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Single-Cell Analysis of Regulatory Gene Expression in Quiescent and Activated Mouse Skeletal Muscle Satellite Cells

D. D. W. Cornelison and Barbara J. Wold
Division of Biology, California Institute of Technology, Pasadena, California 91125

Repair and regeneration of adult skeletal muscle are mediated by satellite cells. In healthy muscle these rare mononucleate muscle precursor cells are mitotically quiescent. Upon muscle injury or degeneration, members of this self-renewing pool are activated to proliferate and then differentiate. Here we analyzed in single satellite cells the expression of a set of regulatory genes that are candidates for causal roles in satellite cell activation, maturation, and differentiation. Individual cells were identified as satellite cells and selected for analysis based on their physical association with single explanted myofibers or their position beneath the basal lamina in unperturbed muscle tissue. Using a multiplex single-cell RT-PCR assay we simultaneously monitored expression of all four MyoD family regulators of muscle determination and differentiation (MRFs) together with two candidate markers of satellite cell identity, c-met and m-cadherin. By making these measurements on large numbers of individual cells during the time course of satellite cell activation, we were able to define which expression states (possible combinations of the six genes) were represented and to specify how the representation of each state changed with time. Activated satellite cells began to express either MyoD or myf5 first among the MRFs; most cells then expressed both myf-5 and MyoD simultaneously; myogenin came on later in cells expressing both MyoD and myf5; and many cells ultimately expressed all four MRFs simultaneously. The results for fiber-associated satellite cells from either predominantly fast or slow muscles were indistinguishable from each other. The c-met receptor tyrosine kinase was also monitored because it is a candidate for mediating activation of quiescent satellite cells (Allen *et al.,* 1995) and because it might also be a candidate molecular marker for satellite cells. A significant difficulty in studying mouse satellite cells has been the absence of molecular markers that could identify them in the quiescent state before expression of MRFs or desmin and distinguish them from fibroblasts. We show here that c-met receptor is present beneath the basal lamina on presumptive satellite cells in intact muscle and that c-met mRNA and protein are expressed by all myofiber-associated satellite cells from the time of explant through the course of activation, proliferation, and differentiation. c-met was not detected in muscle-derived fibroblasts or in other mononucleate cells from healthy muscle explants. When compared directly with m-cadherin, which has previously been suggested as a marker for quiescent satellite cells, m-cadherin mRNA was detected only in a small subset of satellite cells at early times after myofiber explant. However, at late times following activation (by 96 hr in this fiber culture system), c-met and m-cadherin were uniformly coexpressed. From the individual satellite cell expression types observed, a model of the satellite cell population at rest and during the time course of activation was generated.

Key Words: **satellite cell; c-met; m-cadherin; MyoD; myogenin; MRF4; myf-5; HGF; RT-PCR; scatter factor.**

INTRODUCTION

In adult mouse skeletal muscle the majority of myonuclei are located in syncytial myotubes that were formed by myoblast fusion during fetal and postnatal development. These myonuclei are terminally postmitotic. However, a small fraction of myonuclei are in mononucleate precursor cells called muscle satellite cells which are located between the basal lamina and sarcolemma of myofibers (Mauro, 1961; reviewed in Bischoff, 1994). In healthy adult rodent muscle, satellite cells are mitotically quiescent and do not detectably express MRFs (MyoD family muscle regulatory factors) (Grounds *et al.*, 1992). When stimulated by damage to the muscle or by explant and culture manipulations, some fractions of satellite cells are activated to reenter the cell cycle and/or to express myogenic regulatory factors. The resulting myoblasts subsequently differentiate and fuse to form new replacement myofibers (Bischoff, 1986a; reviewed in Bischoff, 1994).

Although all skeletal muscle regeneration in mammals

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is attributed to satellite cells, including recovery phases of neuromuscular wasting diseases, we currently have an incomplete picture of the molecular mechanisms involved in establishing and maintaining the quiescent state, regulating activation and subsequent muscle differentiation, and sustaining the satellite population through multiple rounds of regeneration. This is partly due to technical problems caused by satellite cell rarity within muscle tissue and associated difficulties with identifying them, especially in the quiescent state. For the mouse there are currently no reliable molecular markers that can prospectively identify resting satellite cells or activated cells that do not yet express MRFs or desmin. This work begins to address the problem by evaluating the expression of two candidate markers of satellite cell identity, m-cadherin and c-met, in sets of individual satellite cells during the course of activation and differentiation.

Expression of c-met and m-cadherin is also relevant to muscle regeneration because of their postulated functions. c-met transduces mitogenic, migratory, or morphogenetic signals in a variety of nonmuscle tissues during development and wound healing in response to its ligand HGF/scatter factor (reviewed in Matsumoto and Nakamura, 1996). In muscle development HGF/c-met signaling is essential for proper emigration of muscle precursor cells of the axial lineage during embryogenesis (Bladt *et al.*, 1995). In a mass culture system, exogenous HGF accelerates cell proliferation of freshly isolated rat satellite cells, suggesting that it may be an activation factor *in vivo* (Allen *et al.*, 1995). m-cadherin is a calcium-dependent homophilic cell adhesion molecule that is expressed prominently during fetal myogenesis (Rose *et al.*, 1994). As the member of the cadherin family expressed predominately during skeletal myogenesis, it has been suggested that m-cadherin plays a significant role in alignment and fusion of myoblasts to form and expand developing myotubes (Donalies *et al.*, 1991; Cifuentes-Diaz *et al.*, 1995). It has also been detected in activated satellite cells during regenerative responses after muscle damage (Moore and Walsh, 1993; Irintchev *et al.*, 1994).

