structures, neural tube and notochord, which induce myogenic factor expression. In contrast, hypaxial muscles, such as those of the body wall, derive from the dorsolateral quarter of the somite and myogenesis depends on inductive signals from the dorsal ectoderm and is retarded by lateral mesoderm (see Cossu et al., 1996b for review).
in chick, where invasion of the wing bud is initiated at about the 20-somite stage and is complete at the 36-somite stage (Chevallier et al., 1977; Christ et al., 1977; Solursh et al., 1987). In mammals, information is less precise. In mouse, migratory muscle cells reach the forelimb bud from about the 21-somite stage (Sze et al., 1995; see also Milaire, 1987) and by about the 35-37-somite stage for the hindlimb (Tajbakhsh and Buckingham, 1994).

Transcripts of the Pax3 gene have been shown to mark cells migrating from somites to limbs in avian embryos (Williams and Ordahl, 1994). In splotch (Pax3<sup>−/−</sup>) mutant mice, cells from the lateral dermomyotome do not migrate, limb musculature is absent, and other hypaxial skeletal muscles are affected (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994; Daston et al., 1996). Recent results suggest that Pax3 may act through the c-met receptor, which is also expressed by this cell population and is required for migration (Bladt et al., 1995; Epstein et al., 1996; Yang et al., 1996; Maina et al., 1996). Other markers of the somitically derived cells in the limb bud are N-cadherin, and follistatin (Brand-Saberi et al., 1996).

During early stages of limb morphogenesis, distal ectoderm is induced to differentiate into a transitory epithelial structure, the apical ectodermal ridge (AER). The action of the AER is mediated by diffusible growth factors and maintains the underlying mesenchyme, referred to as the progress zone, undifferentiated and proliferative, permitting distal limb outgrowth (see Tickle, 1996 for review). As a result of this distal outgrowth of the limb bud, proximal cells are removed from the proliferative influence of the AER and allowed to differentiate. When they reach the limb bud, muscle progenitor cells proliferate for at least 24 hours, before they activate the myogenic factor genes Myf5 (Tajbakhsh and Buckingham, 1994), MyoD and then Myogenin (Sassoon et al., 1989) prior to differentiation. Growth factors present in the progress zone probably contribute to the repression of muscle differentiation (Robson and Hughes, 1996).

The homeobox-containing gene Msx1 (previously Hox-7), whose expression is regulated by the AER, is strongly expressed in the nascent limb bud when limb muscle progenitor cells migrate from the somites (Robert et al., 1989; Hill et al., 1989; see Davidson, 1995). Forced expression of this gene blocks terminal differentiation of a myogenic cell line, resulting in a decrease in levels of MyoD (Song et al., 1992); a result subsequently extended to the closely related Msx2 gene, also expressed in the limb bud (Takahashi et al., 1996). Furthermore, experiments in which human chromosomes were transfected into mouse 10T1/2 fibroblast cells showed that activation of the human MyoD gene is inhibited by an additional chromosomal fragment containing the Msx1 locus. Indeed, Msx1 binds to the MyoD enhancer and it has therefore been suggested that it may directly regulate its transcription (Woloshin et al., 1995). However, there has been no direct demonstration that muscle progenitor cells in vivo do actually express Msx1 at any stage; the strong expression of Msx1 throughout the early limb mesenchyme complicates this issue. Embryos with a null mutation in the Msx1 gene show no skeletal muscle phenotype (Satokata and Maas, 1994; Houzelstein et al., 1997). However Msx2 is coexpressed with Msx1 at many sites during embryonic development, and there may therefore be redundancy between these closely related proteins (Catron et al., 1996; Houzelstein et al., 1997).

We have generated mice carrying a null mutation in the Msx1 gene, by insertion of the bacterial nlacZ reporter gene, encoding the β-galactosidase protein with a nuclear localisation signal, into its homeobox. The pattern of expression of β-galactosidase from the targeted Msx1<sup>nlacZ</sup> allele closely mimics that of Msx1 mRNA previously described (Davidson, 1995) and marks cells that normally express the Msx1 gene during development (Houzelstein et al., 1997). The resolution and sensitivity conferred by the nlacZ reporter have permitted us to detect expression in the lateral dermomyotome of somites in the vicinity of forelimbs and in the interlimb region. The timing and extent of this expression on the anteroposterior axis have been compared to that of Pax3, a gene expressed in cells migrating to the forelimb and hindlimb buds. These results show that Msx1 is expressed in a subset of somites some of which contribute cells to limb musculature. By grafting mouse somites in chick embryos, we have been able to demonstrate that cells in the lateral dermomyotome which have activated Msx1 are muscle progenitor cells which migrate into the wing field. Heterotopic grafts show that the wing, but not the leg mesenchyme, is capable of inducing Msx1 expression in mouse somites from different rostrocaudal levels and in cells migrating from them. This may be related to the expression of Bmp4, which is highest in limb mesenchyme at the forelimb level at the appropriate time. The nlacZ reporter gene ceases to be expressed in the mouse limb bud prior to Myf5 activation, consistent with a role for Msx1 in the negative regulation of myogenic differentiation.

**MATERIALS AND METHODS**

**Mouse embryos**

In Msx1 nlacZ-targeted embryos, the nlacZ reporter gene has been inserted into the region encoding the third helix of the Msx1 homeodomain, such that the nlacZ-coding sequences are in phase for translation. This allele was maintained on a C57BL/6J background. Typing of embryos was performed by PCR as previously described (Houzelstein et al., 1997). For Myf5 nlacZ-targeted embryos, the nlacZ reporter gene has been fused into the first exon of the Myf5 gene, 13 codons after the ATG codon such that the nlacZ coding sequence is in phase for translation. The Myf5 gene itself is disrupted, and a small deletion is introduced. This allele was maintained on a mixed (C57BL/6-129-DBA/2) background (Tajbakhsh et al., 1996a).

