In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle

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SUMMARY

Regeneration of adult skeletal muscle is an asynchronous process requiring the activation, proliferation and fusion of satellite cells, to form new muscle fibres. This study was designed to determine the pattern of expression in vivo of the two myogenic regulatory factors, *Myf5* and *MyoD* during this process. Cardiotoxin was used to induce regeneration in the gastrocnemius and soleus muscles of heterozygous *Myf5-nlacZ* mice, and the muscles were assayed for the presence of β-galactosidase (Myf5) and MyoD. Adult satellite cells identified by M-cadherin labelling, when activated, initially express either *MyoD* or *Myf5* or both myogenic factors. Subsequently all proliferating myoblasts express *MyoD* and part of the population is β-galactosidase (Myf5) positive. Furthermore, we demonstrate that activated satellite cells, which express either Myf5 or MyoD, do not accumulate selectively on fast or slow muscle fibres.

Key words: Satellite cell, Regeneration, *Myf5*, MyoD, M-cadherin

INTRODUCTION

The myogenic regulatory genes encode a family of transcription factors comprising four members, *MyoD*, *Myf5, Myogenin* and *MRF4*. The function of these myogenic factors in vivo has been investigated by determining their expression during development and by analysing mice with mutations in these genes. *Myf5* and *MyoD* are expressed in proliferating myoblasts prior to differentiation whereas *Myogenin* and *MRF4* are only expressed in terminally differentiating cells (reviewed by Megeney and Rudnicki, 1995; Yun and Wold, 1996). Mice lacking either MyoD or Myf5 show no severe muscle abnormalities but double mutants lacking both MyoD and Myf5 have no differentiated skeletal muscle, and furthermore the myoblast precursor cell population is absent (Rudnicki et al., 1993). This suggests that not only do MyoD and Myf5 act at an early stage of muscle cell determination but that they also have functionally overlapping roles. The *Myf5* gene is, however, activated prior to *MyoD* during development suggesting that these genes may also have discrete and possibly complementary functions during muscle formation (reviewed by Tajbakhsh and Cossu, 1997).

In the mouse, different myoblast populations with distinct characteristics are present at different times during development. They share a common embryological origin since all skeletal musculature in the body is derived from the somites, which segment from paraxial mesoderm. The first skeletal muscle, the myotome, appears at about embryonic day (E) 9.0 and gives rise to primary fibres from about E11. This is followed by a second generation of muscle fibres at E15-E16 (see Hauschka, 1994). Satellite cells can be identified from about E17 as a stable population, which is responsible for the subsequent growth of the muscle and provides a source of new muscle fibres during regeneration. In the adult these cells are quiescent but re-enter the cell cycle following injury to generate a population of proliferating myoblasts which subsequently fuse and either form new myofibres or are included in surviving injured fibres (reviewed by Bischoff, 1994).

Previous in vitro studies of myogenic regulatory factor expression in murine activated satellite cells have shown that *MyoD* is the first and most abundantly expressed myogenic regulatory gene (Smith et al., 1993). A more recent study by Cornelison and Wold (1997) analysed the expression of the myogenic regulatory factor genes in cultures of mouse satellite cells prepared from isolated myofibres. In this in vitro study they concluded that activated satellite cells begin to express either *MyoD* or *Myf5*; subsequently most cells transcribe both genes. There is currently little knowledge about the in vivo expression of the myogenic genes in adult satellite cells. The in vivo expression of *MyoD* and *Myogenin* during regeneration has been shown to resemble the expression in developing limbs (Füchtbauer and Westphal, 1992). The mRNA and protein for both of these myogenic factors were shown to be present in activated satellite cells a few hours after injury (Grounds et al., 1992; Füchtbauer and Westphal, 1992), in proliferating myoblasts (Yablonka-Reuveni and Rivera, 1994) and in newly formed myotubes (Füchtbauer and Westphal, 1992). Megeney

et al. 1996) have shown that mice lacking MyoD have reduced muscle mass and display marked deficits in satellite cell function, resulting in an increased population of precursor myoblasts and a decrease in the number of regenerated myotubes. These results imply that MyoD plays an important but not exclusive role in activation and/or differentiation of satellite cells. Less attention, however, has been focused on the expression of *Myf5* during regeneration, partly due to the absence of reliable antibodies. In the present study regeneration was induced in hind limb muscles of *Myf5-nlacZ* mice by injection of cardiotoxin. This provokes degeneration of muscle fibres while leaving the nerve and blood supply intact. Following the injury, satellite cells are activated, divide and form new muscle fibres, thus providing a model system for investigating myogenic regulatory factor gene expression. The purpose of this study was to determine the in vivo pattern of expression of *MyoD* and *Myf5* in satellite cells during the course of regeneration.

