Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes

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Received February 25, 1991 / Accepted July 20, 1991

Summary. The activation of mononuclear muscle precursor cells after crush injury to mouse tibialis anterior muscles was monitored in vivo by in situ hybridization with MyoD1 and myogenin probes. These genes are early markers of skeletal muscle differentiation and have been extensively studied in vitro. The role in vivo of these regulatory proteins during myogenesis of mature muscle has not been studied previously. MyoD1 and myogenin mRNA were present in occasional mononuclear cells of uninjured muscle. Increased MyoD1 and myogenin mRNA sequences in mononuclear cells were detected as early as 6 h after injury, peaked between 24 and 48 h, and thereafter declined to pre-injury levels at about 8 days. The mRNAs were detected in mononuclear cells throughout the muscle, with the majority of cells located some distance from the site of crush injury. The presence of MyoD1 and myogenin mRNA at 6 to 48 h indicates that transcription of these genes is occurring at the same time as replication of muscle precursor cells in vivo. At no time were significant levels of mRNA for these genes detected in myotubes. MyoD1 and myogenin provide precise markers for the very early identification and study of mononuclear skeletal muscle precursor cells in muscle regenerating in vivo.

Key words: Skeletal muscle – Myogenesis – Muscle regeneration – MyoD1 – Myogenin – In situ hybridisation – Mouse (Swiss SJL/J)

The recent discovery of a family of skeletal muscle specific genes involved in the early stages of determination and differentiation of muscle cells has provided a powerful approach for investigating important aspects of myogenesis. The most extensively studied of these proteins have been MyoD1 which was originally isolated from mouse myoblasts (Davis et al. 1987; reviewed Weintraub et al. 1991), and myogenin which was isolated from rat myoblasts (Wright et al. 1989).

Until recently, cardiac z-actin was considered to be the earliest muscle specific gene expressed by striated muscle precursor cells (mpc) during differentiation (Gunning et al. 1987; Sassoon et al. 1988; Lawrence et al. 1989), but it now appears that the MyoD1 and myogenin gene products represent earlier and more precise markers for skeletal mpc (Hopwood et al. 1989; Sassoon et al. 1989; Scales et al. 1990). The expression of MyoD1 and myogenin (or their homologous genes) has been studied in vivo during embryogenesis in developing muscles of mice (Sassoon et al. 1989; Buckingham et al. 1991) and other species, but to date there have been no descriptions of MyoD1 or myogenin gene expression during regeneration of mature muscle in vivo.

Muscle precursor cells of mature uninjured muscle are essentially quiescent and are arrested in G0 (or an equivalent state): this contrasts with the situation in embryonic muscle where populations of proliferating mpc are at various stages of differentiation (Sassoon et al. 1989). When mature skeletal muscle is injured or transplanted, the quiescent mononuclear mpc are activated, proliferate and fuse to form multinucleated young muscle cells (myotubes). It is widely considered that the mpc of mature muscle are derived exclusively from satellite cells which lie between the plasmalemma and external lamina of muscle fibres (Mauro 1961; Bischoff 1979; reviewed Mazanet and Franzini-Armstrong 1980); however, the possibility also exists that mpc might be recruited in vivo from cells other than satellite cells (reviewed Grounds 1990, 1991). To date, there are no markers which can readily identify the source of these undifferentiated mpc proliferating in vivo, or which can positively distinguish early mpc from other mononuclear cells present in the regenerating tissue (reviewed in Grounds 1991). It was therefore considered that the mRNA of the MyoD1 or myogenin genes might represent very early and specific markers for the identification of skeletal mpc in vivo in regenerating mature muscle. In this paper the transcription of these genes was examined in regener-
ating adult mouse muscle by use of in situ hybridisation to localise mRNA for MyoD1 and myogenin on tissue sections.

