Molecular cloning of up-regulated cytoskeletal genes from regenerating skeletal muscle: potential role of myocyte enhancer factor 2 proteins in the activation of muscle-regeneration-associated genes

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A subtractive hybridization and cloning strategy was used to identify genes that are up-regulated in regenerating compared with normal skeletal muscle. The gastrocnemius muscle of CD1 mice was injected with a myotoxic agent ($BaCl_2$). A cDNA library was constructed from the regenerating muscle, and was screened with subtracted probes enriched in genes up-regulated during regeneration. Cofilin and vimentin cDNA clones were isolated. Both cofilin and vimentin were demonstrated to be overexpressed in regenerating compared with non-regenerating muscle (17-fold and 19-fold induction respectively). Cofilin and vimentin mRNAs also exhibited an increased expression in C2C12 myoblasts and a decreased expression in differentiated myotubes. Analysis of the regeneration-induced vimentin enhancer/promoter region revealed a consensus binding site for the myocyte enhancer factor 2 (MEF2) transcription factors. Electrophoretic mobility-shift assays and *in vivo* reporter assays revealed that MEF2 DNA-binding activity and transcriptional activation are increased in regenerating skeletal muscle, indicating that they may play a role in the activation of muscle genes during regeneration. These data suggest that both cofilin (an actin-regulatory protein) and vimentin (an intermediate filament) may be key components of the cytoskeletal reorganization that mediates muscle cell development and adult skeletal-muscle repair.

INTRODUCTION

Skeletal-muscle regeneration after injury reflects the plasticity of muscle tissue and its ability to respond to physiological perturbations. Many reports have characterized skeletal muscle as a recapitulation of stages of embryonic muscle development [1]. However, the molecular events regulating muscle regeneration are just beginning to be elucidated [2]. Advances in the area of muscle development have provided a useful framework for the molecular analysis of muscle regeneration *in vivo*. A number of molecules have been identified in conjunction with the regenerative events occurring in the mature animal on the basis of their role during the embryonic muscle differentiation programme. The spectrum of factors identified includes extracellular matrix components, cell surface molecules, cytoskeletal proteins, local growth factors and myogenic transcription factors [1–7]. A complex interplay between these factors is probably responsible for orchestrating muscle repair *in vivo*. Also, there are important differences between the regenerative and developmental processes that necessitate a more direct study of muscle regeneration *in vivo* in order to identify other genes that are associated with the regenerative process [2].

Investigating the changes in gene expression that occur during skeletal-muscle regeneration is one means of identifying and isolating genes, the protein products of which may mediate processes such as muscle precursor cell activation, proliferation and differentiation. The potential for unravelling biochemically complex events by determining the differences in mRNA complement between different physiological states has been used to develop several differential gene expression-based screening strategies. Of these strategies the most common are subtractive hybridization [8], differential display reverse transcription PCR (DDRT-PCR) [9] and representational difference analysis (RDA) [10]. DDRT-PCR relies on random-primed amplification of a subfraction of total mRNA from two populations, running the PCR fragments on a sequencing gel, and isolating bands expressed at different levels. RDA is a process of subtraction coupled to amplification which combines the power of the two approaches. In practice all of these methods are technically difficult, all have been successfully used to isolate differentially expressed genes, and all are prone to mistaken identification of mRNAs that are not differentially expressed [8–10].

In this study, we used a subtractive hybridization and cDNA cloning strategy to identify genes that are differentially expressed in regenerating, compared with normal, skeletal muscle [11,12], A cDNA library was constructed from regenerating murine skeletal muscle, and the library was screened with subtracted cDNA probes. Two cDNA clones were isolated and confirmed to be differentially expressed between regenerating and normal muscles. These cDNA clones encoded the cofilin and vimentin genes. Analysis of the vimentin promoter/enhancer revealed a consensus site for the myocyte enhancer factor 2 (MEF2) transcription factors and subsequent analysis indicated that the transcriptional activity of these factors is increased in regenerating skeletal muscle. The up-regulated expression of the cofilin and vimentin genes during muscle regeneration implies a potentially important role for the cytoskeleton during skeletal-muscle regeneration. Also, our observation of increased MEF2

Abbreviations used: MHC, myosin heavy chain; MEF2, myocyte enhancer factor 2; DDRT-PCR, differential display reverse transcription-PCR; RDA, representational difference analysis; EMSA, electrophoretic mobility-shift assay; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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protein activity in regenerating muscle establishes a hypothetical model of the molecular control of muscle regeneration in which enhanced MEF2 activity is involved in driving the expression of muscle-regeneration-associated genes controlled by the MEF2 *cis* element.