MATERIALS AND METHODS

Animals and induction of regeneration
Six-week-old heterozygous mutant mice (CD1 outbred) where the *nlacZ* gene was introduced into the *Myf5* locus by homologous recombination in ES cells were used (Tajbakhsh et al., 1996a). Mice were anaesthetised by an intraperitoneal injection of Sagatal (0.2 ml of 10% sagatal/gram body weight) prior to manipulation.

Regeneration was induced by an intramuscular injection of 400 μl of cardiotoxin, 10^{-5} M (Latoxan), into the hind limbs of these mice. This concentration and volume were chosen to ensure maximum degeneration of the myofibres.

Treated soleus and gastrocnemius muscles were examined at various times between 3 hours and 42 days after injury. Five animals were used for each time point.

Histological analysis
All experiments were performed in duplicate on 2-5 μm frozen cryostat sections. Sections from six-week-old non-regenerating muscles were initially immunostained with an anti-M-cadherin antibody, an anti-laminin antibody (ICN) and counterstained with Hoechst to determine the ratio of satellite cell nuclei to the total sublaminal nuclear population. Sections were prepared for β-galactosidase staining essentially as described (Tajbakhsh et al., 1996b). Immunohistochemistry was performed using the Vectastain Elite kit (Vector Laboratories) and diaminobenzidine (DAB) for all single labelling experiments, or DAB and alkaline phosphatase for double labelling (MyoD/M-cadherin and MyoD/slow MHC). Monoclonal anti-MyoD (Dako) was used at a dilution of 1/10, polyclonal anti-MyoD at 1/50, anti-slow myosin (Novacastra) at 1/5 and anti-M-cadherin at 1/60. The MyoD and M-cadherin polyclonal antibodies were kindly provided by J. Harris and A. Starzinski-Powitz, respectively.

After X-gal staining, sections were post-fixed with 70% methanol/30% acetone and reacted with anti-M-cadherin or anti-slow MHC antibodies. Sections were either photographed using an Olympus BH2 microscope or digitalized using the DXC-950P 3-CCD colour video camera.

For triple immunofluorescence staining, sections were incubated simultaneously with polyclonal β-galactosidase (Rockland) used at a dilution of 1/5000 and monoclonal MyoD primary antibodies overnight, washed and overlaid with the appropriate secondary antibodies conjugated with rhodamine or fluorescein. Slides were then washed, and mounted in Mowiol (Calbiochem-Novabiochem)

containing bis-benzimide (Hoechst No. 33258, Sigma) to visualise the nuclei (Cossu et al., 1996).

The percentage of cells expressing *MyoD* or *Myf5* and coexpressing *MyoD/Myf5* at each stage of regeneration was scored by counting a total of 1200 nuclei from two different experiments at each time point. Due to the fact that more satellite cells are present in slow than in fast fibres, sections were randomly chosen that contained various ratios of slow/fast fibres to avoid biased results.

Clonal analysis
Cells were isolated from back, body wall and limb muscles of *Myf5-nlacZ* heterozygous mice at postnatal day (P) 2, 7, 14 and 28, by collagenase/dispase treatment and cloned by serial dilution in RPMI 1640 medium (Gibco) supplemented with 20% horse serum and 5% chick embryo extract in collagen coated dishes as previously described (Cossu et al., 1987). In parallel experiments, control for myogenic cells was carried out using an antibody against desmin (D33, DAKO). All clones of round cells stained positively for desmin.

RESULTS

M-cadherin, Myf5 and MyoD expression in non-injured muscle
In six-week-old control muscles, sections were initially analysed for the expression of M-cadherin which is a cell adhesion molecule that has been reported to be expressed in quiescent, activated and proliferating satellite cells (Irintchev et al., 1994) and has therefore been used to distinguish them from myonuclei. Triple staining of these sections with M-cadherin, laminin and Hoechst showed that M-cadherin reactive nuclei in six week old control muscles accounted for 5.3% of the total sublaminal nuclear population (Fig. 1A). This percentage is very similar to the values previously obtained (Table 1) on the basis of morphological analysis using immunohistochemistry on muscle sections (Reimann et al., 1998); electron microscopy on muscle sections (Ontell et al., 1984) or single fibres (Rosenblatt et al., 1998) and immuno-electron microscopy on isolated fibres (Rosenblatt et al., 1999), for the frequency of satellite cells. The c-met receptor tyrosine kinase, another candidate molecular marker for quiescent satellite cells (Cornelison and Wold, 1997) was also analysed on intact and regenerating tissue sections. However, currently available c-met antibodies do not give reliable results on in vivo tissue sections (data not shown, and C. Birchmeier, personal communication). Therefore c-met was not used in this study as a marker for satellite cells.