In this study, the first somite to express Pax3 or Myf5 at the anterior of the embryo was counted as somite 3 (according to Tajbakhsh et al., 1996b; Spörle and Schughart, 1997). This locates the anterior margin of the forelimb between somites 9 and 10, and the posterior margin between somites 14 and 15. In this way, the somite that we are counting as 13 was referred to as somite 11 by Sze et al. (1995) and as somite 12 by Burke et al. (1995).

**In situ hybridisation and staining procedures**

The Msx1 probe corresponds to the 3′ untranslated region of the mouse gene as previously described (Lyons et al., 1992). The Pax3 (Goulding et al., 1991) probe is derived from a 519 bp PstI-HindIII fragment from the 3′ coding end of the mouse cDNA (kindly provided by P. Gruss). To generate a mouse-specific Pax3 probe, two oligonucleotides, 5′-GGGGTATGTTCTTCTGGGAAG-3′ and 5′-TTTCATGTCTAGTCTGTGGAGGC-3′, were used to amplify the 3′
were fixed between 16 and 24 hours following surgery (Fontaine-Pérus et al., 1997). Donor neural tubes were excised from between 16 and 24 hours following surgery. Permutation was performed in the same way. In all series, chick embryos were fixed changing their orientation. Heterotopic and heterochronic grafts were performed according to Henrique et al. (1995) except that mouse embryos were fixed in ethanol diluted 1/1000 in PBS). They were subsequently washed twice in PBS and mounted in Mowiol (Calbiochem). Salmon-gal (Apollo Scientific Ltd, UK) staining was performed following the protocol.

All pictures have been scanned and assembled using Adobe Photoshop.

Somite and neural tube transplantation

Somite grafting was performed as previously described (Fontaine-Pérus et al., 1995) except that Msx1lacZ mice were maintained on a C57BL/6 background. For homotopic grafting, donor somites were excised from 18- to 20-somite stage Msx1lacZ mouse embryos. Somites 8-15 were retained to be transplanted into the 15- to 21-somite region of the chick host, a region previously described to give rise to myogenic progenitor cells of the wing muscles (Chevallier, 1979; Zhi et al., 1996). Mouse somites were replaced without changing their orientation. Heterotopic and heterochronic grafts were performed in the same way. In all series, chick embryos were fixed between 16 and 24 hours following surgery.

Neural tube grafting was performed as previously described (Fontaine-Pérus et al., 1997). Donor neural tubes were excised from the caudalmost part of 15- to 18-somite stage Msx1lacZ mice. Hosts were fixed between 16 and 24 hours following surgery.

RESULTS

Origin and timing of the somitic contribution to the limb buds

Using a probe specific for the Pax3 gene, which is expressed in limb muscle progenitor cells during their migration (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994), we examined which somites contribute muscle progenitor cells to the limb and when this migration takes place in the mouse embryo, based on the detection of delaminating Pax3-positive cells. Lateral dermomyotomal cells were first detected to delaminate in 24-somite embryos (about embryonic day (E) 9.75) from somites 9-11, adjacent to the forelimb bud (data not shown). In 25-somite embryos, delaminated Pax3-positive cells are seen adjacent to somites 9-12 (Fig. 1a; arrowhead). The contribution to the limb bud gradually extends more caudally to include somite 14, in keeping with the anterior-posterior gradient of somite maturation. Migration is most intense in 29-somite embryos (Fig. 1b). In 32-somite embryos, fewer cells are delaminating adjacent to the forelimb bud and somite 9 no longer contributes; by the 33-somite stage only a few cells continue to delaminate from somite 14 (data not shown). At the 38-somite stage forelimb premuscle masses are clearly isolated from the adjacent somites (Fig. 1e). Therefore, Pax3-positive cells delaminating from somites 9 to 14 are detected in the forelimb bud field in 24- to 33-somite embryos (results summarised in Table 1).

Somites contributing to limb musculature exhibit a typical aspect after completion of migration, when compared to somites at the thoracic level. While thoracic somites exhibit a
ventrolateral bud (somitic bud) strongly expressing *Pax3*, this bud is absent from limb muscle contributing somites (Christ et al., 1983). However, somite 13 exhibits an intermediate aspect, the anterior half having the morphology of limb level somites and the posterior half that of thoracic somites (Fig. 1e). This suggests that somite 13 contributes to limb as well as thoracic musculature. Somite 14, which contributes to limb muscle, exhibits a more thoracic aspect.

We detected the first cells delaminating towards the hindlimb bud from somites 26 to 29, in 32-somite embryos (Fig. 1c). By the 35-somite stage, somite 26 no longer contributes to hindlimb muscles (data not shown). It exhibits a structure reminiscent of the thoracic somites, suggesting that it may also have an intermediate status (cf somite 13). Delamination of *Pax3*-positive cells and their detection in the hindlimb bud in 35- to 38-somite embryos extends from somites 27 to 31 (Fig. 1d, cf Table 1). At the 40-somite stage, somite 27 appears to be still contributing; while delamination from more posterior somites does not appear to be continuous with positive cells in the limb, it is nevertheless detectable (Fig. 1f). By the 41-somite stage, delamination was no longer detectable from somites opposite the hindlimb bud. These results are summarised in Table 1.

In addition to *Pax3*-positive cells in the limb buds, we detected cells delaminating from somites 4 to 6 in 28-somite embryos, and from somites 4 to 7 in 29-somite embryos (Fig. 1b). These cells appear to migrate towards the branchial arches where they probably contribute to hypoglossal and tongue muscles, according to results obtained in the chick (Noden, 1983; Mackenzie et al., 1998). Somites 4-7 continue to show such delaminating cells until the 32-somite stage (data not shown).