MATERIALS AND METHODS

Model of muscle regeneration

Outbred CD1 mice (Charles River), 5–15 weeks of age, were used for these studies. The gastrocnemius muscle of the right hindlimb was injected intramuscularly with 2×50 μl of an aqueous solution of $BaCl_2$ (1.2 %, w/v). The gastrocnemius muscle of the left hindlimb served as a control (normal muscle). At 48, 65, 72 h or 96 h after injury, animals were killed with a lethal dose of sodium pentobarbital (MTC Pharma), and the gastrocnemius muscles were dissected out. The muscle specimens were either frozen in liquid nitrogen and stored at -70 °C for RNA extraction, or embedded in O.C.T. gel (Miles) and quickly frozen in isopentane that had been immersed in liquid nitrogen, then processed for histological examination. A series of 10 μm sections was obtained, stained with Haematoxylin and Eosin, then viewed using a light microscope.

cDNA library construction

On histological examination of normal and regenerating muscle at various time points after injury, activated muscle precursor cells were evident 48–96 h after injury. A time point of 65 h was chosen to process regenerating skeletal muscle for cDNA library construction.

Gastrocnemius muscle obtained from the right hindlimb of the injured mice (regenerating muscle, 65 h after injury) was crushed under liquid nitrogen, then used for total RNA extraction by the acid guanidinium thiocyanate/phenol/chloroform extraction method [13]. Selection of polyadenylated RNA was made on an oligo(dT)–cellulose column (Pharmacia Biotech). Single-stranded cDNA was synthesized from 5 μg of polyadenylated RNA as described previously using avian myeloblastosis virus reverse transcriptase and $d(T)_{12}$ oligonucleotide primer (Invitrogen [14]. The second cDNA strand was synthesized by the addition of RNase H and *Escherichia coli* DNA polymerase I (New England Biolabs) as described [14]. The double-stranded cDNA was then ligated to 1 μg of an adaptor oligonucleotide (Invitrogen). The adaptors had a *Bst*XI-compatible site on one end with an internal *Eco*RI site. This allowed the ligation of the adapted cDNAs into *Bst*XI-cut pcDNAI plasmid vector (Invitrogen) using T4 DNA ligase (New England Biolabs). The ligation reaction was then used to transform XL1 Blue cells by electroporation using Cell-Porator Voltage Booster (Gibco-BRL).

Subtraction hybridization

Total cellular RNA was extracted, and polyadenylated RNA was selected from regenerating muscle (65 h after injury) as well as from normal muscle, as described above. Approx. 1 μg of regenerating-muscle mRNA was subtracted from 10 μg of non-regenerating-muscle mRNA using The Subtractor Kit (Invitrogen). Briefly, the regenerating-muscle mRNA was converted into single-stranded cDNA using avian myeloblastosis virus reverse transcriptase and $d(T)_{12}$ primers. The non-regenerating-muscle mRNA was photobiotinylated under 300 W Sylvania clear bulb irradiation. The cDNA was left to hybridize with the biotinylated mRNA for 48 h at 68 °C. Hybridized species were removed by treating with streptavidin followed by phenol/chloroform extraction. The single-stranded cDNA left in the aqueous phase was precipitated with ethanol then dissolved in sterile water [12].

cDNA library screening

cDNAs obtained by subtractive hybridization were used to generate random-primed probes (Ready-To-Go Labelling Kit; Pharmacia Biotech). Colony lifts were performed on primary transformants grown on 14 cm plates (around 10000 transformants per plate), using a standard protocol [14]. The nitrocellulose filters (Nitro Plus; MSI) were prehybridized with formamide-based hybridization buffer (1 M NaCl, 50 mM Tris/HCl, pH 7.5; 1 % SDS, 10 mg/ml calf thymus DNA, 10 % dextran sulphate, 50 % formamide) for 2 h at 68 °C, then the heat-denatured probes were added to the hybridization buffer at a concentration of 1×10^6 c.p.m./ml, and left to hybridize overnight at 68 °C. The filters were then washed initially with 200 ml of $2 \times$ SSC/0.1 % SDS at room temperature for 30–60 min, followed by two more washes in 200 ml of $0.1 \times$ SSC/0.1 % SDS at 68 °C for 30 min each (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Autoradiographs were obtained by exposing the filters to X-ray film (Kodak Scientific Imaging) for 12 h at -70 °C. A secondary screening was performed to obtain purified single cDNA clones.

Northern-blot analysis

Total cellular RNA was extracted from muscle and from C2C12 myoblasts (cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum) and from C2C12 myotubes (5 days after transferring myoblasts to a differentiation medium containing Dulbecco's modified Eagle's medium with 5 % horse serum) as described previously [15]. Approx. 30–35 μg of total RNA was resolved on 1 % formaldehyde–agarose gel, and the RNA was transferred on to nitrocellulose membrane (Nitroplus) using a capillary-transfer technique. The blots were prehybridized in a formamide-based buffer at 68 °C for 2 h. The random-primed labelled denatured probe (prepared as described above for library screening) was added at a concentration of 1×10^6 c.p.m./ml, and the blots were incubated overnight at 68 °C. Washing was performed as described for the colony lifts. The intensity of the signals obtained on the blot were directly quantified using a Hewlett–Packard Instantimager which provides linear quantification of the mRNA bands.