In sections of six-week-old control muscles, no expression of *Myf5* or *MyoD* was detected in quiescent satellite cells (Fig. 1B,C).

Table 1. Summary of prior quantitative studies of quiescent satellite cell number

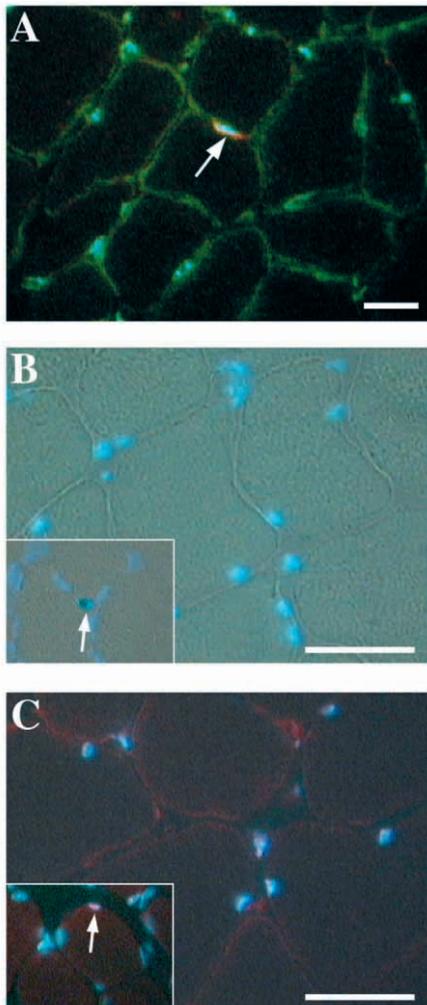| Percentage of satellite cell nuclei (%)	Age of mice	Reference
5-6 (EDL)	4-8 weeks	Ontell et al., 1984
6.2 (cultured soleus fibre)	10 weeks	Rosenblatt et al., 1998
3.6 (cultured EDL fibre)	10 weeks	Rosenblatt et al., 1998
4 (soleus)	4-6 months	Reimann et al., 1998
5.3 (gastrocnemius and soleus)	6 weeks	This study

Fig. 1. Cross section from a six-week-old non-regenerating hindlimb muscle obtained from a *Myf5-nlacZ* mouse. (A) Triple immunofluorescence staining visualising M-cadherin (Texas Red, red) Laminin (FITC, green) and the nuclei, (Hoechst). The arrow points to a sublaminal nucleus which is positive for M-cadherin. (B) Double X-gal/ Hoechst (nuclei, blue) staining to demonstrate no detection of Myf5 in six-week-old mice. The inset shows *that Myf5 expression*, in regenerating muscle, shows up as dark blue β-galactosidase labelling, quenching the Hoechst. (C) Double immunofluorescence staining to demonstrate the complete absence of MyoD (Texas Red, red) in the nuclei (Hoechst) of six-week-old mice. The inset shows a MyoD positive nucleus after 3 hours of regeneration (MyoD, Texas Red and nuclei, Hoechst). Bars, 5 μm.

MyoD is upregulated in activated satellite cells and in myonuclei

In order to investigate the expression of *MyoD* and *Myf5* in activated satellite cells, sections were costained with M-cadherin. As early as three hours after induction of regeneration with cardiotoxin, MyoD was detected in nuclei which were both negative (myonuclei) and positive (activated satellite cells) for M-cadherin (Fig. 2A). It should be noted that the M-cadherin negative nuclei stained less intensely for MyoD compared to M-cadherin positive satellite cells. Unlike the MyoD positive nuclei, all of the β-galactosidase (Myf5) expressing nuclei were M-cadherin positive (Fig. 2B),
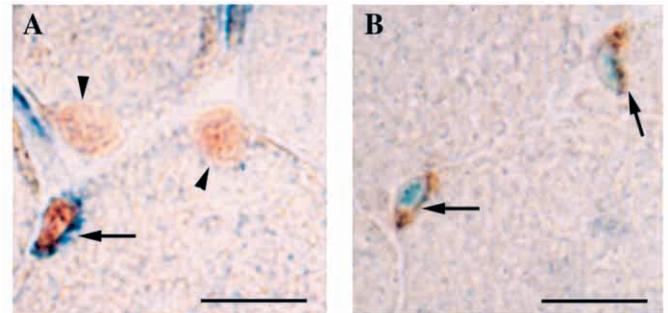

Fig. 2 Double immunohistochemical/X-gal staining of a cross section from the hindlimb of a *Myf5-nlacZ* mouse at 3 hours of regeneration. (A) The arrow points to a MyoD (brown, peroxidase) positive M-cadherin (blue, alkaline phosphatase) positive nucleus, showing that it is an activated satellite cell within the basal lamina. The arrowheads point to two M-cadherin negative MyoD positive myonuclei. (B) All β-galactosidase (Myf5) (blue) positive nuclei were also M-cadherin (brown, peroxidase) positive. Bars, 5 μm.