Materials and methods

Muscle injury

Thirty inbred Swiss SJL/J mice were used in experiments. Animals were housed in a clean, warm animal holding facility. Muscle trauma was induced in the tibialis anterior (TA) muscles of 24 adult (6–8 week old) male SJL/J mice by a single standard crush injury inflicted with artery forceps, as described by Grounds and McGeachie (1989). All surgical procedures were carried out under halothane anaesthesia. Animals were sacrificed at varying times from 0 to 12 days after injury (see Table 1) and muscle tissue examined by in situ hybridisation for transcription of MyoD1 and myogenin mRNA.

Probes

Riboprobe were prepared from a 1.8 kb full length cDNA MyoD1 clone (Davis et al. 1987) and a 1.5 kb cDNA myogenin clone (Wright et al. 1989). A further 0.7 kb myogenin probe was also prepared which lacked the c-myc homology domain present in both MyoD1 and myogenin. Antisense and sense riboprobes of MyoD1 and myogenin, and the antisense myogenin minus c-myc probe were labelled with 35S-UTP (Amersham) to a specific activity of 0.2–1 x 10⁶ cpm/μg of template DNA, then hydrolysed in carbonate buffer (0.4 M NaHCO₃, 0.6 M Na₂CO₃, pH 10.2) at 60°C to reduce the probe size to approximately 150 nucleotides.

In situ hybridisation

Muscle tissue for in situ hybridisation was fixed in 4% paraformaldehyde for about 4 h and processed for embedding in paraffin wax. Sections 5 μm thick were cut and mounted on gelatin/chrome alum-subbed slides (Gall and Pardue 1971). Following removal of wax and rehydration, sections were post-fixed in 4% paraformaldehyde for 5 min, then treated with the following solutions with washes of phosphate-buffered saline (pH 7.2) between each treatment: 0.2 N HCl 2 min; 0.01% Triton X-100 1.5 min; 50 μg/ml proteinase K (Boehringer-Mannheim, Penzberg, FRG) 15 min at 37°C; 0.09 M triethanolamine/0.25% acetic anhydride 10 min. Sections were then dehydrated in graded concentrations of ethanol. RNase control slides were treated with 100 μg/ml RNase A (Sigma, St. Louis, Mo., USA) in 300 mM NaCl, 10 mM TRIS at 37°C for 30 min prior to the acetylation step. Probe was applied to sections at 5 x 10⁶ cpm/μl in hybridisation cocktail (Sassoon et al. 1989) under sealed glass coverslips and hybridised at 50°C for 16 h. Following hybridisation, coverslips were removed and the slides washed in four changes of double-strength saline sodium citrate (SSC) (SSC: 0.15 M NaCl; 0.015 M trisodium citrate, pH 7.0), containing 0.1% Triton X-100, 1 mM EDTA and 5 μM diithiothreitol at 60°C for 15 min each, then in one-tenth-strength SSC with the same additions for a further 30 min at 60°C. To remove nonspecifically bound probe, slides were treated with 40 μg/ml RNase A (Sigma) and 10 U/ml T₁ RNase (Boehringer-Mannheim) for 40 min at 37°C, followed by six changes of double-strength SSC at 60°C for 10 min each. Dehydrated air-dried slides were coated in NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y., USA) exposed for 6–7 days at 4°C, developed in D19 (Kodak), fixed in Hypam (Ilford) and stained lightly with Giemsa.

Analysis of sections

The distribution of mRNA-positive cells throughout the regenerated muscle was analysed in zones as shown in Fig. 1 and Table 1.

Results

In situ hybridisation showed that MyoD1 and myogenin genes had a similar pattern of expression over the time course examined. Cells positive for MyoD1 or myogenin mRNA were infrequent in uninjured adult TA muscle (results not shown), and in TA muscle examined immediately after injury (Table 1, time zero). The small number of positive cells seen were mononuclear.