Immunoblot analysis

Western-blot analysis was performed on whole-cell lysis extracts. Four whole gastrocnemius muscles were pooled for each extract. Extracts were made from non-regenerating and regenerating gastrocnemius muscle at 75 h, 5 days and 8 days after injury. Whole-cell extraction was performed as described by Harlow and Lane [16] for lysis of mammalian cells and tissue, with slight modifications. Briefly, tissue was ground in liquid nitrogen using a mortar and pestle, and 100 μg of powdered tissue was combined with 100 μl of $2 \times$ SDS loading buffer. Samples were then boiled for 5 min, sonicated for 30 s to shear chromosomal DNA and spun for 10 min at 4 °C to remove insoluble material. Total protein concentration was determined using the Bio-Rad Protein Assay Reagent. A 60 μg portion of total protein (or 10 μg for myosin heavy chain) per lane was separated by SDS/PAGE (10 % gel).

After SDS/PAGE separation, proteins were transferred using a semi-dry transfer chamber to nitrocellulose membranes (Micron Separations), blocked with 5 % Blotto for 1 h at room temperature and incubated with primary antibodies overnight at

4 °C. Monoclonal anti-vimentin and anti-(myosin heavy chain) antibodies were used as undiluted supernatant. Blots were washed three times in 5 % Blotto and incubated for 2 h with anti-mouse IgG conjugated to horseradish peroxidase (Sigma) diluted in 5 % Blotto. Western blots were visualized using the ECL reagent (Du Pont–NEN) and Kodak X-OMAT film.

Nucleotide sequence analysis

cDNA clones that displayed positive signals on library screening, and were shown to be overexpressed on regenerating muscle RNA blot, were purified and sequenced using an automated sequencer (Applied Biosystems). The nucleotide sequences of the cDNA clones were analysed for homologous sequences in the gene bank using the University of Wisconsin Genetics Computer Group sequence analysis software package, and the Blast network service of the National Center for Biotechnology Information.

Nuclear protein extraction and electrophoretic mobility-shift assays (EMSAs)

Nuclear protein extracts were made from regenerating and contralateral non-regenerating gastrocnemius muscles at 65 h after injury. Four whole gastrocnemius muscles were pooled for each extract. Nuclear extraction was performed as described by Deryckere and Gannon [17] with one minor modification: the addition of a freeze–thaw step ($\times 3$) after muscle homogenization. Protein concentration of the nuclear extracts was determined using the modified Bradford assay (Bio-Rad), and 3 μg of total protein from each nuclear extract was used in each binding reaction.

EMSAs were carried out as described by McDermott et al. [18]. Briefly, extracts were preincubated with 0.45 μg of poly (dI-dC) and 0.45 ng of a single-stranded oligonucleotide with the EMSA binding buffer (20 μM Hepes, pH 7.8, 50 μM KCl, 1 μM EDTA, 5 % glycerol) to sequester any non-specific binding. Antibodies were added at this point where applicable (see below). For all EMSAs, 0.2 ng of ^{32}P-radiolabelled double-stranded MEF2 oligonucleotide probe (5′-CGCTCTAAAAATAACCCT-3′) was added after the 15 min preincubation. Nucleotides in underlined print conform to the consensus sequence of the MEF2 [19,20]. Binding complexes were separated from the free probe using 4 % non-denaturing PAGE and visualized using autoradiography.

In vivo reporter gene assays

Reporter plasmid constructs were injected into the right gastrocnemius muscle of both non-regenerating and regenerating groups ($n = 5$–6 per group in each experiment) as previously described by Wells [21]. Tissues were then collected, and reporter protein was extracted. For transfection, 20 μg of a β-galactosidase-containing plasmid (rSVβ-Gal), used for normalization of DNA uptake, and 60 μg of the relevant chloramphenicol acetyltransferase (CAT) construct were dissolved in 100 μl of PBS and co-injected into the muscle (purified plasmid DNA constructs were prepared using the Qiagen purification system). CAT reporter constructs consisted of (1) the embryonic myosin heavy chain promoter (MHCemb) from position -109 driving CAT expression (PE102 CAT) and (2) two copies of the MEF2 site inserted in a concatemerized orientation upstream of the -102 position of the MHCemb promoter of PE102 CAT (MEF2 $\times$ 2 PE102CAT). The mice were killed, whole gastrocnemius muscles were excised, and extracts were prepared. Each gastrocnemius muscle was ground in liquid nitrogen, added to 300 μl of 0.25 M Tris/HCl, pH 7.8, frozen and thawed three times, sonicated for 5 s, centrifuged for 10 min at 6000 g/min in a Microfuge at 4 °C, and the supernatant was used for both β-Gal and CAT assays. Before CAT assay, the extracts were heated at 65 °C for 8 min to inactivate any endogenous acetyltransferases. The two-liquid-phase rapid CAT assay described by Neuman et al. [22] was used to quantify CAT levels in the extracts.