Table 2. Percentage of activated satellite cells (scored as M-cadherin positive) expressing *MyoD* and/or *Myf5 nlacZ* in damaged muscle 3 and 12 hours after regeneration

	% Activated satellite cells	
Factor expressed	3 hours	12 hours
MyoD	27.7	29.1
Myf5 nlacZ	20.0	17.5
MyoD and *Myf5* nlacZ	52.3	53.4

The number of nuclei scored was 1200.

indicating that at the onset of regeneration *Myf5* is upregulated only in satellite cells.

Expression pattern of Myf5 and MyoD in activated satellite cells

To examine the expression patterns of *Myf5* and *MyoD* during regeneration, sections were assayed by X-gal staining and either immunohistochemistry or immunofluorescence. The results showed that β-galactosidase (Myf5) and MyoD proteins had a similar pattern of expression over the time course examined. In quiescent satellite cells no expression of *Myf5* or *MyoD* was detected (see Fig. 1B,C) but upon activation both genes were expressed in satellite cells (see Fig. 2). In order to determine whether *Myf5* and *MyoD* were coexpressed in the same activated satellite cell nuclei we performed immunohistochemical staining on 2 μm serial sections using an anti-MyoD antibody on one section and an anti-M-cadherin antibody after X-gal (*Myf5*) staining on the adjacent section. The results showed the presence of activated satellite cells which expressed, exclusively *nlacZ* (*Myf5*) (Fig. 3A,B,C), exclusively *MyoD* (Fig. 3D,E,F) or coexpressed both (Fig. 3G,H,I). Double immunofluorescence analysis confirmed the presence of nuclei positive for both factors (Fig. 4A,B,C). The data in Table 2 shows that at 3 or 12 hours after activation, approximately half of the satellite cells co-expressed both *MyoD* and *nlacZ* (*Myf5*), about 30% expressed only *MyoD* and 20% only *nlacZ* (*Myf5*).

Expression pattern of Myf5 and MyoD in proliferating satellite cells and young myotubes

Proliferating adult myoblasts derived from activated satellite
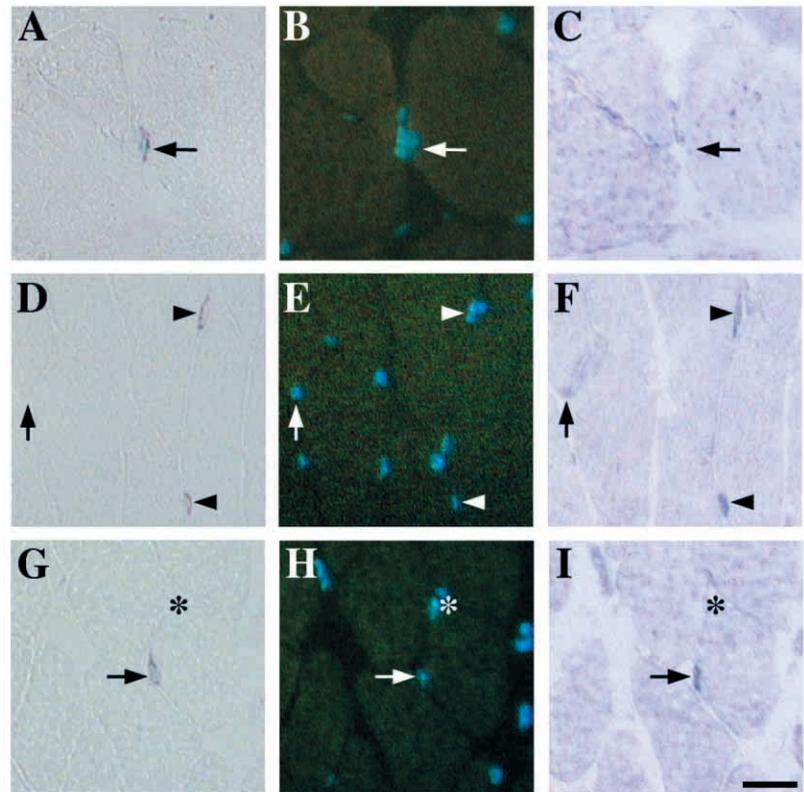
Fig. 3. Cross-section from the hindlimb of a *Myf5-nlacZ* mouse at 12 hours of regeneration. Immunohistochemical staining on 2 μm serial sections using an anti-M-cadherin antibody (brown, peroxidase) after β-galactosidase staining (blue) on one section (A,D,G) and an anti-MyoD antibody (purple, peroxidase) on the adjacent section (C,F,I). The section that was double labelled with X-gal and M-cadherin was counterstained with Hoechst to visualise the nuclei (B,E,H). The arrow in A points to a satellite cell which is positive for β-galactosidase (Myf5) and M-cadherin and negative for MyoD in C. In D, the arrowheads indicate nuclei which were positive for M-cadherin (brown, peroxidase) and negative for β-galactosidase. These nuclei which are indicated by arrowheads in F, were both positive for MyoD. The arrow in E demonstrates a nucleus which is negative for X-gal and M-cadherin (D) but positive for MyoD (F). The arrow in G and I shows a satellite cell which is positive for both MyoD and β-galactosidase (Myf5). The asterisk in H and I indicate a myonucleus (confirmed by the absence of M-cadherin, asterisk in G) which is negative for MyoD. Bar, 5 μm.