Little is known about the nature and extent of cell to cell heterogeneity of gene expression in satellite cells at present, but at least two types can be hypothesized. First, some distinction is expected between satellite cells which will differentiate as myotubes and those which maintain the progenitor pool for subsequent rounds of regeneration. Second, satellite cells from muscles of different fiber types or of different embryonic sublineages may retain distinct identities. The emerging picture for skeletal muscle development during embryogenesis is that specific combinations of regulatory genes, rather than any single myogenic master regulator, are responsible for directing determination and differentiation (reviewed in Yun and Wold, 1996). Genetic analyses of knockout mice have established that the four members of the MRF family of transcription factors are individually and collectively important for muscle precursor development and for terminal differentiation (reviewed in Olson and Klein, 1994; Yun and Wold, 1996). It is generally believed that the MRFs will have similarly important functions in muscle regeneration, and evidence for a specific requirement for MyoD was recently reported for regeneration in dystrophic mdx mice (Megeney *et al.*, 1996). Prior studies have also shown that these regulators are not expressed detectably in unactivated satellite cells, but that all four are transcribed beginning at different times over the course of activation in mass cultures (Table 1 and references therein). However, the combinatorics of MRF family expression at the single-cell level remain only partly known for embryo or satellite cell populations. For the first time we could address this problem because satellite cells are physically identifiable by positional criteria in our fiber cultures and because they are experimentally accessible for single-cell RNA analysis. Thus, coexpression of c-met, m-cadherin, myf-5, MyoD, myogenin, and MRF4 mRNAs was measured in a large number of individual myofiber-associated satellite cells. From these data we were able to reconstruct the satellite cell population expression pattern as the sum of distinct individual expression patterns. The sets of expression types observed over time suggested a simple developmental model for MRFs during satellite cell activation and differentiation.

MATERIALS AND METHODS

Fiber Isolation and Culture

Myofibers from adult mice (>100 days old) were isolated essentially as in (Bischoff, 1986a), with these exceptions: Fibers were isolated from multiple muscles, including the anterior tibialis, soleus, and longissimus dorsi; in our hands it was not necessary to use only short, tendonous muscles. Dissected muscles were treated with 400 U/ml collagenase type I (Worthington) in DMEM at 37°for 60 min. Muscle masses were not triturated, but were manually rocked before individual fibers were harvested with a fire-polished Pasteur pipet, preflushed with medium to prevent sticking. Fiber cultures were grown in DMEM supplemented with 10% equine serum (Hyclone), 5% chick embryo extract (Gibco), penicillin–streptomycin (Gibco), and 2.5 μg/ml amphotericin B (Sigma) at 37°C in a humidified incubator at 5% CO_2. BrdU (10 μM; Boehringer) was supplied continuously in the medium to monitor proliferation history. Fresh individual fibers isolated in this way were up to 1.4 cm in length, averaging 5–7 mm and having 62 $\pm$ 8.6 myonuclei and 0.77 $\pm$0.08 satellite cells per millimeter. To decrease possible contamination from nonsatellite cell types which were carried from the isolation, after 24 and 48 hr of culture individual fibers were repicked with a Pasteur pipet and transferred to a fresh dish of medium (by this time, most contaminant cells emigrate from the fibers, apparently due to higher affinity for the culture dish). To derive muscle fibroblast cultures, cells adhering to the dish after fibers are transferred away which appear to be fibroblasts are identified and surrounded with a cloning cylinder, then trypsinized and removed to a new dish and expanded.

Marcaine Treatment Live/Dead Staining

To identify and harvest individual satellite cells immediately after fibers had been dissociated, fresh fiber preparations were

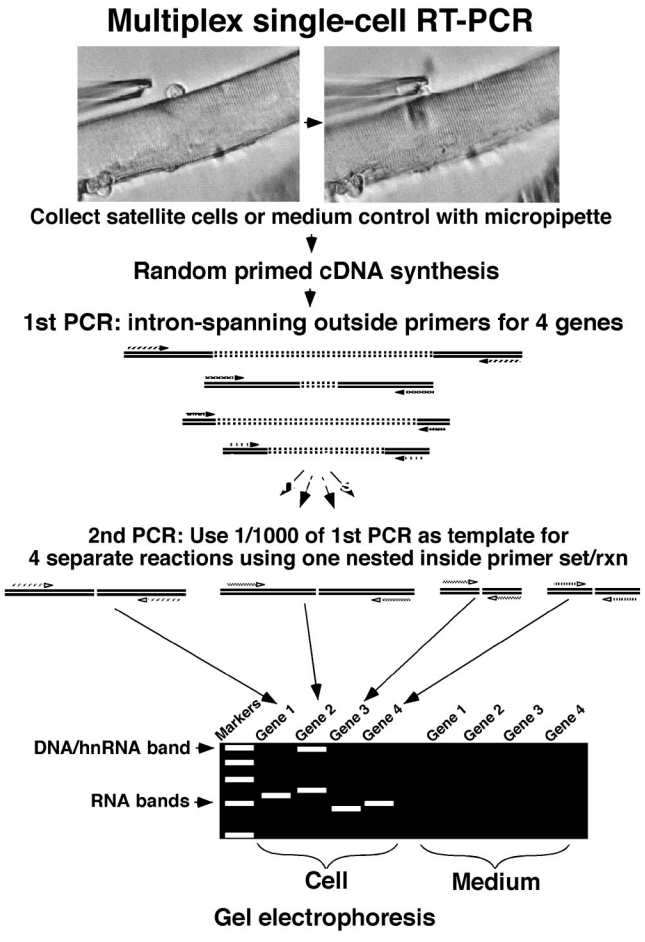

FIG. 1. Schematic depiction of single-cell RT-PCR as used for this work. The top panels show phase images of a satellite cell (typical in appearance for cells from 24 to 96 hr) before and during harvest.

treated with the myotoxic anesthetic Marcaine. The fibers and their satellite cells could then be stained with Live/Dead reagents (Molecular Probes), which consist of calcein AM, a fluorescent vital dye activated only in the cytoplasm of living, viable cells, and an ethidium homodimer, which stains the nuclei of dead cells. Marcaine-treated fibers would hypercontract, and after treatment their cytoplasm could no longer activate calcein AM and their nuclei stained with the dead cell reagent. Satellite cells are not affected by Marcaine treatment and can thus be identified by calcein AM staining. Fresh fiber preparations were treated with Marcaine (Winthrop; 0.05% in PBS) for 20–30 min at 25°C, rinsed twice in PBS, and treated with 2 μM calcein AM (Molecular Probes) for 20 min at 25°C. We note that the fibers in which Marcaine caused most extreme hypercontraction appear to be those with ends broken from the explant surgery.

