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The distal limb environment regulates MyoD accumulation and muscle differentiation in mouse-chick chimæric limbs

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SUMMARY

Differentiation of muscle and cartilage within developing vertebrate limbs occurs in a proximodistal progression. To investigate the cues responsible for regulating muscle pattern, mouse myoblasts were implanted into early chick wings prior to endogenous chick muscle differentiation. Fetal myogenic cells originating from transgenic mice carrying a *lacZ* reporter were readily detected in vivo after implantation and their state of differentiation determined with species-specific antibodies to MyoD and myosin heavy chain. When mouse myogenic cells are implanted at the growing tip of early stage 21 limbs MyoD expression is suppressed and little differentiation of the mouse cells is detected initially. At later stages ectopically implanted mouse cells come to lie within muscle masses, re-express MyoD and differentiate in parallel with differentiating chick myoblasts. However, if mouse cells are implanted either proximally at stage 21 or into the limb tip at stage 24, situations in which mouse cells encounter endogenous differentiating chick myoblasts earlier, MyoD suppression is not detected and a higher proportion of mouse cells differentiate. Mouse cells that remain distal to endogenous differentiating myogenic cells are more likely to remain undifferentiated than myoblasts that lie within differentiated chick muscle. Undifferentiated distal mouse cells are still capable of differentiating if explanted in vitro, suggesting that myoblast differentiation is inhibited in vivo. In vitro, MyoD is suppressed in primary mouse myoblasts by the addition of FGF2 and FGF4 to the culture media. Taken together, our data suggest that the inhibition of myogenic differentiation in the distal limb involves MyoD suppression in myoblasts, possibly through an FGF-like activity.

Key words: MyoD, muscle differentiation, chimæric limbs, chick, mouse

INTRODUCTION

The early chick limb bud consists of at least two distinct populations of mesodermal precursor cells, encased in an ectodermal jacket. Myogenic cells originate by migration from the somites, whereas cartilage and connective tissues derive from lateral plate mesoderm. Premyogenic cells, distinguished by expression of the transcription factor Pax3 (Bober et al., 1994), migrate into the wing-forming region between Hamburger and Hamilton stages 13-18 (Jacob et al., 1978; Chevallier et al., 1978). The myogenic cell population of early limb buds does not at this time express high levels of members of the MyoD family of myogenic basic helix-loop-helix genes. Yet, when early limb bud cells are grown as an explant or as dissociated cells, MyoD and myosin heavy chain (MyHC) are expressed (Sassoon et al., 1989; Wang and Sassoon, 1995; Lin-Jones and Hauschka, 1996). The expression of either MyoD or Myf-5 is required to commit myoblasts to myogenesis (Rudnicki et al., 1993). So although in the limb, migrating somitic cells will form muscle, they appear to be regulated by signals from the limb environment to fulfil that destiny.

Initially, premyogenic cells are dispersed throughout the mesenchyme of the limb bud (Williams and Ordahl, 1994). As the limb develops, potentially myogenic cells become excluded from the tip (Newman et al., 1981) and localised in the dorsal and ventral muscle masses at the periphery of the limb (Schramm and Solursh, 1990). Differentiation of myoblasts, which involves the cessation of division, expression of muscle contractile proteins and fusion to form syncitial muscle fibres, occurs in a proximal-to-distal sequence beginning at stage 25 (Hilfer et al., 1973; Sweeney et al., 1989). The signals that control the localisation of myoblasts to the muscle masses and the timing of myogenic differentiation are at present unknown.

The terminal differentiation of dividing myoblasts into postmitotic multinucleated myotubes can be regulated by many extracellular signalling molecules: PDGF, FGFs, TGFβ, thyroid and sex hormones, and components of fetal bovine serum (FBS) can all inhibit or enhance myoblast differentiation in vitro (reviewed in Florini et al., 1991; Gardahaut et al., 1992; Yoshida et al., 1996). However, in vivo it is unclear what factors are involved in the regulation of myoblast differentiation. Recently, it has been shown that terminal differentiation of C2C12 myoblast cell line can be regulated at two

distinct levels. FGF2 causes repression of differentiation accompanied by MyoD down-regulation (Vaidya et al., 1989), whereas lysophosphatidic acid can inhibit differentiation without loss of MyoD (Yoshida et al., 1996).

To investigate the regulation of muscle differentiation, we have implanted primary cultures of mouse myoblasts into the developing chick wing and determined the fate of the implanted cells. Mouse myogenic cells are inhibited from differentiating in the distal limb environment, but differentiate when located in the normal differentiating chick musculature. Furthermore, we find that the expression of MyoD by implanted myoblasts is controlled by positional signals within the limb. Our findings indicate that the chick limb environment contains signals that regulate the pattern of muscle differentiation, both in regions that do, and those that do not, normally contain myogenic cells.

MATERIALS AND METHODS

Animals

Mice expressing the ROSAβgeo26 transgene on a CBA/129 background (Friedrich and Soriano, 1991) were kindly provided by Martin Raff and bred at King's College London. Chick embryos (Rhode Island Red) were incubated in a humidifying incubator at 37°C.

Myoblast culture

Late fetal (E17-18, E1 being the day of plug) mouse forelimb and hindlimb myoblasts were prepared by the method of Rando and Blau (1994). Briefly, muscle tissue was dissociated and filtered, and cells were plated in growth medium consisting of Ham's F10, 20% FBS, 2.5 ng/ml FGF2 (Boehringer Mannheim) on Nunc tissue culture dishes coated with 0.01% type 1 collagen (Sigma). Cells were passaged after 2 days and every 2 days thereafter and enriched for myoblasts by preplating, i.e. allowing the fibroblast population to adhere to uncoated dishes for 40 minutes before unattached cells were transferred to a fresh collagen-coated dish.

To obtain myoblast clones, primary bulk cultures were split after 2-6 days of culture and plated at clonal density (1 cell per 80 mm^2). Individual clones were isolated using cloning rings when the colonies reached 50-200 cells. Small clones were expanded on 24-well plates for 4-6 days, whereas larger colonies were directly implanted into chick limb.

All bulk populations of mouse cells were over 85% myogenic based on the frequency of nuclei within cells containing sarcomeric MyHC detected immunohistochemically with antibody A4.1025 (Pavlath et al., 1989) on cells that had differentiated in DMEM, 2% horse serum for 3 days. To assess the effects of growth factors, primary cultures after treatment were fixed in methanol at –20°C for 5 minutes, blocked, incubated with rabbit anti-MyoD (a gift from John Harris) and anti-desmin (Sigma) antibodies overnight at 4ºC and detected with goat anti-rabbit IgG-FITC and goat anti-mouse IgG-Texas Red (Vector).

Implantation of mouse myoblasts into chick wing bud

To implant cells into chick limbs, bulk populations of mouse cells were trypsinized off dishes, washed in fresh growth medium and pelleted at 3000 ***g*** for 3 minutes in 1.5 ml Eppendorf tubes. Pellets were carefully removed into a dish containing MEM, 10% FBS and divided into small pieces containing approximately 300 cells. Clones were similarly trypsinized and pelleted in 0.4 ml microtubes (Kartell; 200 cells on average). Bulk or clone pellets were grafted under a loop of ectoderm made at the apex of the apical ectodermal ridge (AER) of stage 21 or 24 chick embryos (Hamburger and Hamilton, 1951). The loop held the cell pellet in place and the ectoderm rapidly healed. Other stage 21 limbs had mouse cells implanted under a flap of dorsal ectoderm cut in the centre 350 μm from the tip.