**Msx-1 expression in limb muscle progenitor cells**

In embryos in which one *Msx1* allele has been targeted with *nlacZ*, we have detected β-galactosidase activity in the lateral dermomyotome. This is shown by whole-mount staining in an E9.5 (23-somite) embryo in Fig. 2a and on a section through
a somite in Fig. 2b, at the level of the forelimb bud. As previously reported for the endogenous gene, there is strong labelling of the neural tube, lateral mesoderm and limb buds, at this stage. This labelling of the lateral dermomyotome is first seen in 22-somite embryos, in somites 11-15. By the 25-somite stage, labelling in heterozygous embryos is detectable in somites 13-16. In 32-somite embryos, it is no longer seen (summarised in Table 2).

In homozygous embryos, where both Msx1 alleles are targeted with nlacZ, more extensive expression is detectable in somites along the anteroposterior axis (Fig. 2c), where it is concentrated in cells of the lateral dermomyotome (Fig. 2d). In the dermomyotome of these somites, the expression domain of Msx1 overlaps with the lateral domain of Pax3 expression (Fig. 2d; see also Fig. 3c). Low-level expression is seen in somites in the interlimb region from the 25-somite stage, extending to somite 26 in 30-somite embryos. By the 32-somite stage, β-galactosidase-positive cells are no longer detectable in most somites, but persist in somites 25-26 until the 32-somite stage.

These results indicate that Msx1 is a marker of the lateral dermomyotome of somites, in the forelimb and interlimb region. Msx1<sup>nlacZ</sup> does not appear to be expressed in somites that contribute the muscle progenitor cell population of the hindlimb, with the possible exception of somite 26, which, as previously noted, has a mixed thoracic/limb morphology; nor is Msx1<sup>nlacZ</sup> expression detectable more anteriorly in somites that will contribute migratory cells to the branchial arches. At the forelimb level, Msx1 expression is maximal in somites 12-16, while somites 9-14 are the main contributors of Pax3-positive cells. The onset of Msx1<sup>nlacZ</sup> expression begins at stage 22, prior to delamination of Pax3-positive cells, and is not detectable in somite 9, which also contributes Pax3-positive cells to the forelimb bud (cf Tables 1 and 2). In contrast, β-galactosidase activity derived from the Msx1<sup>nlacZ</sup> allele is no longer detectable at somite stage 32 from somites opposite the forelimb in embryos with both alleles targeted, when Pax3-positive cells are still delaminating.

In the avian embryo, it is well demonstrated that the dorsolateral zone of the brachial somites provides the myogenic progenitors of the forelimbs, a demonstration relying largely on exchange of somites between quail and chick (Reviewed in Hauschka, 1994; Christ and Ordahl, 1995). In mammals, experiments in which labelled somites were transplanted into cultured embryos also pointed to myogenic progenitor migration from somites to the limb (Beddington and Martin, 1989; Lee and Sze, 1993; Sze et al., 1995). In the Msx1<sup>nlacZ</sup> mice, at the stage when limb muscle progenitor cells migrate from the somites, limb buds strongly express β-galactosidase from the nlacZ-targeted Msx1 allele throughout most if not all the mesenchyme (Fig. 2a,b). It is therefore very difficult to assess against this background whether muscle progenitor cells express the Msx1 gene. To address this issue, murine somites, which would normally contribute to forelimb muscles, were grafted in place of chick somites, which would normally contribute to wing muscles, prior to the onset of migration. Surgery was performed on 27 embryos in which the last 4-6 somites from the brachial level of 18- to 20-somite chick hosts were replaced unilaterally with somites 8-15 from E9 mouse embryos heterozygous or homozygous for the Msx1<sup>nlacZ</sup> allele. In all specimens examined 6 hours after grafting (10 cases), β-galactosidase-positive cells were detected in the dorsolateral border of the implanted somites (Fig. 3a-c). β-galactosidase was detected only in somites 11-13 when somites 8-13 were grafted, implying that 6 hours after grafting the expression of Msx1<sup>nlacZ</sup> in the grafted somites still corresponds to that seen in the mouse. 12 hours after grafting (9 cases), labelled cells were observed located outside the somites in the proximal wing field (Fig. 3d,e). In slightly older chimaeric embryos (5 cases), they were all present within the wing field (not shown).

To further characterise the mouse somitic cells that express Msx1, we investigated whether they also express Pax3. For this

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Number of cases studied:

- Strong expression in homo- and heterozygous embryos
- Weak expression in homo- and heterozygous embryos
- Strong expression in homozygous embryos only
- Weak expression in homozygous embryos only
- Not detectable

Table 2. Spatial and temporal expression of the Msx1-nlacZ gene in the lateral dermomyotome of the somite during development
Fig. 2. Expression of the Msx1nlacZ gene in the lateral dermomyotome of somites. (a) An E9.5 (23 somites) Msx1nlacZ/+ embryo, with β-galactosidase activity revealed on whole mount. The position of somite 13 is indicated. The Msx1nlacZ gene is expressed in the lateral dermomyotome of somites 12-15, and more weakly in somite 11. (b) An E9.5 Msx1nlacZ/+ embryo. This embryo has been whole-mount stained for β-galactosidase, embedded in paraffin and sectioned. A section at forelimb (fl) level is shown, the Msx1 nlacZ/+ dermomyotome of somites. (a) An E9.5 (23 somites) Msx1nlacZ/+ embryo cryostat sectioned at the level of somite 13 is indicated. The position of somite 13 is indicated. The Msx1nlacZ gene is expressed in the lateral dermomyotome. The forelimb bud is strongly labelled. (c) An E9.75 (30 somites) Msx1nlacZ/+ embryo. This embryo has been whole-mount stained for β-galactosidase. The position of somite 13 is indicated. β-galactosidase activity is detected with variable intensities in the lateral domain of somites 10-26. (d) E9.75 Msx1nlacZ/+ embryo cryostat sectioned at the level of the forelimb (fl) and stained for β-galactosidase activity. Strong staining in the forelimb and also in the lateral dermomyotome is seen. The inset shows an adjacent section hybridised with the Pax3 probe. Pax3 and Msx1nlacZ expression domains are superimposed in the lateral region of the dermomyotome. Scale bar (a,c) 250 μm; (b,d) 100 μm.