cells invade the sites of degeneration and start fusing to form young myotubes, characterised by centrally located nuclei. In these proliferating myoblasts and young myotubes only two populations of nuclei were observed, one expressing *MyoD* only and one coexpressing both *MyoD* and *Myf5* (Fig. 5A,B and Table 3). Remarkably, there were no detectable nuclei positive for only β-galactosidase (Myf5).

In vitro analysis of Myf5 and MyoD in cloned satellite cells

In order to investigate whether myogenic cells capable of forming colonies express preferentially *Myf5* or *MyoD* (or both), we cloned mononucleated cells from deep back and limb muscle of *Myf5-nlacZ* mice, at P2, P7, P21 and P60 days. After 5 days of culture, cells were fixed and stained for β-galactosidase activity and labelled with anti-MyoD antibody. Fig. 6 shows the distribution of individual clones from P7 limb muscle. Each bar of the figure represents the total number of cells per clone (all of which expressed *MyoD*), while the black bar represents the number of cells per clone expressing *nlacZ* (*Myf5*). It is clear from this analysis that, as seen at five days in vivo, the majority of myogenic clones (85%) contain β-

galactosidase (Myf5) positive cells albeit in variable proportion within the total population of the clone. However 15% of the clones did not express *nlacZ* (*Myf5*). All of the experimental samples gave similar results in spite of the age dependent variation in clone size (data not shown).

Expression of MyoD or Myf5 positive satellite cells on slow and fast fibres

It has been previously reported that MyoD mRNA is present at a low level in adult fast rather than slow fibres (Hughes et al., 1993). To determine whether there was selective expression of *MyoD* or *Myf5* in activated satellite cells on the edge of fast or slow fibres, sections were double labelled with an anti-slow myosin heavy chain antibody. Fig. 7 shows that activated satellite cells positive for either β-galactosidase (Myf5) (Fig. 7A), or the MyoD protein (Fig. 7B), were similarly distributed on both fast and slow muscle fibres (Fig. 7A,B); in a total of 500 different fibres, MyoD positive nuclei were scored in 51% slow and 49% fast fibres, while the β-galactosidase (Myf5) figures were 54% and 46%, respectively.

DISCUSSION

The objective of the present study was to determine the in vivo expression pattern of the two myogenic regulatory factor genes, *Myf5* and *MyoD,* during muscle regeneration. We first investigated the use of M-cadherin as a marker for the identification of satellite cells. We conclude that in our in vivo study, M-cadherin identifies all satellite cells since the figure we obtained based on this criterion is comparable to that reported on morphological grounds (see Table 1 and Rosenblatt et al., 1999). Therefore in agreement with previous studies, M-

Table 3. Percentage of proliferating myoblasts and young myotubes at 5 days of regeneration expressing *MyoD* and/or *Myf5 nlacZ*

Factor expressed	% Proliferating myoblasts	% Young myotubes
MyoD	61.5	23.5
Myf5 nlacZ	0	0
MyoD and *Myf5 nlacZ*	38.5	76.5