Statistical analysis

Statistical analysis was performed by Student's paired t test. The data are expressed as means $\pm$ S.E.M., and values of $P < 0.05$ were used to indicate statistical significance.

RESULTS

Histochemical characterization of the time course of muscle regeneration

When comparing the non-regenerating and regenerating muscle at 48 h, the regenerating muscle had little myofibre integrity (Figure 1). After 72 h of regeneration small myotubes are present (showing satellite cell activation and differentiation) as indicated by their size in relation to surviving fibres and their centrally located nuclei. By 96 h, myofibres and the muscle structure appear more phenotypically normal. Based on this analysis, 65 h was chosen as the time point for cDNA library construction since this is just before the appearance of new myofibres when considerable regeneration-associated gene expression is occurring.

Screening the regenerating-muscle cDNA library

When approx. 20000 independent clones were initially screened with the enriched DNA probes, a large number of clones displayed positive signals. A smaller number of positive clones was further subjected to a secondary screening in order to isolate pure clones. Further analysis by Northern-blot hybridization was carried out on eight cDNA clones.

Detection of mRNA levels of positive clones and nucleotide sequence analysis

Each of the eight cDNA clones was sequenced and used to prepare a probe for Northern-blot analysis of regenerating and non-regenerating muscle RNA, in order to verify whether the cDNA clone represented a gene that is actually up-regulated during regeneration. Two cDNA clones were demonstrated to represent genes for which the corresponding mRNA is highly up-regulated in regenerating, compared with normal, skeletal muscle. Analysis of the other six candidate cDNA clones revealed their mRNA levels to be approximately the same in normal and regenerating muscle. These mouse cDNA clones were sequenced and by BLAST analysis were found to have the highest nucleotide identity with the following genes: skeletal-muscle actin (99 % identity), human Fus-like protein (92 %), proα_1-collagen type III (95 %), human skeletal-muscle mRNA for MHC light meromyosin region (89 %), *Mus domesticus* hydrophobic protein mRNA (99.5 %) and human neuroleukin mRNA (89 %). All of these identities were based on more than 300 bp of overlapping sequence information. Nucleotide sequence analysis of the first and second up-regulated cDNA clones identified showed that they encoded cofilin and vimentin genes respectively. BLAST analysis showed that clone 1 had an identity of 98 % in a 493 bp region with mouse cofilin mRNA (accession number D00472). Clone 2 had an identity of 100 % in a 307 bp region with mouse vimentin mRNA (accession number X56397). Cofilin mRNA

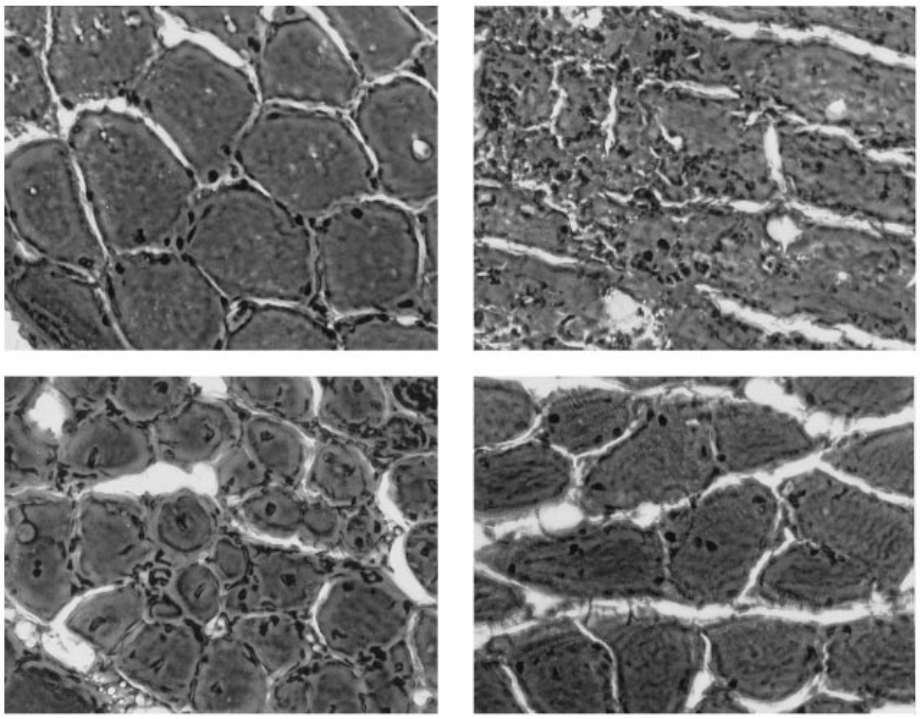

Figure 1 Time course of $BaCl_2$-induced regeneration in skeletal muscle

Animals received two 50 μl injections of 1.2% $BaCl_2$, one in each lobe of the right gastrocnemius muscle. Gastrocnemius muscles were taken from normal mice (top left), and at 48 h (top right), 72 h (bottom left) and 96 h (bottom right) after injury, sectioned and stained with Haematoxylin and Eosin. Representative photomicrographs are shown.