Single-Cell Multiplex RT-PCR

The design of the multiplex single-cell RT-PCR protocol (Fig. 1) was derived from that of Kato *et al.* (1997), in which a patch-clamp pipet was used to harvest a sample of cell cytoplasm. We modified this procedure with the goal of maximizing and normalizing the amount of RNA obtained from each cell. Fibers were transferred to a dish of sterile, RNAase-free PBS immediately before harvest; collection pipets were filled with RNAase-free PBS. The orifice of the micropipet was enlarged to be only slightly smaller than the circumference of a satellite cell, and the entire cell was collected into the micropipet. Each collected cell was used individually as substrate for reverse transcription with M-MLV RT using random primers [7.5 μl per cell RT mix containing 1× (2 μl 5×) RT buffer (Boehringer), 40 mM (4 μl 100 mM) DTT, 0.5 mM dNTPs (0.5 μl 10 mM), 10 U (0.2 μl) RNAase inhibitor (Boehringer), 200 ng random primers (0.4 μl 500 ng/μl) (Boehringer), and 0.4 μl DEPC water; keep mix on ice at all times. Add cells in approximately 2 μl of collection buffer; after all cells have been added to mix tubes, add 10 U (0.5 μl) M-MLV RT (Boehringer) per tube for a final volume of 10 μl; incubate at 37°C for 1 hr]. In some control reactions (specified in text and figure legends) several individual cells were collected and then pooled for analysis of sensitivity and reproducibility. The entire cDNA reaction was then added to a PCR containing an outside primer pair for each gene of interest (for 1.0 cell input, 50 μl PCR containing 1× reaction buffer (Qiagen), 200 nmol dNTPs, 200 μmol each outside primer, 2.5 U *Taq* polymerase (Qiagen); 35 cycles PCR at 60°C annealing). In reactions where more than four genes were monitored from a single cell, the cDNA reaction was first divided in half, and each half was then used in a one-half scale PCR with primers corresponding to a subset of the genes to be assayed. All outside primer sets were designed to cross at least one intron, so that any products derived from unprocessed hnRNA or from genomic DNA could be distinguished from messenger RNA templates; this design feature is critical when using whole cells containing nuclei as the substrate. After the first round of PCR, the reaction was diluted 1:1000 into separate secondary PCR reactions, each of which contained a single set of primers positioned internal to the first set for one of the genes being tested. The second PCR was then executed under the same protocol as the first. Products were analyzed on a 2% agarose gel; representative bands were sequenced to confirm identity.

Primer pairs for GAPDH, c-met, and m-cadherin were written by eye; primers for MyoD, myogenin, myf-5, and MRF4 were selected using Lasergene (DNAStar, Madison, WI). All primer sets were screened using Lasergene for possible interference with each other and then tested empirically alone and in combination with all primers that were to be used within a given multiplex set. Outside primers used in the first PCR were GAPDH, 5′-GTG GCA AAG TGG AGA TTG TTG CC-3′ forward, 5′-GAT GAT GAC CCG TTT GGC TCC-3′ reverse; c-met, 5′-GAA TGT CGT CCT ACA CGG CC-3′ forward, 5′-CAC TAC ACA GTC AGG ACA CTG C-3′ reverse; m-cadherin, 5′-CCA CAA ACG CCT CCC CTA CCC ACT T-3′ forward, 5′-TCG TCG ATG CTG AAG AAC TCA GGG C-3′ reverse; MyoD, 5′-GCC CGC GCT CCA ACT GCT CTG AT-3′ forward, 5′-CCT ACG GTG GTG CGC CCT CTG C-3′ reverse; myogenin, 5′-GGG CCC CTG GAA GAA AAG-3′ forward, 5′-AGG AGG CGC TGT GGG AGT-3′ reverse; myf-5, 5′-TGC CAT CCG CTA CAT TGA GAG-3′ forward, 5′-CCG GGG TAG CAG GCT GTG AGT TG-3′ reverse; and MRF4, 5′-CTG CGC GAA AGG AGG AGA CTA AAG-3′ forward, 5′-ATG GAA GAA AGG CGC TGA AGA CTG-3′ reverse. Primers used for the second PCRs were GAPDH, 5′-GTG GCA AAG TGG AGA TTG TTG CC-3′ forward, 5′-GAT GAT GAC CCG TTT GGC TCC-3′ reverse; c-met, 5′-GAA GGT ATC CGA ATT CAA GAC CGG-3′ forward, 5′-GAA CAT GCA GTG GAC CTC AGA CTG-3′ reverse; m-cadherin, 5′-ACA GCA GCT AGG CAG TGT CAT C-3′ forward, 5′-AAC CTG AGG GCT GCA TTG TCT GTC-3′ reverse; MyoD, 5′-

CCC CGG CGG CAG AAT GGC TAC G-3′ forward, 5′-GGT CTG GGT TCC CTG TTC TGT GT-3′ reverse; myogenin, 5′-CCG TGG GCA TGT AAG GTG TG-3′ forward, 5′-TAG GCG CTC AAT GTA CTG GAT GG-3′ reverse; myf-5, 5′-GAG GGA ACA GGT GGA GAA CTA TTA-3′ forward, 5′-CGC TGG TCG CTG GAG AG-3′ reverse; and MRF4, 5′-TGC GGA GTG CCA TCA GC-3′ forward, 5′-CTC CTC CTT CCT TAG CAG TTA TCA-3′ reverse.

To determine the fidelity of the single-cell RT-PCR when challenged with increasing numbers of primer sets or decreasing amounts of input cDNA or both, singly-harvested satellite cells were pooled prior to cDNA synthesis and reverse-transcribed, and single-cell equivalent aliquots of the resulting homogeneous pool of cDNA were analyzed. When all six primer sets were used together, the reactions tended to fail regardless of the amount of input cDNA, but under the conditions used in this work highly consistent positive results were obtained using between 0.25 and 0.125 of a cell equivalent of input cDNA (Figure 6).

Technical Considerations for Single-Cell Multiplex RT-PCR

This technique has been optimized for sensitivity in order to enable detection of nonabundant transcripts from multiple regulatory genes. A significant trade-off is that the procedure intentionally operates outside the linear range of PCR and is therefore nonquantitative. A series of theoretical and technical considerations argue that reliable and meaningful quantitation (absolute RNA levels or even relative amounts of different transcripts) will not be possible by this approach and will require different techniques. It is also useful to recognize that different primer sets are differentially efficient so that empirical tests on positive samples are required for each new primer set. Moreover, primer compatibility for new multiplex combinations must be tested on a case-by-case basis. Concordance tests such as those in Fig. 6 and in Kato *et al.* (1997) provide a means to evaluate robustness of a given set of measurements.