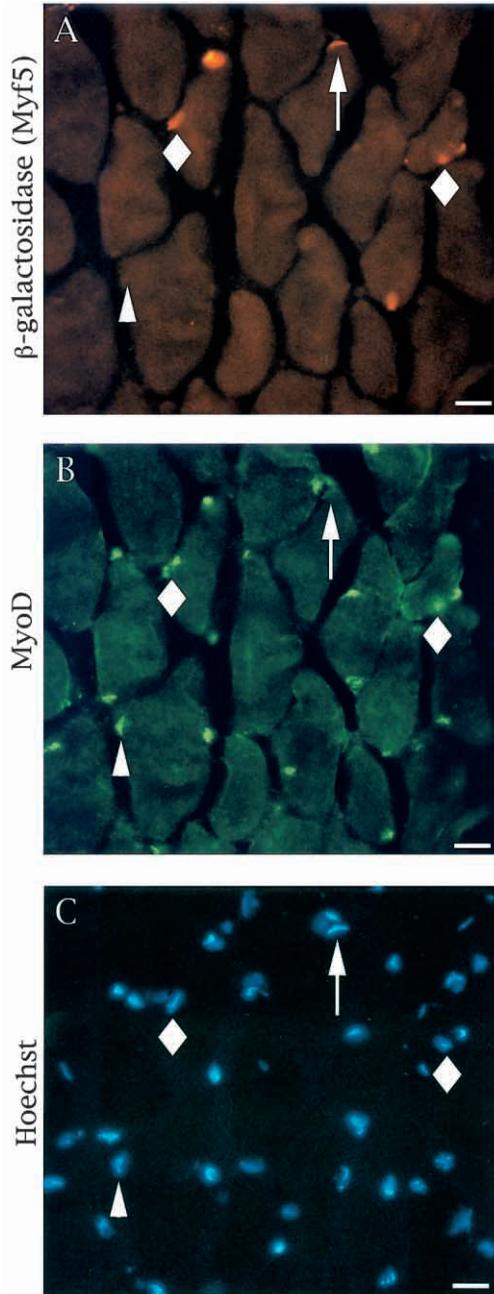The number of nuclei scored was 1200.

Fig. 4. Triple immunofluorescence staining visualising (A) β-galactosidase (Myf5) (TRITC, red), (B) MyoD (FITC, green) and (C) the nuclei, Hoechst). The arrow points to a β-galactosidase (Myf5) positive satellite cell that is negative for MyoD. The arrowhead marks an example of a MyoD positive nucleus that is negative for β-galactosidase (Myf5). The diamonds mark two nuclei, as examples, which are positive for both MyoD and β-galactosidase (Myf5). Bars, 5 μm.

Fig. 5. Double immunohistochemical/X-gal staining of a cross-section from the hindlimb of a *Myf5 nlacZ* mouse at 5 days of regeneration. The majority of young myotubes coexpress *MyoD* and Myf5 *nlacZ* (4 examples indicated by diamonds in A and B). Many MyoD only positive proliferating myoblasts (brown, peroxidase) (example indicated by arrowhead in A and arrow in B) were present at this time. An example of a MyoD only positive myotube (brown, peroxidase) (arrowhead) is shown in B. The arrow in A points to a MyoD/β-galactosidase (Myf5) positive myoblast. Note that there are no β-galactosidase (Myf5) only positive myoblasts or myotubes at this time. Identification of nuclei was verified by haematoxylin staining on parallel sections. Bars, 5 μm.

Fig. 6. Clonal analysis of satellite cells from 7-day post-natal mouse hind-limb muscle cultured for 5 days. Cells were isolated and cloned as described in Materials and Methods. Each bar represents the total number of cells per individual clone (all of which were positive for MyoD). The black bars represent the number of β-galactosidase (Myf5) positive cells per clone.

cadherin can be considered to be a reliable marker for both quiescent and activated satellite cells. The present investigation establishes for the first time that satellite cells in vivo, following activation can enter the myogenic program via two pathways: one initially expressing *Myf5* and the other *MyoD*. The population of activated satellite cells which coexpresses both genes may arise from a rapid transactivation of either
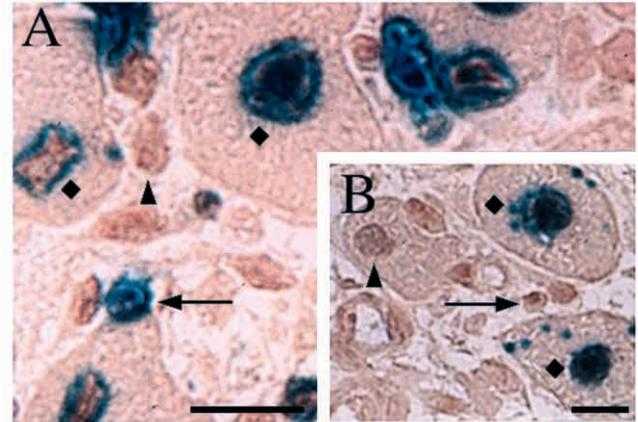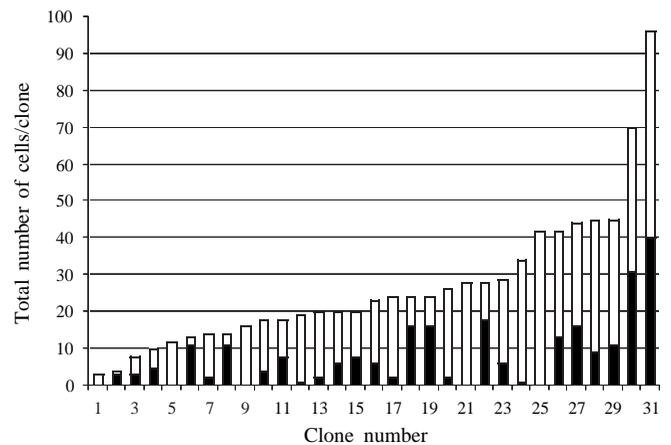
MyoD or *Myf5* in nuclei initially expressing either of these genes, as reported for embryonic muscle progenitor cells in explant cultures (see Cossu et al., 1996). However, we cannot exclude the possibility that some satellite cells may enter activation by expressing both *Myf5* and *MyoD*.