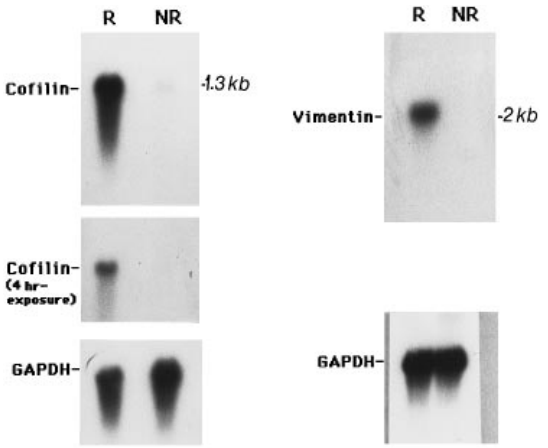

Figure 2 Cofilin and vimentin mRNA expression in non-regenerating and regenerating skeletal muscle

Approx. 35 μg of total RNA from 65 h regenerating muscle (R) or normal muscle (NR) was loaded in each lane. In the left column a cofilin cDNA was used to probe the RNA blot and in the right column a vimentin cDNA was used to probe the blot. The top left panel shows the hybridization signals after a 20 h exposure and the middle panel shows the same blot after a 4 h exposure. The bottom panel shows the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal on the same blot to control for loading and transfer efficiency.

was shown to be minimally expressed in normal skeletal muscle, and its expression was up-regulated 19-fold during regeneration (Figure 2). Vimentin mRNA was detected at a low level in normal muscle and its level of expression was up-regulated during regeneration. Quantitative normalized mRNA levels using an instant imager (Hewlett–Packard) were determined by dividing the specific mRNA signal with that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal (Table 1).

Table 1 Quantification of cofilin and vimentin mRNA levels in regenerating skeletal muscle

Results are means $\pm$ S.E.M. for three determinations. The mean value in the non-regenerating group (NR) was set at 100 and the regenerating muscle (R) values were expressed relative to that value (S.E.M.s were converted on the same scale). Fold difference = R/NR. $^*P < 0.05$ for regenerating versus non-regenerating muscle.

mRNA	NR	R	Fold difference
Cofilin	100 ± 5	$1730 \pm 207^*$	17
Vimentin	100 ± 8	$1956 \pm 160^*$	19

mRNA expression pattern for cofilin and vimentin during muscle regeneration

When cofilin and vimentin mRNAs were analysed at various time points during early stages of muscle regeneration (48, 65 and 75 h, and 5 days after injury), cofilin levels were elevated at 48 h, and reached maximal levels at 65 and 75 h after injury. At 5 days after injury, the level of cofilin mRNA expression declined (Figure 3). Vimentin mRNA levels began to increase at 48 h after injury, peaked at 65–75 h, and decreased at 5 days after injury.

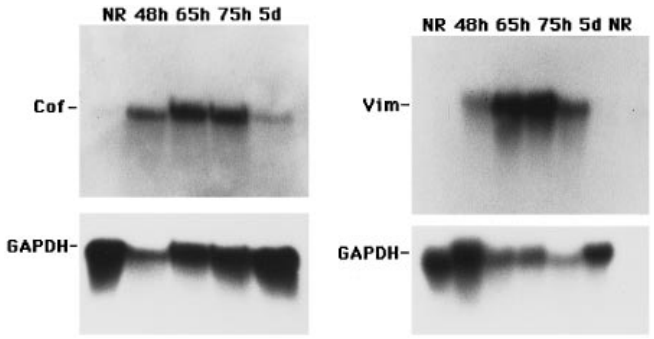

Figure 3 Cofilin and vimentin mRNA expression during the time course of muscle regeneration

Left and right panels illustrate Northern blots for cofilin (Cof) and vimentin (Vim) respectively. Approx. 35 μg of total RNA was loaded in each lane. NR is non-regenerating muscle, and 48 h, 65 h, 75 h, and 5 d are regenerating muscle at different time points after injury. For quantification cofilin and vimentin mRNA signals were normalized to GAPDH signals which are shown below each respective blot.

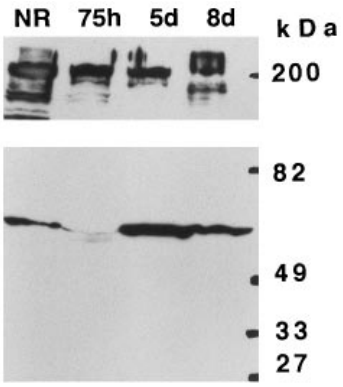

Figure 4 Vimentin protein expression during the time course of muscle regeneration

Immunoblot analysis of vimentin and MHC protein levels in non-regenerating and regenerating skeletal muscle at various time points after muscle injury. The upper panel shows the levels of sarcomeric MHC in non-regenerating muscle and at 75 h, 5 days (5 d) and 8 days (8 d) after injury. The predominant MHC band in muscle is at 200 kDa although other less abundant bands in non-regenerating muscle and regenerating muscle for 8 days are due to other isoforms and post-translational modification. The lower panel shows vimentin expression in non-regenerating muscle and at 75 h, 5 days and 8 days after injury.