We believe that different cell types may require significantly different sample collection techniques. Specifically, we note that attempts to collect cytoplasm from mature myofibers have so far been unsuccessful because of difficulty in recovering sample from the highly structured ''cytosol.'' Also, the variation we used here in which whole satellite cells are collected may not be possible when harvesting cells from whole embryos or from more intact tissues, and in these cases the use of cytosol-specific collection (Kato *et al.*, 1997) may be preferable.

Protein Blotting and Immune Reagent Characterization

Western blotting was performed on protein extracts from pooled satellite cells derived from our fiber cultures. The anti-mouse c-met polyclonal rabbit serum (Santa Cruz, Cat. No. sc-162) specifically detected a single band of appropriate size for c-met (Fig. 2). As anticipated, preincubation of the antibody with the immunizing peptide eliminated this band.

Immunohistochemistry

Cultured myofibers were fixed in fresh 4% paraformaldehyde for 20 min at 25°C, washed three times with PBS, permeabilized in 1% NP-40 for 5 min at 20°C, washed three times with PBS, blocked in 10% normal goat serum, incubated overnight at 4°C with primary antibodies, washed three times with PBS, incubated 1 hr at 25°C with secondary antibodies, washed three times with PBS, and mounted in Vectashield (Vector). Fibers were photographed on a Nikon Optiphot-2 with a UFX camera attachment or imaged using a Bio-Rad 600 confocal microscope with false color added in Photoshop (Adobe).

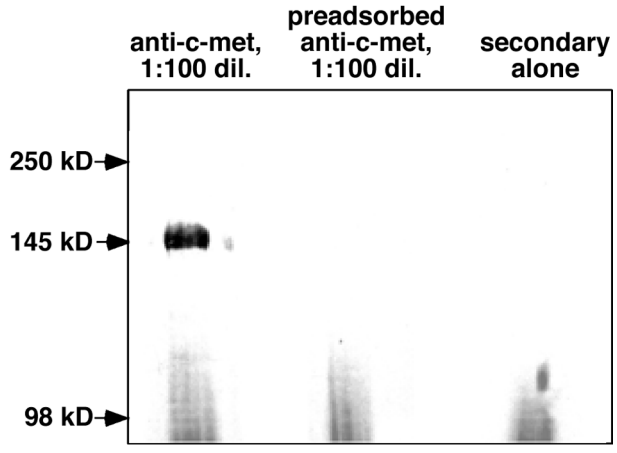

FIG. 2. Western blot of proteins from satellite cells expanded for 2 weeks in mass culture, with all myofibers removed. A single band of a molecular weight appropriate for the β-chain of c-met is detected and was competed away by preincubating the antibody with excess peptide antigen.

Ten-micrometer cryotome sections of unmanipulated quadriceps muscle were double-stained for c-met and laminin sequentially by fixing, blocking, and incubating with rabbit anti-met antibody as above, followed by incubation with monovalent goat anti-rabbit Fab–FITC conjugate (Jackson Immunochemicals) at at 1:50 for 2 hr. Sections were washed in PBS and incubated with rabbit anti-laminin (Sigma) for 4 hr followed by anti-rabbit TRITC and mounted in Vectashield. The sections were then photographed or imaged as above.

Primary antibodies and dilutions used were rabbit anti-m-met (Santa Cruz) at 1:50; F5D (mouse anti-myogenin, Wright *et al.*, described in Cusella-DeAngelis *et al.*, 1992) at 1:5; NCL-MyoD1 (mouse, Novocastra Labs) at 1:10; mouse anti-BrdU (Boehringer-Mannheim) at 1:10; and rabbit anti-laminin (Sigma) at 1:250. Secondary antibodies were raised in donkey (Jackson Immunochemicals) and, except for blocking Fab used for section double-staining, used at 1:100.

RESULTS

Expression of c-met and m-Cadherin mRNAs by Fiber-Associated Satellite Cells

In the first part of this study we used isolated myofiber cultures to test individual satellite cells for the expression of m-cadherin and c-met genes. We began with these genes because each is a candidate ''molecular marker'' for myosatellite cells at rest or in the early stages of activation and because each is of functional interest in muscle regeneration. To serve as a satellite cell marker, expression should encompass all satellite cells and should exclude other mono-

nucleate cells in muscle tissue such as muscle-derived fibroblasts. The prime candidate thus far for a resting satellite cell marker has been the homophilic adhesion molecule m-cadherin, which has been detected by *in situ* hybridization (Moore and Walsh, 1993) and immunostaining (Cifuentes-Diaz *et al.*, 1995; Irintchev *et al.*, 1994) in tissue sections taken from regenerating mouse skeletal muscle. However, RNA expression was not detected in unstimulated satellite cells or in satellite cells stimulated by denervation rather than trauma injury, and the data regarding protein expression in quiescent satellite cells are conflicting. A prior study had shown that transcripts for the c-met receptor tyrosine kinase can be detected by RT-PCR in RNA from pooled cultured rat satellite cells (Allen *et al.*, 1995), indicating that it is expressed in some satellite cells. Measurements of that type could not, however, reveal what fraction of cells are responsible for the positive signal observed.

To evaluate these candidate markers an independent criterion for satellite cell identity was required. In the experiments that follow, a cell was defined as a satellite cell and picked for assay based on its anatomic association with an isolated myofiber. First utilized by Bekoff and Betz (1977), the isolated fiber technique was further refined by Bischoff who focused on the rat flexor digitorum brevis muscle and used it to show that satellite cells identified initially by association with the parental myofiber proliferate in culture and are subsequently myogenic (Bischoff, 1986a). The modification used here was designed for source material from diverse muscles, which enabled us to compare satellite cells from muscles that are mainly fast or slow in fiber type or from muscles of the two major developmental lineages, the axial and appendicular groups (see Materials and Methods).