Since neural crest cells traverse the somite, they may be carried over with the graft and contribute migrating cells to the chick host. Previous work has shown that active emigration of neural crest cells from mouse neural tube occurs after in ovo grafting (Fontaine-Pérus et al., 1997). Under these conditions, neural crest cells from the implant contribute to the development of the host peripheral nervous system and to the melanoblast population. These cells may also express Pax3 at certain levels along the rostrocaudal axis (Goulding et al., 1991; Natoli et al., 1997). The major routes followed by neural crest cells do not traverse the wing bud (Fontaine-Pérus et al., 1997). However, to rule out the possibility that the cells expressing Msx1nlacZ in the somite and proximal wing field are related to the neural crest population, a fragment of mouse neural tube was grafted in place of part of the chick neural tube, adjacent to a level caudal in the segmental plate to the youngest newly formed somite in 15 to 18-somite-stage chick embryos. A total of 10 cases were examined between 16 and 24 hours following surgery. At 16 hours post surgery, neural crest cells had left the mouse neural tube. At this time, Msx1nlacZ transgene expression was restricted to the dorsal area of the grafted mouse neural tube. 24 hours post surgery, the mouse crest cells compacted and constituted the early spinal ganglionic primordia; at this stage enzyme activity was still restricted to the neural tube area. In one chimera, in which in situ hybridisation with the Pax3 probe and β-galactosidase staining were carried out simultaneously, co-expression was seen in the dorsal aspect of the grafted neural tube (Fig. 3f). No staining was observed in the wing bud, demonstrating that mouse neural crest cells do not contribute to the Msx1-expressing population detected in the proximal wing bud after somite grafting.

Mouse somites can be induced to express Msx1 by ectopic grafting

Chick/quail transplantation experiments have demonstrated that unsegmented paraxial mesoderm or somites, not derived from the forelimb region of the mouse embryo, are...
reprogrammed to contribute migratory limb cells when placed opposite the chick wing field (Chevallier, 1979). To assess whether such reprogramming also takes place for Msx1 expression, heterotopic and heterochronic grafts were performed. In the mouse, the anterior-most somites never express Msx1 (see Table 2). When such somites (somite 1-5 level) from E8.5 Msx1\(^{nlacZ}\) heterozygous mice were implanted into the chick at the brachial level, β-galactosidase expression was observed laterally to the grafted somites (Fig. 4a) (4 cases analysed).

Somites from the wing level of 15- to 18-somite chick hosts were unilaterally replaced by the unsegmented paraxial mesoderm from 18- to 20-somite Msx1\(^{nlacZ}\) heterozygous mouse embryos (18 cases). The unsegmented paraxial mesoderm does not express Msx1 in the mouse. In these experiments, it was derived from an area that would later form thoracic level somites, which do not express the Msx1 gene at a high level. Histological analysis showed that the murine segmental plate was able to become segmented in ovo (not shown). After 18 hours of incubation, β-galactosidase-positive cells in the newly formed murine somites were clearly detectable, although migration had not yet been initiated in such less advanced embryos (Fig. 4b).

Conversely, somites from the forelimb region of a mouse embryo were grafted slightly anterior to the chick wing field, such that only the posteriormost somite of the graft replaced a chick somite that would contribute muscle progenitor cells to the wing bud (Fig. 4c). Cells migrating from this somite expressed the Msx1\(^{nlacZ}\) gene, whereas the more anterior mouse somites did not provide β-galactosidase-positive cells. Noticeably, somites adjacent to the anterior area of the limb field in the mouse do not express Msx1 in the lateral part of the dermomyotome (see Table 2), although it has not been demonstrated whether muscle precursors migrating from these somites express the gene. Our results show that signals from the wing somatopleure (Hayashi and Ozawa, 1995) are able to induce strong Msx1\(^{nlacZ}\) expression in the lateral margin of the dermomyotome and in cells migrating from it, but suggest that the distribution of the inducing activity may be different in chick and mouse.

To better define the inductive properties of the limb mesenchyme, mouse somites from the hindlimb level, which