Vimentin protein expression during muscle regeneration

To determine whether the protein levels for vimentin mirror the RNA-expression pattern, we performed immunoblots on regenerating muscle extracts using a specific vimentin antibody. The data show that vimentin is present in normal muscle, is virtually undetectable 75 h after injury, is abundant at 5 days after injury and decreases at 8 days after injury towards normal muscle levels (Figure 4). This indicates that the protein levels lag behind the changes in mRNA expression since peak mRNA expression is observed at 65–75 h when the protein levels are still very low (Figures 3 and 4). However, by 5 days the protein levels are very high, reflecting the large increase in RNA in the period preceding this time point (Figure 4).

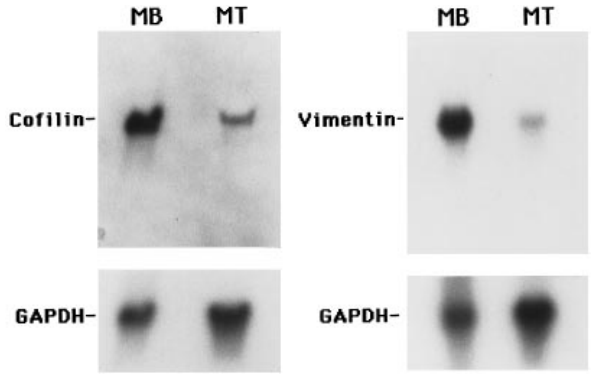

Figure 5 Cofilin and vimentin mRNA expression in cultured C2C12 myoblasts and myotubes

Left panel illustrates cofilin signal on a RNA blot made from C2C12 myoblasts (MB), and C2C12 myotubes (MT). The right panel illustrates the same experiment using vimentin cDNA as a probe.

Table 2 Quantification of cofilin and vimentin mRNA levels in C2C12 myoblasts and myotubes

Results are means $\pm$ S.E.M. for three determinations. The mean value in the myotubes (MT) was set at 100, and the myoblast (MB) values were expressed relative to that value (S.E.M.s were converted on the same scale). Fold difference = MB/MT.* $P < 0.05$ for myoblast versus myotubes.

mRNA	MT	MB	Fold difference
Cofilin	100 ± 14	322 ± 28*	3.2
Vimentin	100 ± 21	371 ± 47*	3.7

mRNA expression pattern for cofilin and vimentin in proliferating myoblasts and differentiated myotubes

To determine whether cofilin and vimentin genes are expressed in myogenic cells, and whether their level of expression is developmentally regulated, a Northern blot containing total RNA from proliferating C2C12 myoblasts and from differentiated myotubes was hybridized with cofilin and vimentin cDNA probes (Figure 5, Table 2). Cofilin mRNA expression was approximately 3-fold higher in myoblasts than in myotubes. Vimentin mRNA expression was 3–4-fold higher in myoblasts than in myotubes (Table 2). This pattern of expression in cultured myoblasts is important since they are considered functionally analogous to muscle precursor cells in regenerating mature muscle [3,4].

Identification of an MEF2 site in the vimentin promoter/enhancer region and increased MEF2 DNA-binding activity in regenerating skeletal muscle

Analysis of the vimentin gene promoter showed a consensus MEF2 site in a region of the promoter that has been shown to be important for high-level expression of the gene [23]. The MEF2 *cis* element has been shown to regulate many muscle-specific genes during developmental myogenesis [24]. Because of our