An initial question was whether either c-met or m-cadherin is expressed in all or only some satellite cells at the earliest times after fibers are explanted from healthy muscle tissue. The mRNA assay used was multiplex single-cell RT-PCR modified from Kato *et al.* (1997) (see Fig. 1 and Materials and Methods). In this experiment, three genes were monitored for each cell: GAPDH, m-cadherin, and c-met. GAPDH is commonly employed as a standard because of its ubiquitous expression and was used here to show that a cell had been harvested successfully and that the reverse transcription and PCR had proceeded properly. Satellite cells associated with freshly harvested myofibers were visualized for collection by treating the preparation with the myotoxic anesthetic Marcaine (Winthrop) and staining with the live-cell specific fluorescent dye calcein AM; at later time points morphological criteria with respect to the associated myofiber were used to define satellite cells for harvest. Satellite cells that migrated away from their fiber of origin in the culture were not picked, because their identity as satellites by anatomical criteria was uncertain.

The result of this analysis for c-met was striking: 100% of GAPDH-positive cell samples also scored positive for processed c-met mRNA at all time points (Fig. 3A). In contrast, only a small fraction (<20%) of satellite cells scored positive for m-cadherin at the early time point. As expected, we never observed a satellite cell that scored negative for GAPDH, but positive for any other gene assayed. We next asked how the expression of c-met and m-cadherin mRNAs changed over a 96-hr time course in which fiber-associated satellite cells are activated to divide, express MRF family regulators, and differentiate (see below). The fraction of cells expressing m-cadherin increased gradually throughout the time course and was 100% at 96 hr. It is important to note that when we examined satellite cell pools rather than groups of individual cells, all time points scored positive for both markers, even though the majority of individual cells did not express m-cadherin at early time points. A final important result was that GAPDH-positive 10T1/2 fibroblasts (data not shown) and muscle fibroblasts derived from muscle fiber cultures were entirely negative for m-cadherin and c-met (Fig. 3C).

The experiments above and prior studies of rodent satellite cells have all used appendicular muscles, which develop from the c-met-dependent migratory cells of the lateral somite (Ordahl and LeDouarin, 1992), as the source of satellite cells. During development the axial muscles represent a distinct population of myoblasts and are not dependent on the c-met/HGF function (Bladt *et al.*, 1995; Daston *et al.*, 1996). This raised the question of whether c-met expression is general for all satellite cells or is restricted to the limb lineage. We therefore repeated the experiment using myofibers prepared from axial muscles of the deep back and found consistent positive mRNA expression in satellite cells from axial muscle fibers. We also determined that satellite cells derived from predominantly fast or predominantly slow fiber types express c-met and m-cadherin similarly (see below). From these data we conclude that m-cadherin expression marks a small subset of satellite cells at early times following explant or activation but that c-met expression appears to include all satellite cells in fiber cultures.

Satellite Cells Express c-met Protein in Fiber Culture

The finding that c-met mRNA is expressed by all satellite cells raised the question of c-met protein expression, which would be required for a biological function during muscle regeneration. We therefore asked whether c-met protein is expressed by fiber-associated satellite cells and how that expression is related to expression of MRF family proteins and to the proliferation status of the cell. Myofiber isolation activates fiber-associated satellite cells to express MRF family regulators, divide, and differentiate over a period of several days in culture. Prior studies have shown that fiber-associated satellite cells begin to enter S-phase after approximately 36 hr in culture (Bischoff, 1986a; Yablonka-Reuveni and Rivera, 1994). When cultured in the continuous presence of BrdU, fixed at 12-hr intervals, and costained with antibodies directed against mouse c-met (Santa Cruz; see Materials and Methods for characterization) and incorporated BrdU (Boehringer), satellite cells in these fiber cultures behaved similarly, entering S-phase of the cell cycle approximately 36 hr after isolation. When costained with the antibody to mouse c-met satellite cells were positive for c-met