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**Fig. 3.** Expression of the Msx1\(^{nlacZ}\) gene in limb muscle precursors from mouse somites grafted into chick hosts. (a) A 20-somite chick embryo examined 6 hours after grafting of somites 8-13 from an E9 Msx1\(^{nlacZ}\) mouse, to replace chick somites at the wing level. Cells from the lateral border of grafted somites 11-13 express β-galactosidase. The limits of the graft are indicated (white bars). (b) Transverse section of a chick embryo into which somites 9-13 of an E9 Msx1\(^{nlacZ}\) mouse have been implanted at the 18-somite stage at the brachial level. 6 hours post surgery, Msx1\(^{nlacZ}\) expression was revealed on the operated side by salmon-gal (red) staining and mouse Pax3 expression by in situ hybridisation (blue staining). Note the faint cross-hybridisation of the mouse-somatic Pax3 probe with chick transcripts in the chick dermomyotome (+). (c) Higher magnification of the operated side in b. The dorsolateral zone of the mouse dermomyotome co-expresses the Pax3 and Msx1\(^{nlacZ}\) genes (blue and red labelling are superimposed – arrowhead) whereas the mediadorsal region only accumulates Pax3 transcripts (blue labelling only – arrow). (d) Expression of the Msx1\(^{nlacZ}\) gene in a chick host embryo examined 12 hours after implantation of somites 8-13 from an E9 mutant mouse at the wing level. A mass of β-galactosidase-positive cells identified by X-gal blue staining is detected outside the grafted somite. (e) Cryostat section from an embryo similar to that shown in d. The β-galactosidase-positive cells revealed by salmon-gal (red staining) appear just outside the grafted somites in the proximal wing (w) field. (f) Section at the wing bud level from a mouse-chick chimaera where part of the neural tube was replaced by a fragment of neural tube from an Msx1\(^{nlacZ}\) heterozygous mouse at the 18-somite stage, adjacent to the segmental plate. Pax3 expression was revealed by in situ hybridisation with a mouse-specific Pax3 probe (pink staining) and Msx1\(^{nlacZ}\) by X-gal (blue) staining of β-galactosidase activity on the day following surgery. X-gal staining is restricted to the upper part of the grafted neural tube (nt), which also expresses Pax3 in a broader domain. No β-galactosidase-positive (blue) cells can be detected in the wing field (w). The myotome (m) is revealed by in situ hybridisation with a chick MyoD probe (also as pink staining), nc, notochord. (g) Transverse section of a chick embryo implanted at the 18-somite stage at the brachial level with somites 9-13 of an E9 Msx1\(^{nlacZ}\) mouse, stained with bis-benzimide 24 hours post-surgery. Mouse cells in the somite (s) and in the wing field (w) appear brighter, as do the compacted cells of the chick neural tube (nt). (h,i) Adjacent section to that in g, corresponding to the boxed region. This section was first treated with salmon-gal to reveal β-galactosidase activity and, after photography (h), the same section was hybridised with the mouse Pax3 probe and re-photographed (i). Comparison of h and i clearly shows that most mouse cells are co-labelled. Occasional cells express only Pax3 (arrow). Scale bars, (a,d) 500 μm, (f,g) 100 μm, (b,e) 50 μm, (h,i) 25 μm.
never express Msx1, were grafted either at the forelimb (wing) or at the hindlimb (leg) level in a chick host. This was the first attempt to graft mouse somites at a posterior level in chick embryos older than the 20-somite stage; all grafts were successful. In accordance with experiments described above, mouse somites from the hindlimb level, when grafted to the wing level in the chick, were reprogrammed to express Msx1\textsuperscript{nlacZ} and provide \(\beta\)-galactosidase-positive cells to the wing mesenchyme (Fig. 4d) (7 cases). In contrast, when grafted at the leg level (somite 26-32), these somites and the cells migrating from them never expressed Msx1 (4 cases analysed) (data not shown). This shows that, in the chick, the inductive capacity resides in forelimb, not hindlimb territory, in accordance with the endogenous pattern of expression observed for Msx1 in the mouse somites. However, when mouse somites from the brachial level were transplanted, before detectable Msx1\textsuperscript{nlacZ} expression, to the leg level of a chick host, \(\beta\)-galactosidase-positive cells were observed in the lateral margin of these somites (10/11 cases) and migrating from them (4/11 cases) (Fig. 4e). Therefore, induction of a potential for Msx1 expression must take place before expression itself is detectable and the leg mesenchyme then constitutes a permissive region for Msx1 expression, as opposed to the neck mesenchyme (Fig. 4c). Cells migrating to the hindlimb were confirmed to be muscle precursors by hybridisation with the mouse-specific Pax3 probe (not shown).

These results show that signals emanating from the wing somatopleure are able to induce Msx1\textsuperscript{nlacZ} expression in the lateral margin of the dermomyotome of grafted somites and in cells migrating from them into the wing field, which would not have expressed the gene in the mouse embryo. BMP4 has been shown to induce Msx1 expression in mouse dental mesenchyme (Vainio et al., 1993), and is expressed in the limb bud and lateral plate mesenchyme (Pourquié et al., 1996). To investigate whether BMP4 might be the Msx1 inducer in the lateral margin of the dermomyotome as well, we performed in situ hybridisation with a Bmp4 probe on mouse embryos at the 20- to 25-somite stages (Fig. 4f). This shows that Bmp4 expression is highest in the limb mesenchyme facing somites 11-15, which correspond to those somites in which Msx1 expression is maximal by the 22-somite stage (Table 2). The same pattern has been observed at slightly earlier stages by Hogan et al. (1994). This is therefore consistent with the proposition that BMP4 induces Msx1 expression in the lateral dermomyotome of these somites. 

### Msx-1 exclusion from differentiated muscles

Expression of the myogenic factor gene Myf5 serves as an early marker of myogenic cells (Ott et al., 1991; Tajbakhsh and Buckingham, 1994). We have used Myf5\textsuperscript{nlacZ}-targeted mice (Tajbakhsh et al., 1996a) to detect the onset of myogenesis in the limb. At the 32-somite stage, when most of the muscle progenitor cells are already present in the proximal forelimb (see Table 1), the whole bud expresses the Msx1\textsuperscript{nlacZ} gene (data not shown). We have generated mice heterozygous for both Msx1\textsuperscript{nlacZ} and Myf5\textsuperscript{nlacZ}. In such a double heterozygous embryo shown in Fig. 5a (35 somites), the \(\beta\)-galactosidase-positive cells of the progress zone, expressing the Msx1\textsuperscript{nlacZ} allele, are already restricted to the distal domain of the forelimb bud. Cells in the proximal part of the limb, located more than 300 \(\mu\)m away from the AER, no longer express the Msx1\textsuperscript{nlacZ} gene. It is in this proximal area that myogenesis will be initiated as shown by the activation of the Myf5\textsuperscript{nlacZ} allele from the 37-somite stage (Tajbakhsh and Buckingham, 1994). At the same stage (39 somites) shown in Fig. 5d, Msx1 is not expressed in this proximal area (Fig. 5c). The Msx1\textsuperscript{nlacZ} gene is downregulated proximally prior to any sign of Myf5\textsuperscript{nlacZ} activation in the forelimb bud (Fig. 5a,b). The delay between Msx1\textsuperscript{nlacZ} downregulation and the detection of Myf5\textsuperscript{nlacZ} expression is about 5 hours in the C57BL/6 mouse strain. These observations point to exclusion between the expression of Msx1 and that of the early myogenic factor gene Myf5.