Increased amounts of MyoD1 and myogenin transcripts were apparent in mononuclear cells as early as 6 h after injury and the numbers of positive cells increased markedly by 24 h. The positive cells were initially concentrated in an area lying about 1.6–2.6 mm proximal to the site of injury. Fig. 1 shows MyoD1 expression in a longitudinal section 24 h after crush injury, and illustrates the zonal analysis used in the localisation of mPC showing positive probe hybridisation. Zones were defined at increasing distances proximal from the site of injury: they related only to distance and not to the histological appearance of the tissue. The numbers of cells in each zone giving positive signal were counted and are tabulated in Table 1. Representative areas for MyoD1 mRNA expression illustrated in Fig. 2A–D correspond to the grading system used in Table 1. Essentially the same distribution of myogenin-positive cells was seen for all time points. RNase treatment of consecutive sections (Fig. 2E) removed hybridisation signal. After 48 h, numbers of positive mononuclear cells declined and showed an apparent shift toward the site of crush injury (Table 1). After 8 days the number of positive cells was similar to pre-injury levels. Positive hybridisa-

Table 1. Distribution of cells expressing MyoD1 mRNA at various times after crush injury to the tibialis anterior muscle of mice. —, < 3 cells/zone; +, 3–15 cells/zone; ++, 16–30 cells/zone; ++++, > 60 cells/zone. Results were averaged from duplicate slides in the same experiment and three independent in situ hybridisation experiments.

<table>
<thead>
<tr>
<th>Time post injury (h)</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
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<td>288*</td>
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* Positive cells at these time points had a markedly weaker signal. Expression of myogenin followed a similar time-course pattern.
tion was observed only in mononuclear cells and was very weak or absent in newly formed myotubes (Fig. 3). A myogenin probe that lacked the c-myc homology domain gave very similar results (Fig. 2F) to probes for MyoD1 and myogenin which both contained the homology region.

**Discussion**

The use of in situ hybridisation shows the rapid induction and repression of MyoD1 and myogenin genes in mononuclear mpc in regenerating muscle. The low levels of mRNA for these genes in uninjured mature skeletal muscle are consistent with data of Wright et al. (1989) and Eftimie et al. (1991) who reported 15-fold less myogenin mRNA in adult compared with foetal mouse muscle.

The parallel transcription of the genes for MyoD1 and myogenin in regenerating muscle suggests co-expression of these genes within individual mpc, although the data cannot exclude the possibility that MyoD1 and myogenin genes are being expressed in different populations of mpc. If the genes are being co-expressed within the same cell this indicates either a simple co-activation, or the in vivo transcriptional cross-activation that has been observed between MyoD1 and myogenin in vitro (Braun et al. 1989; Thayer et al. 1989). Transcripts of both genes appear at the same time in developing muscles of mouse embryo limbs, although myogenin is expressed before MyoD1 in myotomal cells of the somites (Sassoon et al. 1989).

In regenerating mouse muscle our previous autoradiographic studies show that mpc in vivo do not start to synthesise DNA before 24 h after injury, and that replication peaks around 3 days and is essentially finished by 6 days (Grounds and McGeachie 1989). The present study shows a large number of cells transcribing MyoD1 and myogenin by 24 h, a period too short for significant mpc replication to have occurred. It would appear that these positive cells are probably initiating replication at the same time as they are expressing MyoD1 and myogenin genes. The data suggests that these genes are expressed by activated mpc, and that most of these mpc undergo at least one cell division before fusion, since myotubes are rarely seen before 60–72 h after injury. Studies in embryonic mice also indicate that the expression of MyoD1 occurs in replicating mpc of developing muscles (Sassoon et al. 1989; discussed in Choi et al. 1990). The early appearance of MyoD1 and myogenin mRNA thus does not seem to represent a commitment by mpc in vivo to imminent terminal differentiation. Tissue culture studies indicate a complex interaction between the myogenic regulatory gene products and other helix-loop-helix proteins; one possibility is that a negative regulator such as Id (Benezra et al. 1990) might form heterodimers with the MyoD1/myogenin gene products and thereby affect DNA binding during the replicative phase of mpc in vivo.