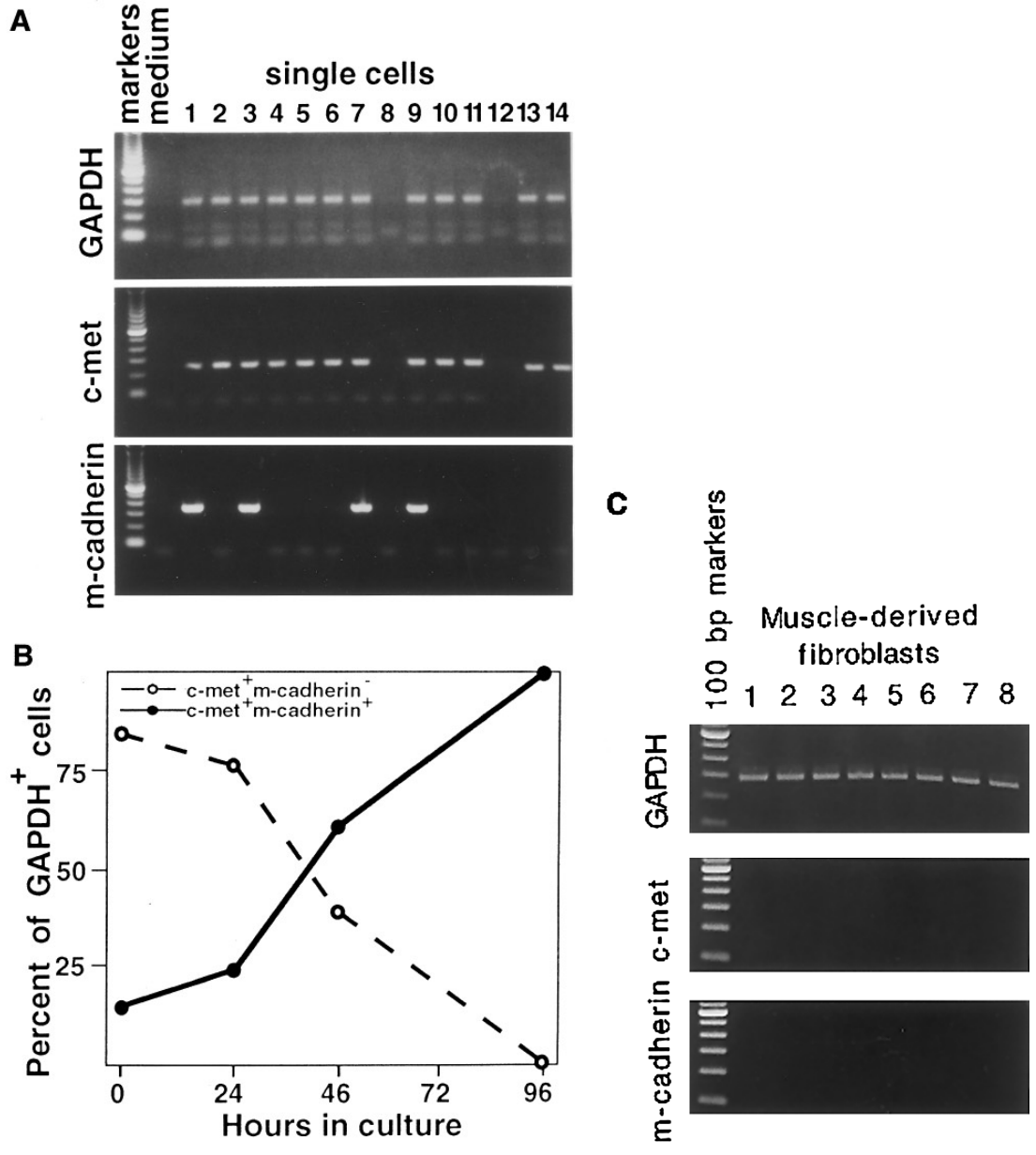

FIG. 3. (A) Sample single-cell RT-PCR gel from 48 hr in culture samples showing GAPDH (expected product 290 bp), c-met (expected product 249 bp), and m-cadherin (expected product 316 bp); marker, 100 bp ladder (Gibco). (B) Line graph illustrating the population shift from c-met$^+$ m-cadherin$^-$ (dashed line) to c-met$^+$ m-cadherin$^+$ (solid line) over the first 96 hr in culture of satellite cells on isolated myofibers. (C) Single-cell RT-PCR of muscle-derived fibroblasts harvested from the surface of a tissue culture dish. All GAPDH-positive cells were negative for both c-met and m-cadherin.

immunoreactivity both before and after BrdU incorporation was detected, indicating that c-met expression continues even after the regeneration program has been activated and cell proliferation has begun (Fig. 4A).

Prior studies have shown that MRF proteins are not expressed in quiescent satellite cells, but accumulate in activated satellite cells (Grounds *et al.*, 1992; Smith *et al.*, 1994; Yablonka-Reuveni and Rivera, 1994; summarized in Table 1). When satellite cells from fiber cultures were costained with immune reagents for c-met and either myogenin (Wright *et al.*, described in Cusella-DeAngelis *et al.*, 1992) or MyoD (Novocastra Labs), c-met expression was observed on all cells that expressed MyoD at or before 12 hr in culture (Fig. 4B) and myogenin by approximately 72 hr in culture (Fig. 4C); no MyoD- or myogenin-positive mononucleate cells were ever seen that were not also positive for c-met protein. This result

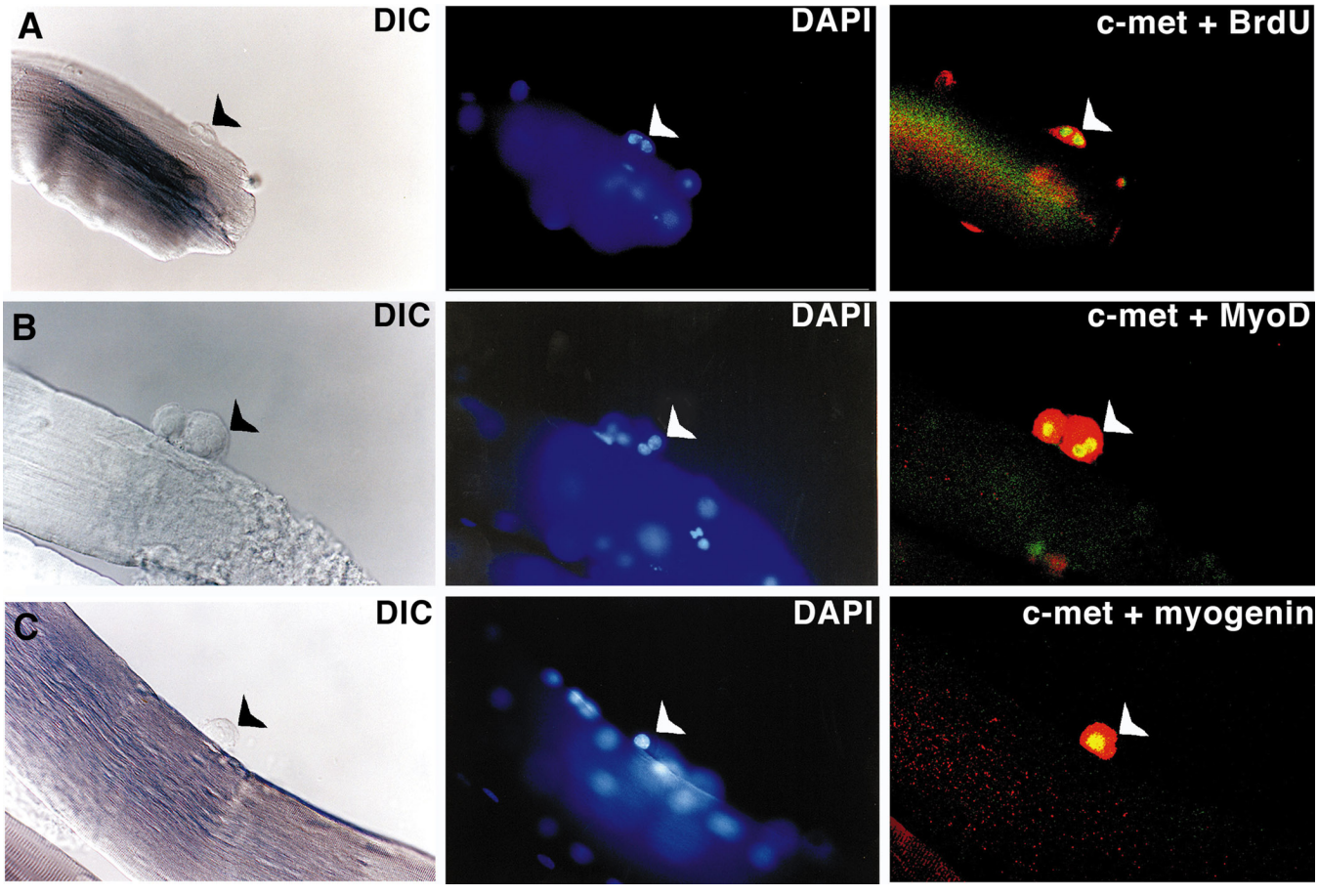

FIG. 4. (A) Myofiber stained and photographed after 48 hr in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing BrdU incorporation in nuclei of c-met-positive cells (400×). (B) Myofiber stained and photographed after 96 hr in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing expression of MyoD in nuclei of c-met-positive cells (400×). (C) Myofiber stained and photographed after 96 hr in culture shown in phase and with nuclei visualized with DAPI; composite confocal image showing expression of myogenin in the nucleus of a c-met-positive cell (400×).