Later when proliferating satellite cells are present, nuclei which are β-galactosidase (Myf5) positive only are no longer detectable, suggesting that there is a rapid activation of *MyoD* in these nuclei. This observation is consistent with in vitro results with rat satellite cells reported by Allen et al. 1998) in

Fig. 7. Double immunohistochemical/X-gal staining of a cross section from the hindlimb of a *Myf5-nlacZ* mouse at 3 hours of regeneration. Myonuclei and satellite cells were randomly localised on fast and slow muscle fibres as revealed by anti-slow myosin antibody (brown, peroxidase) and β-galactosidase (Myf5) (blue, arrows) in A and anti-slow myosin antibody (purple, alkaline phosphatase) and MyoD (brown peroxidase, examples shown by arrows) in B. Identification of nuclei was verified by haematoxylin staining on parallel sections. Bars, 5 μm.
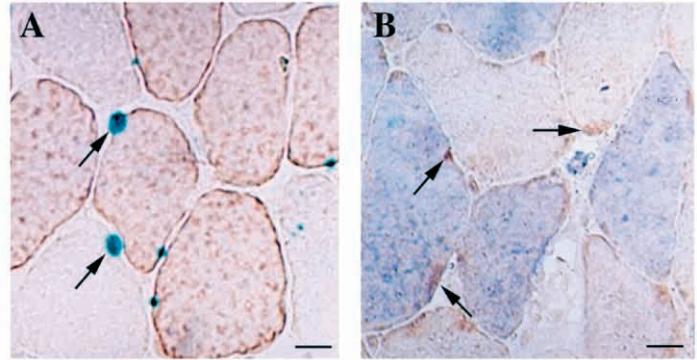
that about 90% of the proliferating cells were MyoD positive while only 30 to 40% were Myf5 positive. Later, when myotubes begin to form, both MyoD only and MyoD/β-galactosidase (Myf5) positive nuclei are present. This phenomenon, also seen during muscle formation, is again presumably a result of cross activation between genes of this family (see Tajbakhsh and Cossu, 1997). Later in more mature fibres β-galactosidase activity is no longer detectable. Smith et al. 1994) also found that *Myf5* expression could not be detected by RT-PCR in pure myotube cultures derived from rat satellite cells. This is analogous to the developmental situation (Ott et al., 1991). These data would suggest that a proportion of the satellite cells which participate in regeneration could proceed through the process of activation, proliferation and early steps of differentiation without ever activating *Myf5*. We have devised a model outlining the expression of *Myf5* and *MyoD* in satellite cells, from quiescence through to fusion, during the course of regeneration (Fig. 8).

The present study extends the work of Cornelison and Wold (1997) who used a multiplex single-cell RT-PCR assay to simultaneously monitor the expression of all four MRFs. They found that in activated single satellite cells from fibres isolated and cultured in vitro, either *Myf5* or *MyoD* is initially expressed. In addition they also observed that fibre-associated

satellite cells from either predominantly fast or slow muscles were indistinguishable from each other. An important difference between the two studies is that in contrast to their study we do not detect *Myf5* or *MyoD* expression in quiescent satellite cells in normal adult skeletal muscle (see Fig. 1), whereas Cornelison and Wold (1997) detect a low level of quiescent satellite cells positive for *Myf5* or *MyoD* transcripts. This may reflect differences in sensitivity of the techniques used. The possibility that a small subset of satellite cells were activated in vivo before myofibre explant should be disregarded because in 3.5- to 4-month-old mice all of the satellite cells are in the quiescent state and do not express *Myf5* or *MyoD*. It is possible that the fibre isolation procedure used by Cornelison and Wold (1997) may induce low level satellite cell activation.