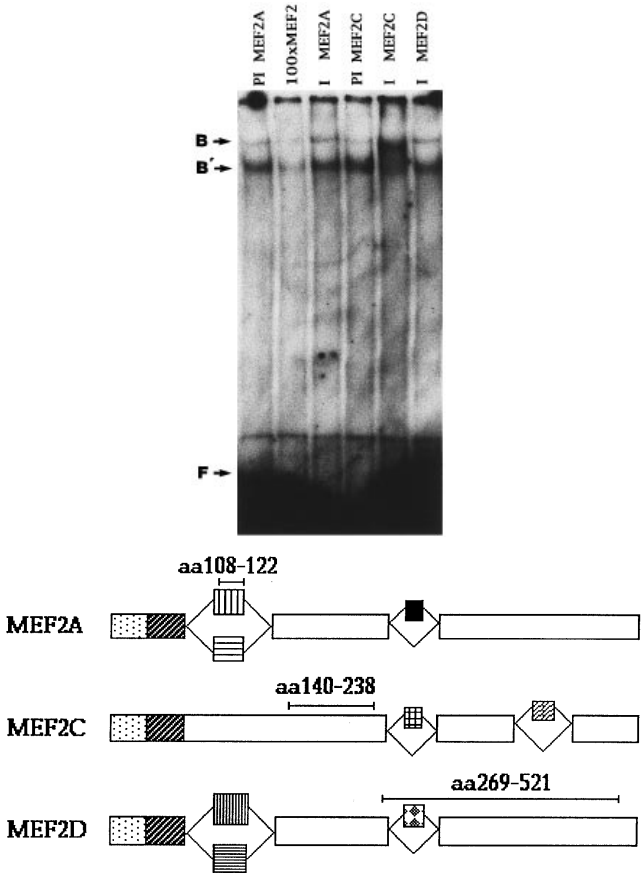

Figure 6 MEF2-specific DNA binding in regenerating adult skeletal muscle

Top, Radiolabelled MEF2 oligonucleotide probe was incubated with equal amounts of protein from nuclear extracts made from regenerating and adult skeletal muscle, in the absence (lane 1) or presence (lane 2) of a 100-fold molar excess of unlabelled competing oligonucleotide (competitor specified at top). B and B′ indicate bound MEF2 complexes. B′ is induced during regeneration and is not present in non-regenerating muscle. To detect which MEF2 proteins are present in the binding complexes the extracts were preincubated with specific MEF2A, C and D polyclonal antisera (I) (lanes 3, 5 and 6) serum along with preimmune (PI) control serum (lanes 1 and 4). The high-mobility complex (B′) that appears during regeneration is competed for by the unlabelled binding site (lane 2) and supershifted by the MEF2C antibody (lane 5) and not by the preimmune serum (lane 4). Bottom, regions of the MEF2 proteins that the polyclonal antisera were raised against. Boxes with the same pattern are regions of identity between the proteins; the remainder of the proteins are less conserved, aa indicates the amino acid regions used for antibody production.

observation that the vimentin gene is highly up-regulated in regenerating muscle and also contains a consensus MEF2 site in its regulatory region (-1341 to -1332), we were interested in determining whether MEF2 activity is increased in regenerating muscle.

To analyse the DNA-binding properties of the MEF2 factors in adult skeletal muscle and during regeneration, EMSAs were performed on nuclear extracts made from the contralateral and regenerating limbs at 65 h after injury. To determine if the MEF2 DNA–protein interactions change throughout the course of regeneration, EMSAs were performed on nuclear extracts made from the contralateral and regenerating gastrocnemius muscles. A constitutive binding complex (B on Figure 6, top) was present in normal tissue and remained unchanged in regenerating muscle. This complex was very low in abundance and required 4–5 days of autoradiogram exposure to be detected. This complex comprises a *bona fide* MEF2-binding activity since competition

Table 3 MEF2-site-dependent activation of transcription in non-regenerating and regenerating skeletal muscle

CAT activity in regenerating (R) or non-regenerating (NR) muscle injected with either the basal CAT reporter (containing the embryonic MHC promoter, PE102CAT) or the same promoter with two concatemerized copies of the MEF2 site upstream of the basal promoter (2 × MEF2 PE102 CAT). Values are means ± S.E.M. for experiments performed three or four times on five animals in each group. * $P < 0.05$ for regenerating (R) versus non-regenerating (NR) conditions. † $P < 0.05$ for PE102CAT versus 2 × MEF2 PE102 CAT for each condition. Fold activation = 2 × MEF2 PE102CAT/PE102CAT.

CAT reporter	Relative CAT activity		Fold activation
	R	NR	
PE102CAT	2670 ± 310	2360 ± 60	1.1
2 × MEF2 PE102CAT	7630 ± 1123*†	2320 ± 204	3.3

analysis with various mutated MEF2 DNA-binding sites showed the same nucleotide specificity as we have previously reported [15,18]. However, a second higher-mobility abundant MEF2 binding complex (B′) was present in regenerating muscle (Figure 6, top). To determine which MEF2 proteins form these binding complexes (B and B′, Figure 3), the nuclear extract from regenerating muscle was incubated with the various anti-MEF2 immune and preimmune (Figure 6, bottom). The higher-mobility regeneration-induced DNA-binding complex (B′) was supershifted using the MEF2C antibody (Figure 6, top, lane 5), indicating that MEF2C, or a complex involving MEF2C, is primarily responsible for the major MEF2–DNA binding complex (B′) in regenerating muscle. We previously found that two distinct MEF2–DNA binding complexes are present in myogenic and neuronal cells, and the higher-mobility complex in these cell types is also comprised of MEF2C [18].