### DISCUSSION

The results reported here document the expression of Msx1 in the lateral dermomyotome of a subset of somites, which include most of those that will contribute migratory muscle cells to the forelimb, as defined here by the presence of Pax3 transcripts. Orthotopic grafting experiments show that somites from Msx1\textsuperscript{nlacZ} mice contribute \(\beta\)-galactosidase-positive cells to the chick wing field. The fact that most of the migratory mouse cells are labelled strongly suggests that these include muscle progenitor cells, rather than representing a small population of endothelial progenitors that are also somite-derived, or of mouse neural crest cells co-transplanted with the somite (Beddington and Martin, 1989; Wilting et al., 1995). Double labelling for Msx1\textsuperscript{nlacZ} and Pax3 expression confirms that the great majority of these cells are muscle precursors. Direct comparison of Msx1\textsuperscript{nlacZ} expression with that of Myf5\textsuperscript{nlacZ} demonstrates that the former is downregulated in the limb bud before expression of this first myogenic factor gene. These results are consistent with a role for the Msx1 gene in the initial repression of myogenesis in muscle progenitor cells in the limb.

### Origin and timing of limb muscle progenitor cell migration in the mouse; comparison with chick

We have used the expression of Pax3 as a marker of cells that migrate from the lateral dermomyotome into the limb buds, in order to define which somites contribute this cell population and when migration occurs. Pax3\(^{3\delta}\)\(^{\delta}\)\(^{\delta}\) (splotch) mutant mice lack limb musculature, clearly indicating that expression of this gene marks muscle progenitor cells, which indeed make up the bulk of the migratory population (see Christ and Ordahl, 1995). It is not clear whether Pax3 is also expressed by other somite-derived migratory cells. However, the apparent absence of an
endothelial phenotype in the limbs of splotch mice suggests that Pax3 may not be a marker of this cell type. In the region of the forelimb bud, we detect Pax3-positive cells delaminating from somites 9-14 between somitic stages 24-33 (approximately 12-15 hours in the C57BL/6J background). The onset of migration is similar to that deduced previously (21-somite stage which corresponds to our 23-somite stage – see Material and Methods) (Sze et al., 1995; see also Milaire, 1987). Interestingly, we detect the onset of delamination of cells from more anterior somites (4-7) in the field of the branchial arches rather later, in 28- to 29-somite embryos, continuing to the 32-somite stage. In quail/chick chimaeras, Mackenzie et al. (1998) have shown that myogenic cells migrating from anterior somites to colonise the hypoglossal musculature do express Pax3. In splotch mutants, muscle progenitor cells which contribute to the hypoglossal chord and to some throat and tongue muscles are absent (Tajbakhsh et al., 1997). Thus, we conclude that Pax3-positive cells delaminating from the anterior somites correspond in part to this population. On the basis of experiments with hindlimb bud explants where the appearance of Myf5-expressing myogenic cells was monitored, we had previously deduced that migratory cells had left the somite by the 35- to 37-somite stage (Tajbakhsh and Buckingham, 1994). Consistent with this, we show here that somites 26-32 contribute Pax3-expressing cells to the hindlimbs in 32- to 40-somite embryos.

Somites that contribute migratory cells to the limbs have a distinct lateral morphology; they lack the ventral somitic bud, clearly labelled by the Pax3 probe, which is typical of interlimb somites. Interestingly, somites 13 and 26 have an intermediate morphology suggesting differences in specification between rostral and caudal halves of the lateral dermomyotome. Such rostral/caudal differences have been reported for somitic neural crest colonisation (reviewed in Christ and Ordahl, 1995) or motor nerve innervation (Keynes and Stern, 1984). Furthermore each hemivertebra is composed of two adjacent hemisclerotomes, the caudal half of the rostral one and the rostral half of the caudal one (Aoyama and Asamoto, 1988). Our results suggest that the anterior half of somite 13 contributes to muscles of the forelimb bud, while muscle progenitor cells from the posterior half of this somite form intercostal and body wall muscles. This resembles observations on chick/quail chimaeras where it has been shown that somite 20 contributes both to limb and intercostal muscles (Zhi et al., 1996).

In comparing the data reported here on the somite contribution to limb muscle with those for chicken, it is necessary to take into account differences in axial formulae. While mice have 7 cervical and 13 thoracic vertebrae, chicken have 14 cervical and 7 thoracic vertebrae, with the consequence that wing buds in chick form at the 15- to 20-somite level (Burke et al., 1995). In both species, the first limb muscle progenitor cells leave the somites at about the same stage (22-24 somites in chick) (Christ et al., 1977; Jacob et al., 1978; Solursh et al., 1987). However, due to their different position along the anterior-posterior axis, the chick somites are less mature. If the most recently formed somite is counted as I (see Christ and Ordahl, 1995), this means that wing muscle progenitor cells begin to migrate from stage VII somites in chick, compared to stage XIV-XVI somites for the mouse forelimb buds. Chicken and mouse hindlimbs are at approximately the same somite level. In both cases, migration takes place from relatively less mature somites; in mouse from stage IV-VII somites and in chicken, even more strikingly, from the most recently formed somites (I-III), even before a distinct dermomyotome is formed (Jacob et al., 1979). These differences in timing indicate that the initiation of delamination and onset of cell migration are not an intrinsic property of somites, but may be induced at different stages of their maturation, by external factors. Indeed in chick, it has been shown that the migration of muscle progenitor cells is induced by contacts between the medial region of the limb field and the lateral margin of somites at the wing level (Hayashi and Ozawa, 1995). The delay in migration of mouse limb muscle precursors towards the limb bud, compared to chick, may be due to a difference in the timing of the lateral rotation of the embryo, which brings limb presumptive mesoderm into contact with the lateral margin of the somite as shown in chick (Solursh et al., 1987). As a result, in birds, the wing field is still immature when migration begins and the apical ectodermal ridge (AER), which is important in determining the direction of migration, is not present (see Hauschka, 1994), whereas in mice the forelimb bud already has a well-developed AER.