In previous in vivo studies MyoD mRNA and protein were shown to be present in activated satellite cells, however little or no expression was reported in the newly formed myotubes of regenerating mouse skeletal muscle (Grounds et al., 1992; Koishi et al., 1995). In this study we clearly show that MyoD protein is present in the centrally located nuclei of young myotubes and in addition we demonstrate that β-galactosidase (Myf5) is also detected in the majority of these myotubes. The rapid down regulation of *MyoD* with no detectable expression in myotubes observed in the studies of Grounds et al. (1992)
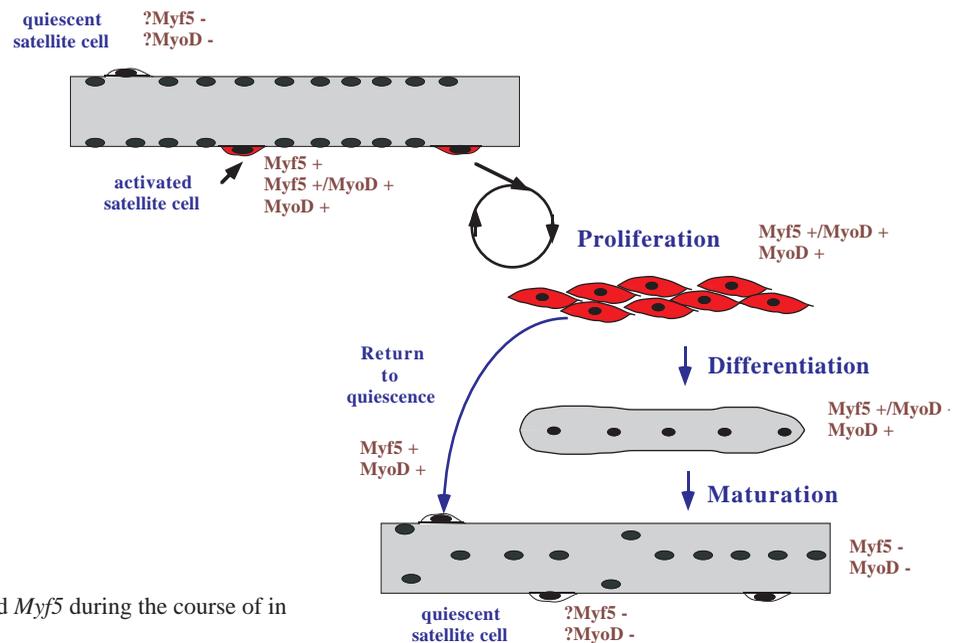

Fig. 8. Model for the expression of *MyoD* and *Myf5* during the course of in vivo regeneration in the mouse.

and Koishi et al. (1995) could be partly explained by the nature of the injury used to induce regeneration. Our observations suggest a major role for both MyoD and Myf5 in the early events of the myogenic program during regeneration.

The results obtained by Megeney et al. (1996) showed that muscle from *MyoD* null mice does regenerate, but not efficiently following trauma. They observed decreased rates of satellite cell activation and reduced myoblast proliferation. The reduced efficiency in regeneration can be partly explained by the fact that in these *MyoD* (−/−) mice the satellite cells that express only *MyoD* will probably not participate in regeneration. Assuming that the MyoD/Myf5 positive population can function in the absence of *MyoD*, this would represent the major class contributing to regeneration in *MyoD* null muscle. In our study, at three hours after injury 20% of the activated satellite cells express only *Myf5* and by 12 hours there is already a decrease in this population correlating with an increase in the MyoD only population at this time. We suggest that it is this Myf5 only population of activated satellite cells which may account for the increased propensity of stem cell renewal proposed for regeneration in *MyoD* null muscle. It has been shown in certain cell lines that MyoD is necessary for cell fusion (Miller, 1990). Therefore it is possible that myoblasts that would normally coexpress both factors would continue to proliferate because in the absence of MyoD the fusion into myotubes would be impaired. This would account for the persistence of mononuclear cells seen in *MyoD* null muscle.

Our results using an in vivo model for muscle regeneration confirm and extend recently reported results on single satellite cells from isolated fibres demonstrating, the initial existence of a Myf5 only sub population and pointing to differences in the kinetics of expression of the two 'upstream' myogenic factors. In conclusion, the study presented here establishes for the first time that adult satellite cells in vivo can be activated into the myogenic programme via two distinct pathways: one expressing *MyoD* only and the other *Myf5*. Furthermore, this pattern of expression is independent of fibre type.

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