Analysis of wings

Limbs were dissected at various times postimplantation, frozen and cryosectioned (Robson et al., 1994). Initially sections were stained unfixed for β-galactosidase activity in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% Nonidet P40 in PBS overnight at 37ºC. Once the location of the blue mouse cells was identified, sections flanking those containing mouse cells were incubated with anti-MyHC antibodies F1.652 (Cho et al., 1994), HV11 or EB165 (Gardahaut et al., 1992; diluted 1:5000) followed by goat anti-mouse IgG (γ specific) FITC (Vector). Sections were mounted in 2.5% 1,4 diazobicyclo-(2,2,2)-octane polyvinyl alcohol to prevent fading. Several wings were double labelled with the anti-MyHC antibodies and anti-MyoD (Koishi et al., 1995) visualised by goat anti-mouse IgG (γ specific) Texas Red (Sera-labs) and goat anti-rabbit IgG FITC (Vector). The number of X-gal-reactive cells was counted under DIC optics with a 40× objective on a Zeiss Axiophot. For bulk myoblast implants, alternate sections were labelled with X-gal and F1.652 or X-gal and HV11. For clones of myoblasts, alternate sections were labelled with X-gal and HV11 or F1.652 alone. For analysis of MyoD, every third section was stained for X-gal alone, MyoD and F1.652, or MyoD and HV11. The generally weak X-gal labelling did not interfere with FITC detection of MyHC. Numbers of cells labelled with each reagent were summed across all sections and corrected for the proportion of sections labelled. To avoid counting the same cell twice in separate sections, we compared the location of mouse cells in serial sections. That our counting system is accurate is shown by the similarity in the numbers of cells present within the implanted pellets (analysed by pellet dispersion and counting in a haemocytometer) and the number of cells scored when sections of limbs were analysed at zero hours postimplantation (data not shown and Fig. 6G). However, our estimates of differentiated cell number are probably slight underestimates when cells are extensively fused, as sometimes occurs within muscle masses. Such systematic counting errors would only decrease the estimated size of the differences detected in our experiments. Thus, values for cell numbers represent estimates of the number of implanted cells labelled with each reagent after taking account of the proportion of sections labelled, and are comparable within an experiment.

Immunohistochemistry

Wings from staged embryos were measured using a graticule before fixation in 90% methanol, 10% DMSO overnight at 4°C. Endogenous peroxidases were blocked with methanol, 5% H_2O_2 for 5 minutes before rehydration in PBS for 30 minutes at room temperature. Non-specific antibody binding was blocked in PBS 0.05% Tween 20 with 2% HS and 0.025% BSA, for 30 minutes with gentle rocking. Embryos were placed in antibody A4.1025 at 1:10 in blocking solution overnight at 4ºC, washed and incubated in sheep anti-mouse IgG-conjugated horseradish peroxidase (1:300, Amersham) for over 6 hours. After washing in PBS, differentiated muscle was visualised in 0.5 mg/ml diaminobenzidine for 15-30 minutes. Some late-stage wings were stained for cartilage in 1% alcian blue for 30 minutes, washed in acid alcohol, dehydrated and cleared in methyl salicylate.

RESULTS

Timing and location of muscle differentiation

To investigate differentiation of myoblasts when implanted into the distal tip of the limb, it is necessary to know the

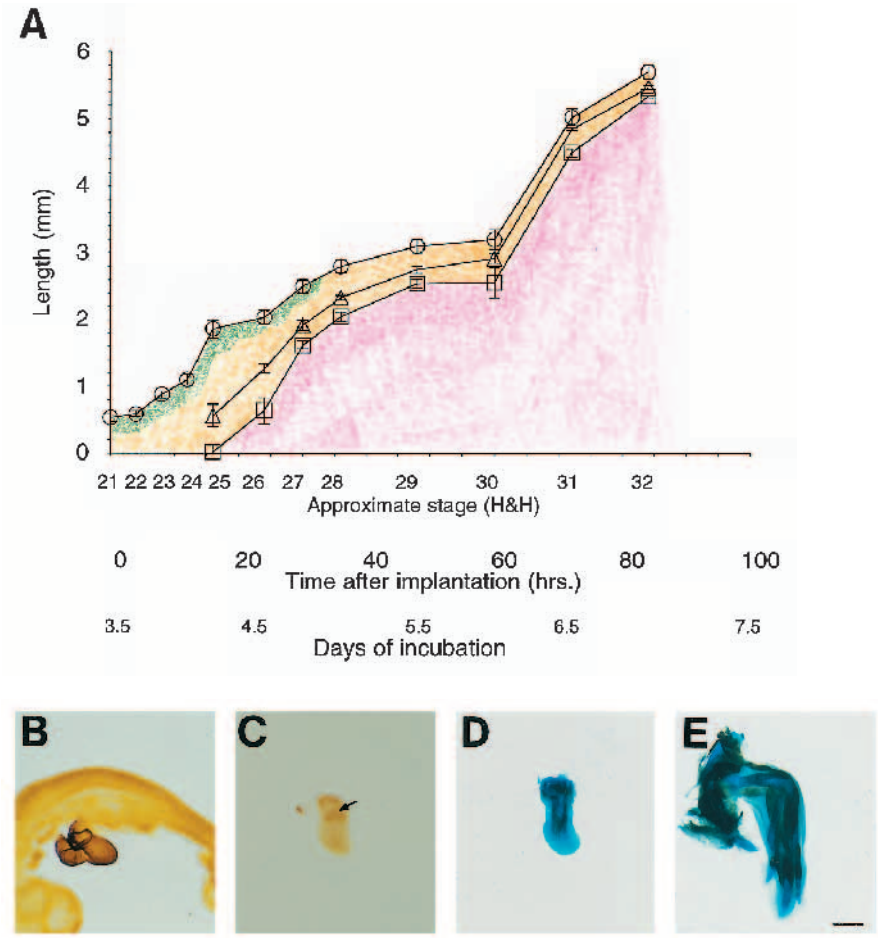

Fig. 1. Proximodistal pattern of muscle formation in the chick wing bud. The maximum extent of muscle (squares), cartilage (triangles) and whole wing bud (circles) was measured perpendicular to the body axis from the posterior border of the wing between stages 21 and 32 (A). Individual embryos vary slightly in their rate of development, so the ordinate represents the embryonic stage but is plotted on an approximately linear timescale. The progress zone (PZ, green), premuscle zone (PMZ, amber) and muscle zone (MZ, pink) are indicated. Dorsal views of representative wings are shown (B-E) labelled with monoclonal antibody A4.1025 in whole mount for all sarcomeric MyHCs with immunoperoxidase (brown) and alcian blue for cartilage (blue, D and E). Distal is at bottom and anterior at left. At stage 21 (B), there is no cartilage or muscle differentiation within the limb, although MyHC is readily detected in heart and somites. By stage 25 (C), the first myogenic cells begin to differentiate but are barely discernible within the humerus region of the wing (arrow). At stage 27 (D), the dorsal and ventral muscle masses are differentiated as far as the wrist and cartilage extends into the autopod. By stage 31 (E), splitting of the muscle masses is almost complete and there is a full skeleton. Bar, 500 μm.

sequence of events that lead to the final pattern of the limb musculature. We therefore determined the location and timing of definitive cartilage and muscle differentiation throughout limb development in relation to the growth of the whole limb (Fig. 1). As reported previously (Summerbell, 1976; Sweeney et al., 1989), there is a proximodistal sequence in the differentiation of both the cartilage and muscle. The growth rate of the wing is not constant: growth is initially rapid and steady over stages 21 to 27 when the pattern is laid down, slows during stages 28 to 30, before finally increasing rapidly after stage 30 as the limb elongates. Chondrocytes start differentiating before myoblasts, so cartilage is always found distal to MyHC-containing cells. The humerus and ulna become detectable almost simultaneously with alcian blue staining at stage 25 (Fig. 1A). The radius condenses later and is detected from stage 27. Digit cartilage is detectable around stage 27-28 (Fig. 1A,D) and a complete complement of skeletal elements is present by stage 31 (Fig. 1E). Myoblast differentiation follows a proximodistal sequence, starting at stage 25 in the muscle masses of the proximal stylopod. A wave of differentiation passes distally, with mononucleate undifferentiated cells remaining at the distal tip of each mass (Williams and Ordahl, 1994). The muscle masses of the zeugopod begin to differentiate around stage 26-27. Muscles of the autopod differentiate from stage 29 onwards. Thus, cartilage differentiation precedes myogenic differentiation at all limb levels.