is important because it suggests that all cells in the culture that are activated satellite cells by the criterion of MRF expression also express c-met. Myogenin protein expression was first detected after the time at which most satellite cells had begun to divide, but it was detected in both BrdU-positive and BrdU-negative cells in experiments where BrdU had been provided continuously (data not shown). Thus, a subset of satellite cells begin to express myogenin before they divide or, perhaps, without ever dividing. Myogenin expression was also observed in the nuclei of cells in the act of cytokinesis, so its expression is clearly not restricted to satellite cells that have already exited the cycle. c-met immunoreactivity was maintained when satellite cells fused with each other to form nascent myotubes on the surface of the cultured myotubes or on the dish (data not shown).

Satellite Cells Express c-met Protein in Intact Muscle

To determine whether the c-met expression observed in culture is a very rapid consequence of activation or is also a property of presumably quiescent satellite cells in intact muscle, sections of undisturbed leg muscle were examined for c-met immunoreactivity. c-met-positive cell outlines were detected at the edges of muscle fibers in transverse sections and were shown to reside beneath the basal laminae by costaining with anti-laminin (Fig. 5A). Costaining with DAPI showed that these outlines contain single nuclei, an observation which rules out the possibility that the c-met immunostaining belonged to small blood vessels rather than cells (Fig. 5B). Unactivated satellite cells do not express MyoD or myogenin (Grounds *et al.*, 1992); when costained with antibodies to these proteins no c-met-positive cells in muscle sections were seen to express either MRF. Thus, by anatomical criteria and by MRF status, c-met antigen was detected on quiescent satellite cells in intact muscle, as well as on satellite cells associated with myofibers immediately after isolation and during the course of fiber culture.

Because c-met RNA and protein were not detectable in muscle-derived fibroblasts or other mononucleate cells in our preparations, we believe that c-met will prove to be a

TABLE 1

Summary of Prior Studies of MRF Expression in Activated Satellite Cells

			MyoD					Myogenin					myf5					MRF4				
Reference	Satellite cell source	Assay	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96
Maley *et al.* (1994)	Primary MPC mass culture, adult SJL/J or Balb/c mice	DIG *in situ* Immunohist	−	+/−	+	+		−	+/−	+	+											
Smith *et al.* (1994)	Primary MPC mass culture, adult rat	RT-PCR	−	+	+	+	+	−	−	+/−	+	+	−	−	+	+	+	−	−	+	+	+
Yablonka-Reuveni and Rivera (1994)	Cultured adult rat fibers	Immunohist	−	+	+	+	−	−	−	+	+	+										
This study	Cultured adult mouse fibers	Immunohist single-cell RT-PCR	+/−	+	+	+	+	−	−	+	+	+	+/−	+	+	+	+	−	−	+	+	+

Note. For this table only those studies in which satellite cells were either isolated as a highly enriched population or identified individually by morphological means were included, since MRF gene expression in other preparations cannot be unequivocally assigned to satellite cells.

useful molecular marker for satellite cells within the context of myofiber cultures derived from healthy adult muscles. However, the uses of any molecular marker must be carefully matched with the context in which the putative marker was characterized. Additional studies in other muscle contexts will be needed to extend the range of application beyond those tested here. For example, while no cells other than satellite cells stained strongly for c-met protein in transverse sections through the midsection of healthy muscles, it remains possible that there will be other muscle-associated cell types that express c-met. In particular, Schwann cells are neuroglial cells present at neuromuscular synapses, and it has been reported that rat Schwann cells are responsive to HGF (Krasnoselsky *et al.*, 1994) so it is likely that they express the c-met receptor. We also do not know how the population of c-met-positive cells changes in damaged muscle where other cell types may become prominent, in addition to satellite cells (see Discussion).

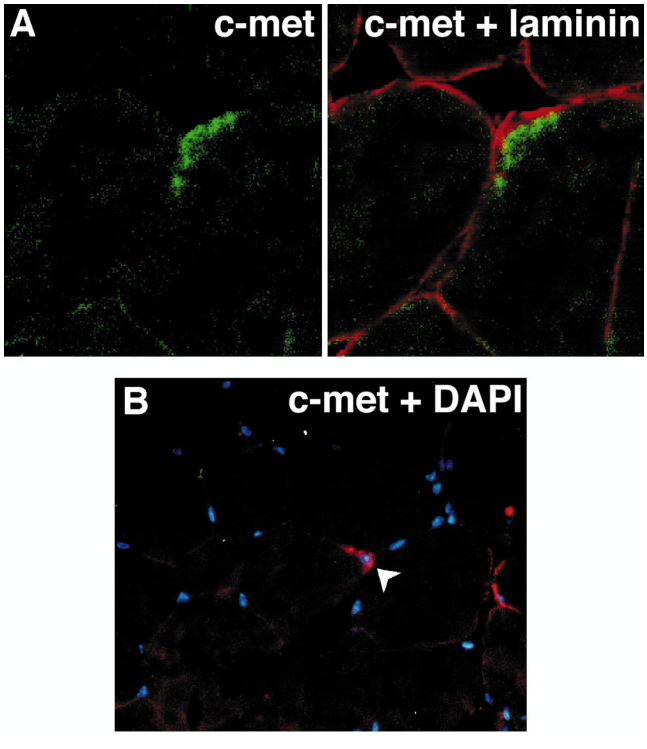

FIG. 5. (A) Confocal images of c-met and c-met + laminin in an unmanipulated muscle section showing localization of c-met expression (green) under the basal lamina (red) of a myofiber (1600×). (B) Composite photograph of a muscle section similar to B showing a c-met-positive (red) satellite cell and its nucleus (blue) (400×).