Restriction of Msx1 expression to a subgroup of somites along the anteroposterior axis

We detect expression of the targeted Msx1 nlacZ allele in the lateral part of the dermomyotome, where it coincides with Pax3 expression. Recently, somite expression has been reported for Msx1-nlacZ transgenes, and then detected by in situ hybridisation for the endogenous gene (Mackenzie et al., 1997; Pereira et al., 1999). A striking feature of the expression of Msx1, which we only detect in the lateral part of the dermomyotome, is that it is not uniform in all somites on the rostrocaudal axis, in contrast to Pax3. β-galactosidase labelling from the targeted Msx1 nlacZ allele is particularly strong in somites 11-14 which contribute Pax3-positive cells to the forelimb. Labelling in somites 9 and 10, which also contribute migratory muscle progenitor cells, is very weak (10) or not detectable (9), indicating that Msx1 expression may mark a subpopulation of these cells. Within the limb, different somites will contribute to more or less anterior muscle masses (see Hauschka, 1994), indicating that Msx1-positive muscle progenitor cells may preferentially colonise a subset of muscles. With the exception of somite 26, where weak β-galactosidase labelling is detectable, Msx1 is not expressed in the lateral dermomyotome at the level of the hindlimb. Differences in gene expression between forelimb and hindlimb have been reported notably for the Tbx4 and Tbx5 genes; Tbx4 is predominantly expressed in the embryonic hindlimb, and Tbx5 in the forelimb. However, these genes are not normally expressed in the interlimb region (Chapman et al., 1996; Gibson-Brown et al., 1996). In contrast, β-galactosidase is detectable, when both alleles of Msx1 are targeted with nlacZ, in the lateral dermomyotome of interlimb somites, which will contribute to intercostal and body wall muscles (see Christ and Ordahl, 1995), and is particularly strong in somites 15 and 16. Differences in gene expression on the anterior-posterior axis are classically identified for the Hox genes (see Krumlauf, 1994). Although somites show flexibility in terms of their
adaptation to changes in axial position, they do register positional information. This is demonstrated by mice carrying the MLC1F-CAT myosin transgene, which show an unexpected caudal-rostral gradient of expression as muscles form (Donoghue et al., 1991). Differences in the extent of Msx1 expression in somites along the axis may well be a response to anterior-posterior positional information, perhaps via Hox genes.

**Msx1 in muscle progenitor cells of the limb**

In keeping with the observation that somites can be reprogrammed to produce limb muscle progenitor cells when transplanted ectopically (Chevallier, 1979), mouse somites can be reprogrammed to express Msx1 when transplanted from a non-expressing level to the wing level in the chick. Expression can be induced in somites grafted at this level whether they come from the most rostral region (somites 1-5) or from the hindlimb level (somites 26-32), and even in somites that form after grafting of the segmental plate. This suggests that the mesenchyme from the brachial level has the capacity to induce Msx1 expression. In accordance with Msx1 expression in the mouse, chick leg mesenchyme does not induce expression of Msx1 in somites grafted at this posterior level (somites 26-31). However, mouse somites from the forelimb level, at a stage prior to detectable Msx1 expression, when grafted at the hindlimb level in the chick do express Msx1, while they do not when grafted rostrally to the forelimb level. This suggests that two different signals lead to Msx1 expression in the somite: one that is instructive, for the induction of Msx1, present in the forelimb (and thoracic) mesenchyme, and another that is permissive, present also at the hindlimb level but not in neck mesenchyme. The forelimb, hindlimb and interlimb mesenchyme, in contrast to that at more rostral or caudal positions on the axis, is also distinguished by its capacity to respond to FGFs and form limb buds (Cohn et al., 1995). It is probable that the initial activation of Msx1 depends on BMP4. This has been demonstrated during tooth morphogenesis (Vainio et al., 1993), and for the mesenchymal cells that are recruited to form the dorsal part of the vertebrae (Monsoro-Burq et al., 1996). In the case of the somite, expression of BMP4, which has a dynamic pattern during mouse embryogenesis (Hogan et al., 1994), is highest in lateral plate mesoderm adjacent to the forelimb/thoracic somites at the time when Msx1 is activated in the lateral dermomyotome at this level. Furthermore, BMP4 has been shown to lateralise the somite, thus preventing or delaying the onset of myogenesis, as evidenced by the inhibition of