Taken together with information from the literature, these data allow us to divide the wing bud into three zones depending on the differentiation state of mesenchymal cells within each zone. The PZ extends 250 μm in from the tip of the limb, contains rapidly dividing, undifferentiated mesenchyme that is capable of chondrogenesis, and is largely free of myogenic cells well before muscle differentiation is initiated (Newman, 1981; Paulsen et al., 1994). The premuscle zone (PMZ) extends from the end of the PZ to the distal-most differentiated muscle cells in the limb bud and contains both differentiated and undifferentiated chondrogenic cells and undifferentiated myogenic precursors. The most proximal region of the limb is the muscle zone (MZ), which contains differentiated myogenic cells within the muscle masses dorsally and ventrally, cartilage elements and intervening mesenchymal connective tissue. These three zones will be referred to below to identify the location of implanted cells. The PMZ increases to a large region just before stage 25, then rapidly decreases as the wave of muscle differentiation moves distally, so that by stage 28 it has reached a stable size (Fig. 1A). The delayed initiation and then rapid advance of the MZ down the limb during the crucial period when muscle is patterned and differentiation commences in the stylopod and zeugopod suggests that muscle differentiation is regulated by signals from the limb environment.

Differentiation of mouse myoblasts is inhibited in distal chick limb

To investigate whether extracellular signals can control muscle differentiation in developing limbs we implanted fetal-stage

myoblasts from E17-18 mice into early chick wing buds. E17-18 myoblasts would not normally be exposed to an early limb environment (see Cusella-De Angelis et al. (1994) for a discussion of the developmental heterogeneity of murine myoblasts). In order to detect the implanted cells, we employed myoblasts isolated from ROSAβgeo26 transgenic mice that expresses βgeo (a fusion protein of β-galactosidase and neomycin phosphotransferase) in many tissues, including muscle, due to a random insertion with a promoter trap construct (Friedrich and Soriano, 1991; Fig. 2A). Myoblasts isolated from ROSAβgeo26 mice also express β-galactosidase activity in vitro (Fig. 2B) and continue to express detectable β-galactosidase activity in many cells after implantation into chick limb buds (Fig. 3). We could determine the fate of implanted myoblasts because differentiated mouse cells can be distinguished from chick muscle fibres with a pair of species-specific monoclonal antibodies to embryonic MyHC. F1.652 detects only mouse embryonic MyHC that is expressed in all muscle fibres until after birth, but it does not react to any chick MyHC that we have tested (compare Fig. 2C,E). In contrast, HV11 recognises an embryonic MyHC isoform that is expressed in all early muscle fibres of the chick, but does not react with mouse MyHC (compare Fig. 2D,F). The use of mouse myoblasts thus allows us to investigate the differentiation of implanted cells and their relationship to the differentiation of endogenous cells.

We isolated bulk populations of ROSAβgeo26 mouse cells containing mostly myoblasts as shown by (a) the presence of 90% desmin-containing cells (data not shown) and (b) the rapid differentiation of greater than 85% of the cells into MyHC-containing myotubes when switched to differentiation medium containing low serum (Fig. 2G). Around 5% of the bulk cells were differentiated even under growth conditions. Small pellets of these bulk myoblast cultures were implanted under the AER into the PZ of stage 21 embryos (Fig. 2H). When limbs were recovered 24 hours later, serial sections frequently revealed that the majority of β-galactosidase-containing mouse cells were still located in a fairly compact mass in the PZ (Fig. 3A). Despite the presence of substantial numbers of β-galactosidase-containing cells, only very few differentiated mouse cells could be detected upon staining serial sections for embryonic mouse MyHC (Fig. 3B). This region of the limb contained no detectable chick embryonic MyHC (Fig. 3C). At this stage, the MZ is the most proximal region of the stylopod, approximately 1000 μm from the implanted cells (Figs 1, 3B). Substantial numbers of mouse cells survive, assessed by X-gal staining, for 65 hours, and mouse cells are still detectable after 6 days in vivo (Fig. 3J and data not shown). Few differentiated implanted cells are detected before 24 hours. During this period, the mouse cells increased in number, indicating that they proliferated in the chick limb, and the vast majority of implanted mouse cells remained undifferentiated in the PZ and PMZ regions. We are unable to determine whether the slight increase in cell number reflects little proliferation of implanted cells, or a near-steady state balance between proliferation and cell death. At 24 hours postimplantation, quantification of myoblast differentiation in five wings revealed that, in some cases, substantial numbers of implanted cells had begun to differentiate, whereas other limbs were essentially devoid of differentiated cells (Fig. 3B,J). We

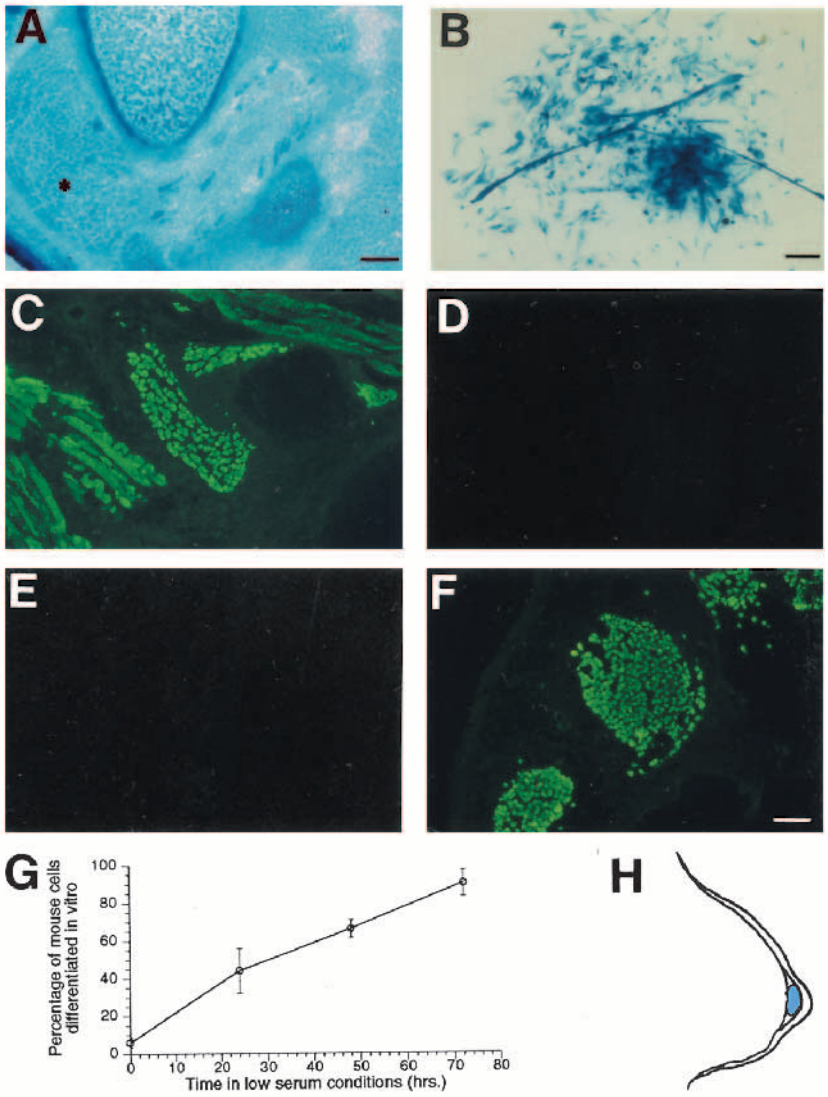

Fig. 2. Mouse and chick myogenic cells can be distinguished in vitro and in vivo. Muscle (asterisk) from E18 ROSAβgeo26 mice contains β-galactosidase activity after staining with X-gal on cryostat sections (A). Most mouse myoblasts grown in vitro also express β-galactosidase activity (B). The specificity of monoclonal antibodies to embryonic MyHCs was tested on unfixed adjacent cryosections of E18 mouse leg muscle (C,D) and stage 36 chick wing (E,F). F1.652 detects all mouse (C) but no chick (E) muscle fibres. HV11 (and EB165, data not shown) is unreactive with mouse (D) but detects chick muscle fibres (F). In vitro, ROSAβgeo26 mouse myoblasts differentiate in low serum conditions (G). A schematic diagram (H) indicates the location of a mouse myoblast pellet (blue) after implantation into chick limbs, under the AER at stage 21. Bar, 50 μm (A, C-F), or 20 μm (B).