Activation of transcription by endogenous MEF2 factors is increased during regeneration of adult skeletal muscle

To determine if the MEF2 factors are transcriptionally active during regeneration, an *in vivo* reporter assay was used in which plasmids containing reporter genes were injected into regenerating skeletal muscle. In these experiments a control basal reporter (PE102 CAT) or an MEF2-site-containing reporter construct (2 × MEF2 PE102 CAT) was injected into regenerating and non-regenerating gastrocnemius muscles (see the Materials and methods section). No difference in the activation of the reporter gene expression with the basal (control) promoter (PE102 CAT) was observed between the non-regenerating and regenerating muscle, whereas there was a significant increase in the level of reporter expression when the MEF2 sites were present (2 × MEF2 PE102 CAT) in regenerating muscle compared with non-regenerating muscle (Table 3; $P < 0.05$). Injection of parental CAT reporter that does not contain any upstream regulatory sequences gave no measureable CAT activity. The experiments described above show that the DNA-binding and transcriptional activity of the endogenous MEF2 factors is significantly enhanced in regenerating skeletal muscle.

DISCUSSION

In this investigation, we identified three criteria that needed to be met in order to designate a gene a candidate regeneration-associated gene. These criteria were: (i) the candidate clone is identified from a regenerating-muscle cDNA library using a

subtracted probe; (ii) the candidate clone is differentially expressed, at the mRNA level, between regenerating and normal skeletal muscle; (iii) the mRNA corresponding to the cDNA clone is abundantly expressed in cultured myogenic cells with a differential expression pattern between myoblasts and myotubes. We were able to identify two cDNA clones that satisfied the above criteria in our initial screening.

The first was a 1.1 kb cDNA clone which encoded the mouse cofilin protein. Cofilin is a 21 kDa actin-binding protein which has been shown to modulate actin polymerization during development and in response to stress (e.g. heat shock and DMSO treatment) [25,26]. Cofilin also exhibits *in vitro* inhibition of binding of tropomyosin to F-actin and actin–myosin interaction in a dose-dependent manner [25]. Our finding that cofilin mRNA is minimally expressed in normal adult skeletal muscle, and that it becomes markedly overexpressed early in regeneration is in agreement with the pattern of cofilin mRNA and protein expression during myogenic cell development and in dystrophic and regenerating muscle reported by others [27,28]. Cofilin was previously shown to be enriched in embryonic and cultured skeletal-muscle cells during development. The presence of cofilin protein is mainly associated with actin filament organization in the cytoplasm as well as the translocation of actin fibres to the nucleus in response to heat shock and DMSO treatment. Cofilin levels in muscle decline gradually on differentiation and continue to decline postnatally. Its expression is kept at a low baseline level in mature muscle. Hayakawa et al. [28] examined the expression of cofilin in dystrophic chicken muscle and in *dy*/*dy* mouse muscle (a dystrophic mouse model). Cofilin protein expression was enhanced significantly in dystrophic chicken and mouse regenerating muscle fibres [28]. These data, along with our observation, suggest a role for cofilin during the early stages of proliferation of muscle precursor cells, as well as in subsequent stages of differentiation.

The second cDNA clone isolated was a 0.9 kb cDNA clone that encoded the mouse vimentin gene. The vimentin gene encodes a 53 kDa protein which belongs to the intermediate filament multigene family that is composed of over 40 related proteins. Vimentin is expressed in cells of mesenchymal origin (e.g. muscle and epithelial cells), and its pattern of expression has been shown to be developmental-stage-specific [29]. Vimentin cDNA was previously identified in ts13 cells (a G_1-specific temperature-sensitive mutant derived from Syrian hamster BHK cells) that were induced to enter the cell cycle (G_1 phase) serum induction [30,31]. Vimentin is detected in somitic premyoblasts, and peak level of expression is observed during embryonic myoblast proliferation. We observed an expression pattern for vimentin mRNA that is in agreement with that reported during muscle cell growth and differentiation *in vitro* and *in vivo* [29].

The MEF2 families of transcription factors have been implicated as key regulatory molecules in the myogenic cascade (reviewed in [24]). Originally the MEF2-binding site was characterized by deletion analysis and mobility-shift assays as an important *cis* element in numerous muscle promoters and it is now recognized that this sequence and the *trans*acting factors which bind to it are fundamental regulators of muscle-specific gene expression [24]. The presence of a binding site for the MEF2 proteins in the vimentin promoter/enhancer region suggested to us that these transcription factors may be important regulators of muscle gene expression during regeneration.

Two lines of evidence in our experiments suggest that MEF2 activity is augmented in regenerating skeletal muscle. First, we observed an increase in the DNA-binding activity of MEF2 in regenerating muscle nuclear extracts by EMSAs. Secondly, analysis of transcriptional activation by endogenous MEF2 factors using an *in vivo* reporter assay demonstrated enhanced MEF2 transcriptional activity in regenerating muscle. Thus a simple interpretation of these data suggests a testable hypothesis for the activation of muscle genes during regeneration in which the MEF2 proteins, along with other transcription factors, drive the expression of structural, metabolic and contractile protein-encoding genes necessary for the re-establishment of the muscle architecture. Future studies will be directed towards understanding the molecular regulation of the vimentin promoter/enhancer during regeneration in order to potentially identify other transcriptional regulatory proteins that are involved in the activation of muscle genes during regeneration.

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