The rapid activation (by 6 h) of mpc distant from the site of direct injury suggests a response to a change in the environment of the muscle fibre as a result of injury which is rapidly translated throughout the length of the fibre (e.g., altered electrical properties, ion fluxes, release of proteases), before the influx of inflammatory cells and associated changes in growth factor availability occurs. It has been demonstrated that mpc can move both longitudinally down muscle fibres towards a site of injury (Schultz et al. 1985) and between regenerating fibres across connective tissue barriers (Phillips et al. 1990). In agreement with these observations, our results suggest that with time the population of activated mpc moves towards the site of injury (Table 1), and encounters changes in growth factor levels capable of stimulating replication (reviewed: Grounds 1991).
Fig. 2A–F. In situ hybridisation on muscle sections illustrates signal representative of the five grading levels — to ++++ shown in Table 1. A + 6 h post injury in zone 1 with antisense MyoD1 probe; B ++ 36 h post injury in zone 1 with antisense MyoD1 probe; C +++ 24 h post injury in zone 2 with antisense MyoD1 probe; D +++ 24 h post injury in zone 3 with antisense MyoD1 probe; E RNase control 24 h post injury in zone 3 with MyoD1 probe.

A to E are all at the same magnification. The grading system assesses the numbers of labelled cells, but there is also a variation in signal intensity which is evident in these photos. In A and B, arrows indicate the labelled mpc. F 48 h post injury with antisense myogenin probe, showing a zonal trend similar to that of MyoD1 expression.
In our study, strong positive hybridisation was observed only in mononuclear cells and not in newly formed multinucleated myotubes. This result suggests that in vivo the transcription of these genes has ceased or has been strongly down-regulated at the time of the fusion event. This contrasts with tissue culture studies where both MyoD1 and myogenin mRNA have been reported to persist in myotubes (Montarras et al. 1989; Thayer et al. 1989; Wright et al. 1989). Such differences between our in vivo and the in vitro data may be accounted for entirely by the marked differences between the environment and history of myogenic cells in regenerating muscle, and those of transfected and myogenic cell lines in tissue culture. In addition, an important aspect of fusion between myogenic cells in vivo is fusion of myoblasts or myotubes with damaged myofibres (Robertson et al. 1990) and the influence of innervation (Efthimie et al. 1991), a situation that does not arise in tissue culture.

The transcription of the MyoD1 and myogenin genes provides a powerful tool for identifying activated mPC in vivo. Assuming that all of the mPC do express MyoD1 and myogenin, one important implication is that the majority of mPC that contribute to regeneration may come from sites distant from the actual site of injury, which itself contributes relatively few cells. The movement of mPC is particularly relevant to potential myoblast transfer therapy which is an active area of research with clinical implications (Partridge et al. 1989; Hughes and Blau 1990; Partridge 1991). Present knowledge of mPC behaviour in vivo is only rudimentary and further work is required to define the source and nature of these cells. The observation of a rapid decrease in MyoD1 and myogenin mRNA upon myotube formation during regeneration, emphasises the importance of monitoring the myogenic process in vivo where the control of events would appear to be more tightly regulated than indicated by in vitro studies.

Acknowledgements. The excellent technical assistance of Marilyn Vague, and typing of the manuscript by Mia Seats within the University Department of Pathology is gratefully acknowledged. We thank Terry Partridge for early discussions in relation to this work. This research was supported by grants from the National Health and Medical Research Council of Australia (M.D.G., M.W.B.), and an Australian Postgraduate Research Award (K.L.G.).

References


Fig. 3. High-power view of MyoD1-positive cells in zone 1 at 4 days post injury. Strong hybridisation is present in some mononuclear cells but this signal is clearly very low or absent in newly formed myotubes (arrows)
Scales JB, Olson EN, Perry M (1990) Two distinct Xenopus genes with homology to MyoD1 are expressed before somite formation in early embryogenesis. Mol Cell Biol 10: 1516–1524

Note added in proof. In later experiments using digoxigenin-labelled riboprobes, some persistence of MyoD1 and myogenin mRNA in newly formed myotubes was noted.