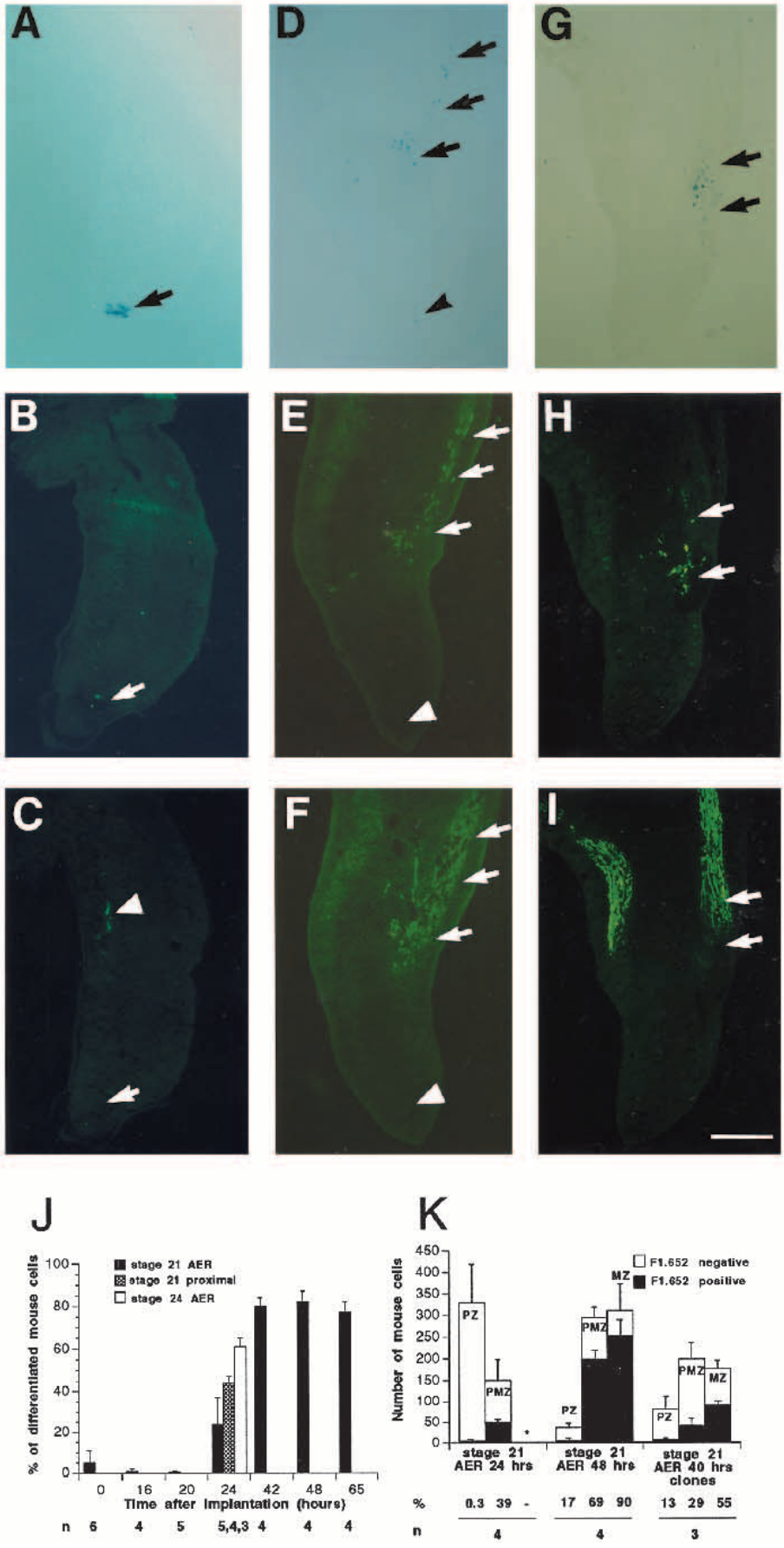

Fig. 3. Mouse myoblasts in chimæric limbs differentiate primarily in the muscle zone. Pellets containing a bulk population of mouse myogenic cells were implanted into the developing chick limb under the AER at stage 21 (A-F) or at stage 24 (G-I). Limbs were recovered 24 hours (A-C, G-I) or 48 hours (D-F) later, serially sectioned and reacted for either β-galactosidase activity with X-gal (A,D,G) to detect implanted cells, F1.652 (B,E,H) to detect mouse MyHC or HV11 (C,F,I) to detect chick MyHC. When cells were implanted at stage 21 and analysed 24 hours later, most cells remained as a coherent mass in the PZ (arrow, A) but some blue cells were detected in adjacent sections under the AER or short distances proximally in the PMZ (not shown). Most mouse myoblasts are undifferentiated (arrow, B). Chick muscle differentiation is commencing in these limbs in the humerus region of the wing (arrowhead, C) but is not occurring in the region of the implanted cells (arrow, C). After 48 hours, the mass of blue mouse cells has dispersed from the PZ, with many coming to lie in the dorsal muscle mass (arrows, D). Most implanted cells are differentiated in the MZ (arrows, E), but not in the distal tip of the limb (arrowhead, D-F). Chick muscle differentiation is well advanced in the region of mouse cell differentiation (arrows, F). When mouse cells are analysed 24 hours after implantation under the AER at stage 24, they lie as a mass of cells under the dorsal ectoderm in the MZ (arrows, G). Many mouse cells have differentiated into muscle (arrows, H), and these lie within the differentiating chick muscle mass (arrows, I). Sections of control or mock-implanted limbs showed no β-galactosidase activity or F1.652 reactivity. The PZ, PMZ and MZ were determined from analysis of the full series of sections. Bar, 250 μm. Quantification of total numbers of differentiated and undifferentiated mouse cells were performed on serial sections of several limbs (J,K). The proportion of detectable mouse cells expressing F1.652-reactive MyHC increases with time after implantation (J, black bars). At 24 hours after implantation under the AER of stage 21 limbs, mouse cells are less differentiated than after the same period implanted either more proximally (J, chequered bar) or under the AER at stage 24 (J, open bar). Error bars represent s.e.m.proportion in J and s.d. in K for n limbs. The differentiation of implanted cells depends on their location within the limb bud (K). Between 24 and 48 hours after implantation, most cells are left behind the outgrowing PZ (K). Yet those cells that remain in the PZ are less differentiated (%, indicated below the graph) than those in more proximal regions at each stage (K). Three separate clones of mouse myoblasts show the same behaviour as the bulk population 40 hours after implantation under the AER at stage 21. Asterisk: 24 hours after implantation there are no mouse cells proximal of the PMZ because differentiating chick myoblasts are restricted to the proximal stylopod.

suppose that limbs showing more differentiation had myoblasts implanted slightly more proximally, because analysis of such limbs revealed that mouse cells were present within the proximal PMZ or MZ but very few were located in the distal PMZ or PZ (Fig. 3K). Even including these cases, the overall frequency of differentiation across all limbs analysed (26%) was significantly below that observed when the same cells were differentiated in vitro for 24 hours (45%, Figs 2G and 3J). Thus, location appears to be the major factor influencing whether mouse cells differentiated within the chick limb.

To test whether the location regulated differentiation of implanted mouse cells, we introduced cells directly into the PMZ by implanting a pellet of myoblasts under a flap of dorsal ectoderm at stage 21. After 24 hours in vivo, mouse cells were mainly located in the MZ and showed markedly enhanced differentiation compared to myoblasts implanted under the AER. The proportion of MyHC-containing mouse cells (44%) was comparable to their behaviour when differentiated in vitro (Fig. 3J). A second test revealed similar results: mouse cells were grafted under the AER of the chick wing at stage 24 and analysed 24 hours later when most implanted cells were located well within the MZ in the developing zeugopod (Fig. 3G). Substantial numbers of mouse cells were differentiated in the MZ (Fig. 3H,I), and counts revealed that around 60% of detectable implanted cells had differentiated, a marked enhancement compared to differentiation in vitro (compare Figs 2G and 3J). Therefore, neither the length of time within the limb nor implantation under the AER per se is responsible for the lack of differentiation of myoblasts implanted under the AER at stage 21.