**Fig. 4.** Mouse somitic cells are reprogrammed to express Msx1 by grafting mouse somites in the chick at the brachial level. (a) A mouse-chick chimaera produced by implantation of the anteriormost (1-5) somites from an E8.5 Msx1nlacZ mouse at the brachial level of an 18-somite chick embryo. In toto salmon-gal treatment shows red staining in cells migrating towards the wing field (arrow), lateral to the grafted somites. (b) A mouse-chick chimaeric embryo, 18 hours after grafting. Unsegmented paraxial mesoderm from an Msx1nlacZ mouse embryo at the thoracic level has replaced chick somite 16 and part of the segmental plate in a 16-somite chick embryo. The unsegmented paraxial mesoderm has started to segment and β-galactosidase-positive cells are detected in the lateral margin of the newly formed mouse somites (11-15) from an E8.5 chimaeric embryo, 24 hours after grafting. Five somites (11-15) from an Msx1nlacZ mouse embryo have been grafted to replace chick somites just anterior to the wing level in a 19-somite chick embryo. Only cells migrating from the most posterior grafted somite, which is adjacent to the wing field, express the Msx1nlacZ gene and are detected by β-galactosidase staining (arrow). The dorsalmost aspect of the chick neural tube is lightly labelled blue by the β-galactosidase reagents, as is sometimes observed for the most external structures. (d) Lateral view of a mouse-chick chimaera, 20 hours after grafting. Mouse somites 26-32 (hindlimb level) were grafted to an 18-somite chick embryo at the brachial level. Msx1nlacZ expression is induced in the mouse somites and in cells migrating from them, as viewed through the apical region of the chick wing bud (w). (e) Dorsal view of a mouse-chick chimaera, 19 hours after grafting. Somites 8-13 from an E9 mouse were grafted to replace presumptive somites 26-32 (hindlimb level) in a 25-somite chick embryo. In toto salmon-gal treatment shows red staining in cells migrating towards the wing field (arrow). (f) In situ hybridisation of a 25-somite mouse embryo with a Bmp4 probe. Expression of Bmp4 is maximum in the forelimb mesenchyme facing somites 11-15 (arrows), which are those where expression of Msx1 is first detected. Somites 9 and 14 are numbered. Scale bars, 500 μm.
MyoD expression in the chick (Pourquié et al., 1996). A negative effect of lateral mesoderm on myogenesis is also seen in mouse explants from the interlimb level (Cossu et al., 1996a). We suggest that, in part at least, this may be due to BMP4 induction of Msx1.

Based on ex vivo observations (Song et al., 1992; Woloshin et al., 1995), which show that Msx1 represses the onset of myogenesis, and the demonstration here that Msx1 is downregulated in the proximal region of the limb bud before activation of Myf5, it is possible that Msx1 acts as an inhibitor of myogenesis in most, if not all muscle progenitor cells in the forelimb. At other levels on the axis, other homeobox genes (see below) may perform a similar function. It also potentially fulfills this role in the lateral dermomyotome in somites of the forelimb and thoracic region. It has been proposed that the distal limb environment is able to repress MyoD expression through an FGF-mediated pathway (Robson and Hughes, 1996). Furthermore, it has been shown that FGF2 and FGF4 are able to maintain Msx1 expression in murine limb buds grown in culture (Vogel et al., 1995; Wang and Sassoon, 1995). It is tempting to propose that the inhibitory action of FGFs in the limb may be mediated by Msx1 in vivo. Although it has been shown that Msx1 can bind to sequences in the MyoD enhancer (Woloshin et al., 1995), no information is available for Myf5. The lag of 3-4 somites (about 5 hours) before activation of Myf5<sup>nlacZ</sup>, and after downregulation of Msx1<sup>nlacZ</sup>, might suggest that this is not a direct effect of derepression, particularly since activation of MyoD in the limb occurs slightly later than that of Myf5 (Ott et al., 1991). Indeed the large number of sites where Msx1 is expressed in the embryo suggests that it may act as a general repressor of terminal differentiation, while maintaining proliferation, in various cell types.

It may also be playing a role in apoptosis; BMP4 induction of Msx2 has been associated with apoptosis of neural crest cells, for example (Graham et al., 1994).

Limb muscle progenitor cells migrate from the somites for a short period of time (10 hours in chick; 13-15 hours in mice). In chick, 30-100 cells migrate from each dermomyotome (reviewed in Hauschka, 1994). Since 6-7 somites contribute, only about 700 cells are progenitors of all the limb musculature. Thus, amplification of this population prior to the onset of differentiation and formation of primary fibres, must be considerable. Msx1 may be one of the factors involved in the initial phase of cell division. Other genes expressed in the lateral dermomyotome are also potentially involved in migration/proliferation/repression of differentiation of the muscle progenitor cells. These include Pax3, probably acting via the c-met receptor, which is clearly essential. Lbx1, homologue of the Drosophila ladybird homeobox gene, shows expression in the lateral margin of somites at the level of the forelimb bud in the mouse, and at later stages, in the dorsal and ventral pre-muscle masses of the forelimb and hindlimb (Jagla et al., 1995). In Pax3-deficient (splotch) mice, Lbx1 transcripts are not detectable in somites (Mennerich et al., 1998). In the chick, it has been shown recently to mark cells in the lateral lip of dermomyotomes from occipital, cervical, forelimb and hindlimb, but not interlimb, somites, and in cells migrating from these (Dietrich et al., 1998). This expression pattern therefore differs from that of Msx1, which is detectable only in forelimb and, more weakly, interlimb somites on the axis.

Another homeobox gene, Mox2, is also expressed in limb muscle progenitor cells and mutation of this gene gives a partial limb muscle phenotype (Mankoo et al., 1999). Although migration and proliferation of muscle progenitor cells are most marked in the limb buds, these phenomena also apply to the interlimb somites where the onset of myogenesis is initially delayed in the lateral somitic bud (see Tajbakhsh and Buckingham, 1999). The relative roles of these different proteins, and that of the bHLH protein Sim1 also expressed in the lateral dermomyotome, remains to be determined.

Mox2, the sister gene to Mox1, has also been reported to inhibit myogenesis (Takahashi et al., 1996). Transcripts of Mox2 were not detectable in somites by in situ hybridisation (data not shown), but it should be kept in mind that Mox1 transcripts were not detected either in the dermomyotome prior to introduction of an nlacZ reporter gene. Targeting of Mox2 with a reporter gene is underway in our laboratory; and this should, as in the case of Mox1, facilitate detection of its expression. The Mox1 null mouse (Satokata and Maas, 1994;...
Houzelstein et al., 1997) does not have a limb phenotype. Double mutant analyses should clarify the potential role of the Msx genes, and also the involvement of the other potential regulatory factors present in the lateral dermomyotome and the muscle progenitor cells which migrate from it.

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