Failure by the mouse cells in the PZ and distal PMZ to differentiate could be due to a change in the myoblasts that rendered them incapable of differentiation. We ruled out this possibility by permitting wings to develop for 48 hours after mouse cells were implanted under the AER at stage 21. Mouse cells were dispersed along the limb, with most of the mouse cells within differentiated chick muscle in the MZ (Fig. 3D). Large numbers of mouse cells had differentiated in the MZ (Fig. 3E,F): around 80% in vivo compared to 70% after 48 hours differentiation in vitro (Figs 3J,K and 2G). However, the few mouse cells that remained in the PMZ distal to the differentiated host musculature were still largely undifferentiated, as were cells outside muscle regions in the MZ (Fig. 3E,K and data not shown). So the undifferentiated cells in the PMZ at 24 hours postimplantation are capable of giving rise to many differentiated cells after they become located in the MZ.

As the grafted cells proliferate within the limb it was possible that a minor population of implanted cells generated the differentiated cells detected in the MZ at 48 hours postimplantation and the reason for the lack of differentiated cells in the distal limb at 24 or 48 hours was that much of the β-galactosidase activity detected derived from dead or dying cells. To address this issue, we implanted pellets of myoblasts under the AER at stage 21, isolated the distal 200 μm of the limb buds 40 hours later and assayed for the presence of mouse myogenic cells in dissociated cell culture (Fig. 4A,B). Numerous viable β-galactosidase-containing cells were recovered and after differentiation in vitro for 3 days in medium containing few growth factors a high proportion (76%) of β-galactosidase-containing cells expressed mouse MyHC, just as did mouse cells recovered from the PMZ and MZ (Fig. 4C). Therefore,

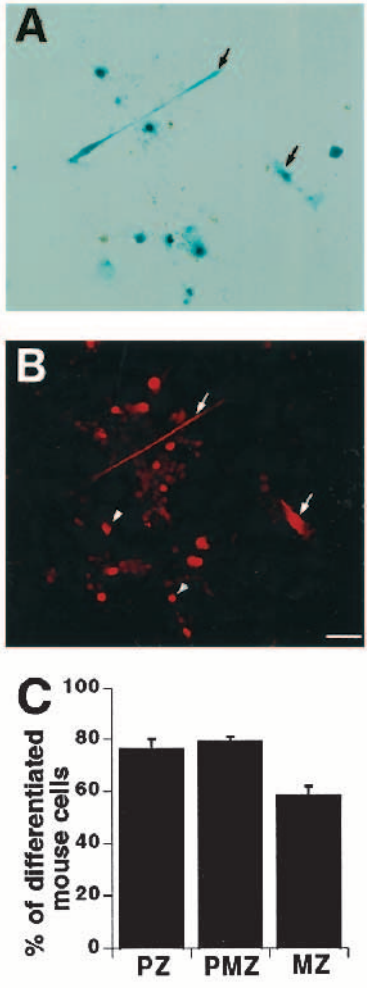

Fig. 4. Mouse cells in distal chick limb remain viable and myogenic. A mouse clone was implanted under the AER of a chick limb bud at stage 20-21. After 40 hours, the distal 250 μm (PZ), the next 250 μm (PMZ) and the remainder (MZ) was excised, dissociated and cultured for 3 days in low serum conditions. Most mouse cells in the cultures (revealed with X-gal, arrows in A) contained detectable mouse MyHC (revealed with F1.652, B). The intense β-galactosidase reaction in cultured cells prevents detection of mouse MyHC in a few mouse cells, however, some differentiated mouse cells do not have detectable X-gal reactivity (arrowheads). Therefore, differentiation was quantified in parallel dissociated cultures of mouse cells recovered from each region of the limb (C). The ratio of mouse MyHC-reactive and X-gal-reactive cells was similar in cells from all limb regions, although the absolute numbers of recovered cells varied: from four limbs the PZ, PMZ and MZ yielded an average of 63 ± 14, 129± 25 and 27 ± 13 β-galactosidase-reactive cells, respectively. Low recovery of cells from the MZ reflects the fact that only a sample of the numerous MZ cells were plated combined with our probable failure to recover the many differentiated mouse cells present in this region. The total yield of 381 ± 41 mouse cells per limb compares well with the numbers of undifferentiated mouse cells detected within limbs and may reflect some proliferation during the culture period. Error bars, s.e.m. proportion. Bar, 20 μm.

many of the undifferentiated mouse cells present in distal limb grafts are viable and capable of differentiation. Taken together with the lack of differentiation of mouse cells in the distal limb, these data suggest that signals in the distal PZ and PMZ prevent differentiation of myoblasts at stage 21.

Myoblast clones selectively differentiate along the proximodistal axis of the chick wing

When a heterogeneous population of mouse myoblasts is implanted into the developing chick wing, the differentiation of all myogenic cells present is suppressed in the distal limb environment. However, this behaviour could be a result of selective survival within the mixed population of implanted myogenic cells. Alternatively, a minor population of non-myogenic mouse cells within the pellet could be responsible for suppressing differentiation. To examine these possibilities,

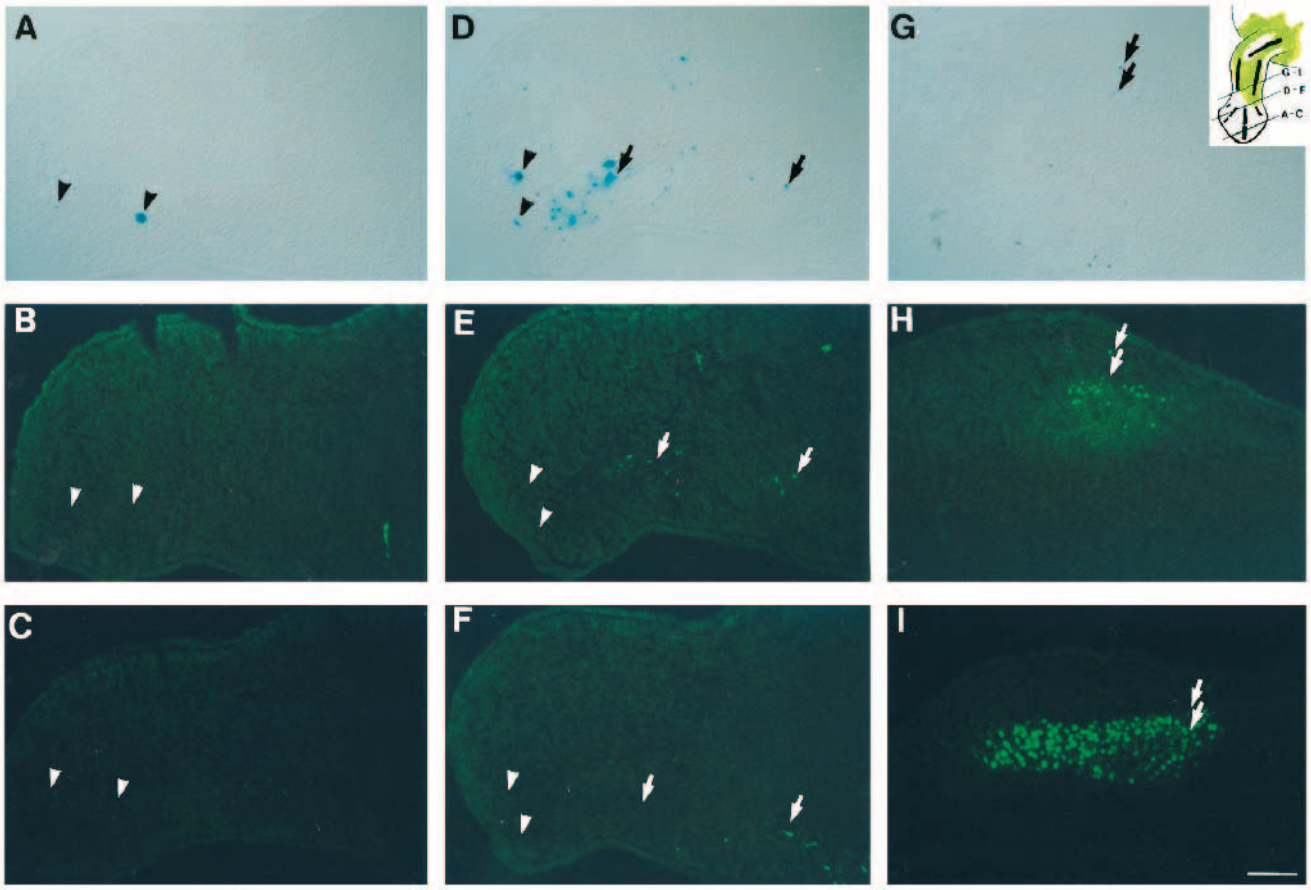

Fig. 5. A mouse myoblast clone responds to the chick environmental signals. A single clone of mouse myoblasts implanted under the AER at stage 21 and analysed 40 hours later gave rise to X-gal-reactive mouse cells (A,D,G) at three proximodistal levels within the limb: PZ (A-C), PMZ-MZ border (D-F) and MZ (G-I). Planes of section are indicated in the inset in G with the relationship of cartilage (black) and muscle (green) elements. Small numbers of mouse myoblasts close to the distal tip of the limb (A) are still undifferentiated (arrowheads), but the majority of cells are found proximally in muscle-forming regions (D,G). Serial adjacent sections were reacted with monoclonal antibody F1.652 to mouse MyHC (B,E,H), or HV11 to chick MyHC (C,F,I). At the PMZ-MZ border, there are both undifferentiated mouse cells (arrowheads, E) in regions lacking chick differentiation (F), and differentiated mouse myoblasts (arrows, E) where chick differentiation has commenced (F). In the MZ where chick muscles are differentiating (I), there are also differentiated mouse cells (H, arrows), elongated parallel to the chick fibres making the β-galactosidase less easy to detect (G). Bar, 100 μm.

we isolated clones of E17-18 mouse myogenic cells by limiting dilution so that we could investigate the behaviour of a homogeneous cell population. To minimise the chance of changes in the cloned myoblasts, clones were reimplanted 14-19 days after isolation from the animal. Pellets containing 200 cells were implanted into the chick wing bud under the AER at stage 21, and followed for up to 65 hours. Myoblast clones show a similar spatially regulated differentiation pattern to the bulk myoblast populations (Fig. 5). Mouse cells are located in all three zones after 40 hours (Fig. 5A,D & G). Mouse cells in the PZ are rarely differentiated (Fig. 5B,C). A few mouse cells within the PMZ differentiated, but the majority of the cells do not (Fig. 5E,F). The number of cells with detectable β-galactosidase activity within the MZ was low (Fig. 5G), but when an adjacent section was stained with F1.652 many fibres within the dorsal muscle mass contained mouse MyHC (Fig. 5H,I). Similar results were obtained for several other clones examined at 24, 40 and 48 hours postgrafting (Fig. 3K and data not shown). As there is considerable fusion of mouse myoblasts with chick fibres, dilution of the β-galactosidase in chimæric fibre cytoplasm may explain why we detect more fibres containing mouse MyHC, which will remain localised near to the nuclei that encode it (Pavlath et al., 1989). We conclude that the randomly selected clones of mouse myoblasts that we have tested behave similarly to bulk populations of myogenic cells: they differentiate poorly in the distal limb but well in muscle-forming regions.

MyoD expression is inhibited in myoblasts when implanted into early limbs distally

MyoD is a member of a family of muscle transcription factors required by myoblasts for their commitment to myogenesis (Rudnicki et al., 1993). MyoD is expressed at high levels in myogenic cells prior to differentiation (Sassoon et al., 1989). To investigate the role of the chick limb environment in the inhibition of differentiation and possible changes in the levels of MyoD protein in implanted mouse myogenic cells, we used an antiserum that detects mouse, but not chick, MyoD protein (Fig. 6). In vitro around 39% of our bulk-cultured myoblast population expressed MyoD. A similar proportion of the

pelleted cells expressed detectable MyoD and this expression was maintained for at least 5 hours after implantation under the AER of stage 21 chick wing buds (Fig. 6G). However, by 24 hours after implantation the proportion of MyoD-reactive mouse nuclei had fallen to only 12% of the total detectable mouse cells (Fig. 6A-C,G). Many of the remaining MyoD-positive nuclei were within the small proportion of F1.652-reactive differentiated cells, suggesting that many of the residual MyoD-positive nuclei were in cells that were differentiated prior to implantation into the PZ. By 48 hours after implantation, the suppression of MyoD was no longer detectable (Fig. 6D-G), suggesting that the repression of MyoD expression in myoblasts is reversible when the myoblasts leave the distal limb environment and enter the MZ. To test this, we implanted myoblast pellets either into proximal limb regions or under the AER at stage 24, so that the mouse myoblasts are located within differentiating chick muscle after 24 hours in vivo. In neither case could suppression of MyoD expression be detected (Fig. 6G). Thus, there is a correlation between the regions of the wing bud in which MyoD is repressed and the regions in which myoblast differentiation is inhibited.

Mouse myogenic cells do not form ectopic muscles within

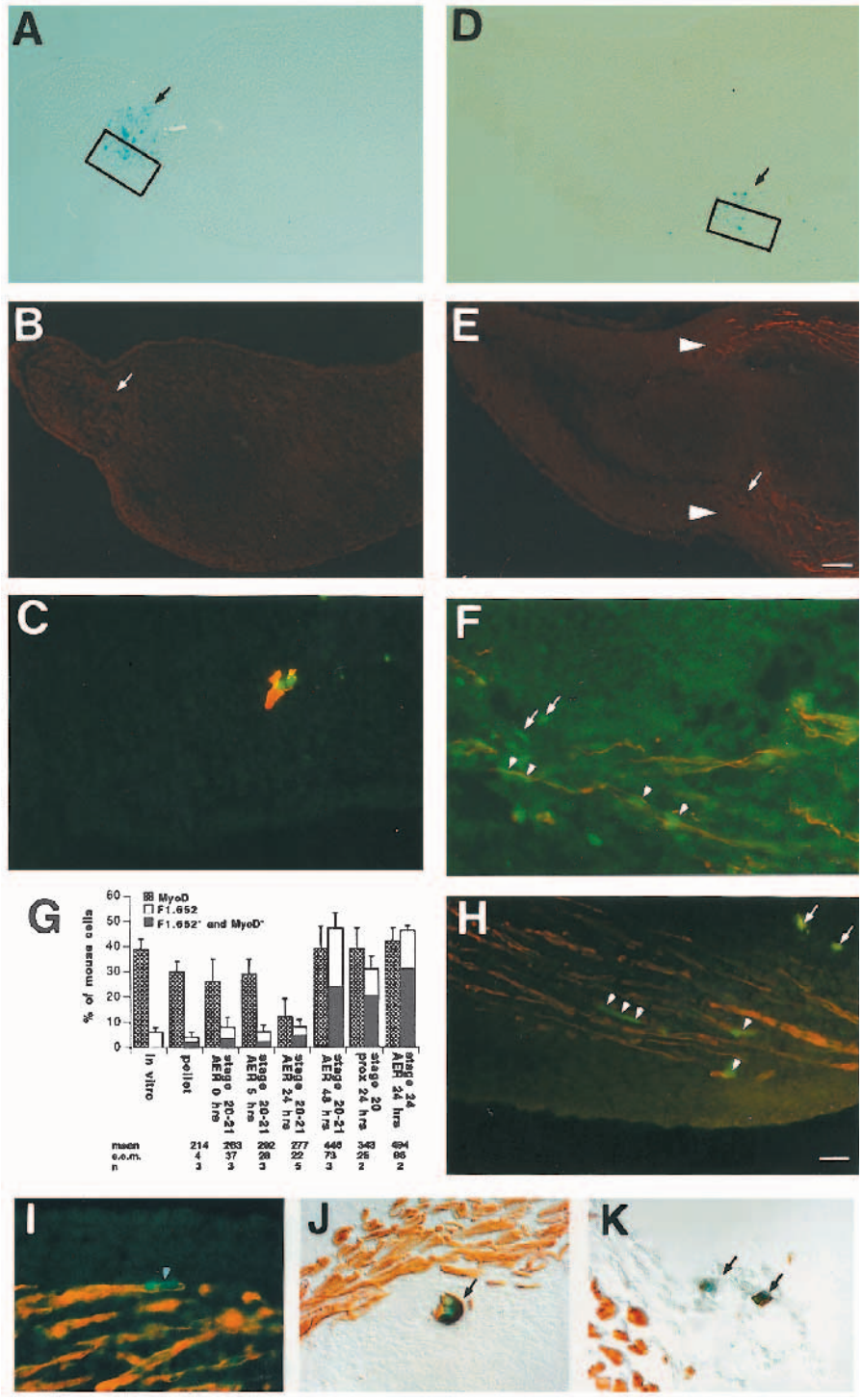

Fig. 6. The expression of MyoD protein is regulated by factors within the limb bud. Pellets of bulk cultured mouse myoblasts were implanted under the AER at stage 20-21. Limbs were sectioned and analysed 24 hours (A-C) or 48 hours (D-F) later. Alternate sections were reacted with X-gal to detect β-galactosidase-containing mouse cells (arrows, A,D), or HV11 to chick MyHC (B,E). Subsequently, anti-MyoD and F1.652 were used on sections containing mouse cells to reveal MyoD-containing nuclei (green) and differentiated mouse MyHC-containing cells (red, boxed areas in A, D shown at higher magnification in C and F, respectively). After 24 hours in vivo implanted cells in the distal limb are generally undifferentiated and MyoD-unreactive (A-C). However, after 48 hours, many mouse cells (D) are located within differentiating chick muscle masses (E) and now express both detectable MyoD and mouse MyHC (F). Note that mouse cells that are MyHC-reactive after 24 hours in vivo tend to express MyoD (C). However, after 48 hours in vivo, both differentiated MyHC-reactive (arrowheads, E) and undifferentiated (arrows, E) mouse cells contain MyoD. The frequency of mouse MyoD- and MyHC-containing cells was determined by analysis of all sections stained in a series of limbs into which mouse cells had been implanted at various locations and stages (G). Values beneath each column represent the mean total number of mouse cells detected, the s.e.m. and the number of limbs analysed (n). 2 days after implantation, limbs (H,I) reacted for mouse MyoD (green) and chick MyHC (HV11, red) contain chimæric muscle fibres with both mouse and chick nuclei (arrowheads). Some mouse MyoD reactive-nuclei are not associated with chick MyHC (arrows). Mouse cells were observed to fuse with chick myoblasts when sections were reacted for both β-galactosidase with X-gal and either HV11 (J) or EB165 (K) to detect chick MyHC (arrows). Chick MyHC was observed in 271 cells of 397 expressing mouse-derived gene products within muscle regions in six limbs analysed 48 hours after implantation at stage 21. Bar, 10 μm (C,F,H), 75 μm (A,B,D,E), 6 μm (I) and 12 μm (J,K).

the chick wing (data not shown), but in general become integrated into chick muscles. Moreover, close examination reveals that mouse myoblasts frequently fuse with chick myogenic cells forming chimæric fibres containing both mouse MyoD and chick MyHC in the same cell (Fig. 6H,I). In a series of limbs analysed 48 hours after implantation of mouse cells at stage 21, we observed that 68% of mouse-derived β-galactosidase and/or F1.652-reactive cells labelled with chick-specific MyHC antibodies (Fig. 6J,K). Within differentiating muscle masses, MyoD is expressed both in differentiated and undifferentiated mouse cells (Fig. 6F). However, not all mouse MyHC-positive cells express detectable mouse MyoD (Fig. 6G). Thus, myoblast differentiation and the expression of MyoD show complex and independent regulation within muscles.

MyoD expression is suppressed by FGFs in vitro

We do not know what the inhibitory signal(s) are that prevent myoblasts from expressing MyoD and differentiating prematurely in the distal limb bud. However, several growth factors are present in the AER and among such factors the FGFs have been shown to maintain outgrowth of limb buds from which the AER has been removed (Niswander et al., 1994). The FGFs are also known to inhibit myoblast differentiation by inducing proliferation (Seed and Hauschka, 1988). We tested two members of the FGF family for effects on the expression of MyoD in primary fetal mouse myoblasts in vitro. After 2 days in a defined basal medium without growth factors, myoblast proliferation ceased and most of the myogenic cells, identified in our cultures by the expression of desmin, expressed detectable nuclear MyoD (Fig. 7). In contrast, when cells were grown for 2 days either in our standard growth medium or in basal medium supplemented with high levels of either FGF2 or FGF4, we detected about threefold more myogenic cells and only around 30% expressed detectable MyoD. If myoblasts were cultured in defined medium with both FGF2 and FGF4, although the total number of myogenic cells was not further increased, MyoD was more strongly suppressed to a level comparable to that in distal implanted cells 24 hours after implantation under the AER at stage 21. Thus, FGF2 and FGF4, both of which are expressed in the AER of early limb buds, are capable of acting together to suppress MyoD expression in proliferating primary myoblasts.

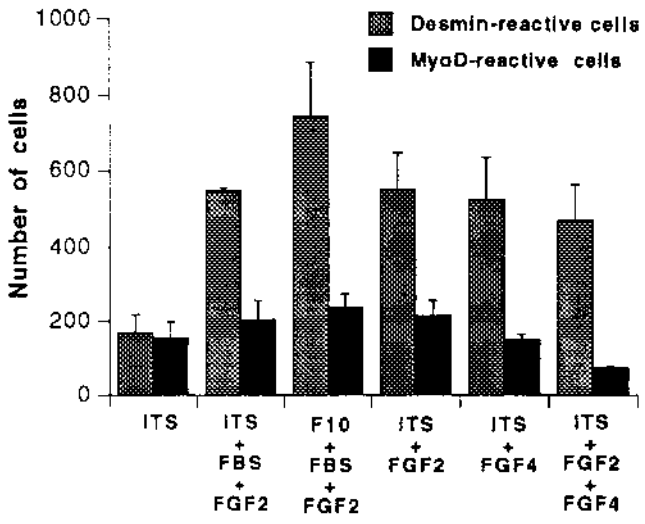

Fig. 7. Regulation of MyoD expression in cultured primary myoblasts by growth factors. Bulk populations of E17-18 mouse myoblasts were grown for 3 days in normal growth medium (F10 20% FBS, 2.5 ng/ml FGF2), then passaged onto 16 mm collagen-coated glass coverslips in the indicated media for a further 2 days. MyoD and desmin were detected by immunofluorescence. Serum-free ITS medium (DMEM supplemented with insulin, 10 μg/ml; transferrin, 5 μg/ml; sodium selenite, 10 nM, caused little myoblast proliferation and many cells expressed MyoD. A variety of other media (normal F10 growth medium; ITS 20% FBS 2.5 ng/ml FGF2; ITS 10 ng/ml FGF2; ITS 50 ng/ml FGF4; ITS 10 ng/ml FGF2 50 ng/ml FGF4) led to increased numbers of desmin-reactive cells but a lower proportion containing detectable MyoD.

DISCUSSION

We have shown that the limb environment can regulate the timing and location of myoblast differentiation during development. Currently, we are unable to determine exactly how this control relates to cell proliferation because we do not know the relationship between proliferation and cell death in the implanted population compared to endogenous cells. Nevertheless, differentiation is inhibited in implanted myoblasts before endogenous differentiation has commenced within the muscle masses. We suggest that endogenous myoblasts are similarly regulated by environmental influences within the early limb bud. Pax3-positive myogenic precursor cells enter the limb early and initially lie close to the apical ectoderm but later become distributed in dorsal and ventral muscle masses. These myogenic cells do not differentiate for several days (Sassoon et al., 1989; Williams and Ordahl, 1994). Yet if early limb buds are dissociated in cell culture, muscle differentiation is readily detectable (Seed and Hauschka, 1988; Wang and Sassoon, 1995). Signals that repress muscle differentiation persist even at later stages because, in limbs in which both endogenous and implanted cells are differentiating within the muscle masses, the implanted myoblasts rarely differentiate if they are located in ectopic sites outside the muscle masses (Fig. 3). These same cells will differentiate efficiently upon reisolation in vitro (Fig. 4). These data suggest that environmental inhibition of muscle differentiation has two functions. First, to prevent myoblast differentiation in early limb buds when myoblasts are close to the PZ and, later, when most myogenic cells are located within the muscle masses, as a safeguard against differentiation outside these masses.

We observed that the distal limb environment causes a suppression of MyoD protein accumulation in implanted cells. This raises the possibility that the suppression of MyoD, a transcription factor known to induce terminal muscle differentiation in cells in vitro (Buckingham, 1994; Molkentin and Olson, 1996), causes the lack of myoblast differentiation in ectopic sites in vivo. Strikingly, when implanted cells enter differentiating muscle masses and begin to differentiate themselves, MyoD is up-regulated again indicating that the correlation between the lack of differentiation and MyoD suppression is robust. However, MyoD knockout mice generate correctly patterned limb muscles (Rudnicki et al., 1993) and we observed that many undifferentiated mouse cells within muscle masses express MyoD (Fig. 6). So MyoD

protein is neither necessary nor sufficient to generate differentiated muscle fibres. Nevertheless, because it has been suggested that, in MyoD knockout mice, a distinct population of 'Myf-5-dependent' myoblasts may substitute for the loss of 'MyoD-dependent' myoblasts (Braun et al., 1994), it remains possible that MyoD expression is required for differentiation of normal fetal myoblasts. The uncertainty over the relationship of MyoD to myoblast differentiation (for a review see Buckingham, 1994) is compounded by the reversed order of expression of avian Myf-5 and CMD1, the chick MyoD homologue. CMD1 is up-regulated at stage 24 just prior to muscle differentiation at stage 25 (Williams and Ordahl, 1994). Resolution of these issues will await a more detailed analysis of the expression and role of MyoD in both species.

We do not know how MyoD levels are regulated (but see below), nor if MyoD is ever entirely lost from the implanted cells. Recent evidence suggests that early limb myogenic cells express low levels of Myf-5 and MRF4, members of the MyoD family, but not MyoD itself (Lin-Jones and Hauschka, 1996). So it is intriguing that our data suggest that the up-regulation of MyoD during limb development (Sassoon et al., 1989; Lin-Jones and Hauschka, 1996) is a reversible process. Msx1 protein can down-regulate MyoD expression in vitro (Woloshin et al., 1995). However, the lack of muscle phenotype in Msx1 knockout mice (Satokata and Maas, 1994) leaves open the questions of whether Msx proteins are expressed in myoblasts, or repress MyoD, in vivo. It will be interesting to determine whether the precursors of migratory myogenic cells that enter the limb field during stages 14-18 ever express MyoD or Msx proteins prior to migration.

We found that FGFs are capable of mimicking the actions of the distal limb environment on our myoblasts. As reported by Vaidya et al. (1989) and Yoshida et al. (1996) in studies on the C2C12 mouse myoblast cell line, our primary myoblasts are induced to proliferate and down-regulate MyoD upon exposure to FGF2. We show that FGF4 elicits a similar response and that, together, FGF2 and FGF4 cooperate to suppress MyoD still further. FGF2 protein and FGF4 and FGF8 transcripts have been shown to be present in the distal limb (Fallon et al., 1994; Niswander and Martin, 1992; Crossley and Martin, 1995) and it is suggested that FGF-like factors from the AER are necessary and sufficient to maintain limb outgrowth (Niswander et al., 1994; Fallon et al., 1994; Mahmood et al., 1995). However, we have not determined whether implanted myoblasts in the distal limb are induced to proliferate extensively. Implanted cell numbers do rise slightly in the first 24 hours postimplantation but the rate of cell death within this population is unknown. So the mechanism by which members of the FGF family of growth factors could inhibit differentiation and/or induce proliferation of distal myoblasts in vivo is unclear.

The FGF receptor 1 is expressed in myoblasts but is down-regulated upon differentiation, consistent with the ability of FGF2 and FGF4 to affect myoblast behaviour (Olwin and Rapraeger, 1992). Interestingly, if FGF receptor 1 is overexpressed in myoblasts in vivo differentiation is inhibited (Itoh et al., 1996), suggesting that the up-regulation of MyoD in myoblasts in muscle masses may be due to the loss of FGF responsiveness in this new environment rather than to the absence of FGFs per se. Indeed, several FGFs are expressed in the muscle masses of chick and mouse embryos (deLapayriere et al., 1993; I. Mason, personal communication). Several other families of signalling molecules, such as TGFβs, IGFs and BMPs, that are known to affect myoblast differentiation in vitro are also expressed in limbs (Cusella-De Angelis et al., 1994; Payton et al., 1996; Duprez et al., 1996). Taken together, these data suggest that a complex interplay between myoblasts and environmental factors, including FGFs, is likely to regulate myoblast differentiation in vivo.

Our studies have employed mouse myoblasts taken from late foetuses and cultured in FGF-containing medium, which probably correspond to the 'late', 'fetal' or 'secondary' myoblasts that are thought to form secondary muscle fibres and satellite cells (Seed and Hauschka, 1988; Cossu et al., 1983, 1988; Cho et al., 1993). Yet the early chick wing buds into which we implant our cells contain mostly 'early', 'embryonic' or 'primary' myoblasts that give rise to primary muscle fibres (Miller et al., 1985; Crow and Stockdale, 1986). The early and appropriate differentiation of both bulk cultures and clones of E17-18 myoblasts suggest a wide range of myoblasts are capable of responding to the patterning signals present in early limbs. Indeed, in separate experiments, we observed that *lacZ*-labelled C2C12 and C7 'satellite cell' myoblasts (Hughes and Blau, 1992) showed similar appropriate spatial regulation of differentiation (data not shown). Based upon the morphology of differentiated myotubes in culture, our E17-18 myoblasts probably correspond either to the 'late' myoblasts that generate secondary fibres or satellite myoblasts in vivo. These cells are not inhibited from early differentiation by factors located within early muscle masses when primary fibres are forming. Moreover, these E17-18 myoblasts fuse with endogenous chick primary fibres suggesting that there is no mechanism that restricts fusion of these myoblasts in vivo. In addition, on occasion the implanted myoblasts formed the majority of differentiated cells within a region of a muscle mass. In this situation, many fibres contained only mouse MyHC (data not shown), yet appeared otherwise indistinguishable from chick primary fibres. The appropriate differentiation of E17-18 myoblasts suggests that they are not irreversibly committed to the formation of secondary fibres or satellite cells.

For clarity, based on our experiments and the work of others, we suggest a simple testable model for limb muscle patterning, which extends the model of Song et al. (1992). Somitic myogenic cells migrate to the limb field early in limb development, where they remain proliferative under the influence of FGF-like factors from the apical ectoderm. They fail to differentiate due to the suppression of MyoD (which may or may not be linked to their continued proliferation). As the limb bud grows out, myogenic cells fall behind the PZ, leave the influence of the FGF-like factors, re-express MyoD and initiate differentiation. At this point the myoblasts are one step nearer to differentiation but other environmental factors now act within the muscle masses to regulate the rate and location of myoblast differentiation. Continued presence of FGF-like activity outside the muscle masses prevents ectopic differentiation in the event of chance misplacement of myogenic cells.

The chick-mouse chimæra technique that we have developed greatly extends the utility of the classic chick-quail system that has occasionally been used to examine muscle cell differentiation (Womble and Bonner, 1980; Antin et al., 1991; DiMario and Stockdale, 1995). Species-specific cDNAs and antibodies simplify the analysis of behaviour of donor tissue

in such experiments and our data suggest that donor mouse myoblasts respond appropriately to cues present within the chick wing bud. Thus, many of the signals involved in controlling limb muscle pattern appear to have been conserved across the evolutionary distance from mammals to birds.

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