Six-Gene Multiplex Single-Cell RT-PCR: Rationale and Feasibility

As summarized in Table 1, it has been reported that all four MRF family regulators are expressed at the RNA level in mass cultures of activated satellite cells (Smith *et al.*, 1994). At the individual cell level, immunostaining studies with reagents directed against MyoD and myogenin showed positive cells on isolated myofibers and *in vivo* (Grounds *et al.*, 1992; Yablonka-Reuveni and Rivera, 1994). Because of the number of genes in the family and the absence so far of powerful immune reagents for MRF4 and myf5 proteins, the patterns of fourfold coexpression are not known for either embryo or adult muscle systems. However, our ideas about the mechanisms of MRF action as deduced from genetic and biochemical assays are strongly attached to and bounded by our knowledge of which members of the family are coexpressed at the cellular level. We therefore expanded the multiplex single-cell RT-PCR to characterize coexpression of all four MRFs together with m-cadherin and c-met.

The determination of six genes for each cell is a higher level of multiplexing than has been used previously with this protocol and so raises the important question of how

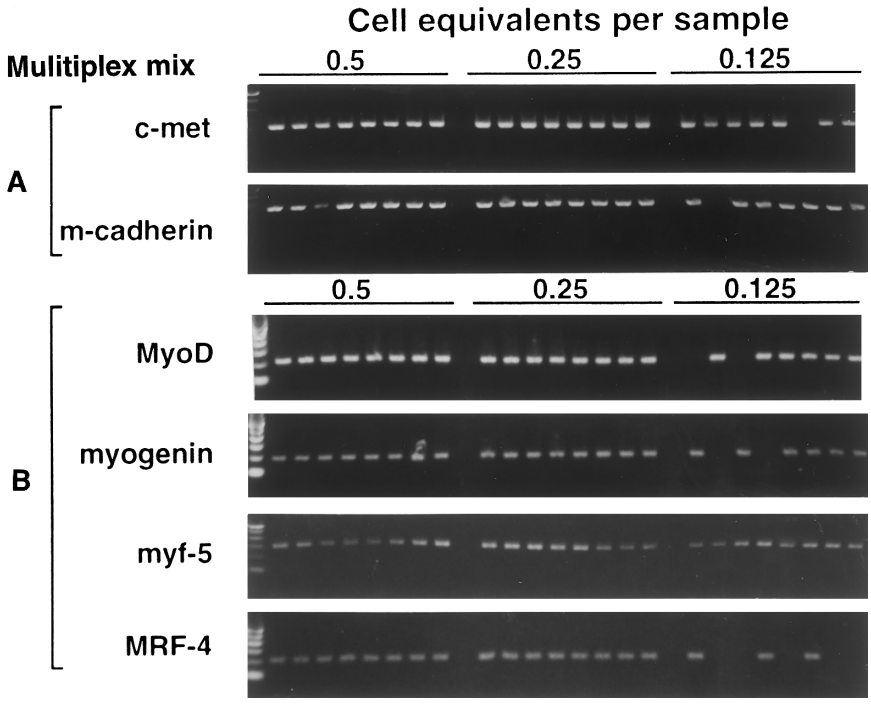

FIG. 6. Analysis of fidelity of multiplex single-cell RT-PCR when challenged with decreasing input cDNA per reaction. A set of individual satellite cells after 96 hr in culture were harvested, pooled, and reverse-transcribed; the resulting homogeneous cDNA was used in varying cell equivalents in first PCR containing primers for c-met + m-cadherin (Mix A), MyoD + myogenin + myf-5 + MRF4 (Mix B), or all six primer sets (Mix C, data not shown). Second reactions were run as usual and the products analyzed. Consistent positive results were obtained using from 0.25 to 0.125 of a cell equivalent of cDNA as input. It should also be noted that, even if every cell put in the pool had been harvested successfully (the usual yield is about 80%), only about half would be expected to express myogenin or MRF4, thereby increasing the effective dilution of the mRNAs for those genes.

robust these measurements are. Using this method, we have found that the question of fidelity and sensitivity must be investigated for each combination of genes to be used together, presumably because of sequence-specific interactions among primers. To evaluate fidelity for this gene set, we collected individual satellite cells from the 96-hr time point and pooled the cells together. The purpose of pooling was to create a single homogeneous master sample from which identical aliquots could be drawn and tested with the expectation that each sample should deliver the same answer. We then used this pool to make a series of identical measurements on samples containing 0.5 to 0.125 cell equivalents of starting material. We chose the 96-hr time point because we knew from our own data and from studies of satellite cell mass cultures that all four MRFs are expressed in at least some cells at this time. The assay scored positive in all samples for each of the six genes from 0.5 to 0.25 cell equivalents per reaction (Fig. 6). However, at 0.125 cell equivalents per reaction, individual gene determinations from different samples began dropping out, indicating that at this level there would be false-negative results, especially for mRNAs which are not expressed by all cells that made up the pool. We also note that if the same set of genes was used with all six primers grouped together (rather than split into group A and group B, as shown), the reactions began to fail even at the 1.0 cell equivalent input level. Further control experiments suggest that this failure is tied to the overall concentration of primers per reaction in the first-round PCR as well as overall sequence complexity of the primer mix. We also noted that for some primer sets that cross short introns, PCR bands corresponding to genomic DNA or to unprocessed hnRNA were sometimes detected. This indicates that the assay is sensitive to templates present in the range of one to a few copies per cell. False positives from nuclear DNA were easily distinguished from true positives by primer design as noted above, while other possible false positives from molecular or cellular contamination were controlled by taking control samples of fiber culture media at the beginning and end of each experiment (see Materials and Methods). Any experiment in which a media sample scored as positive was eliminated in its entirety. A PCR product band was harvested and sequenced for each gene and was shown to be the expected sequence, so we are confident that the bands shown represent specific amplification from the intended target. We conclude that for this six gene set, the assay has high fidelity and sensitivity down to at least 0.25 cell equivalents of input material, and all measurements reported here were

performed at 0.5 cell per reaction (half of each cell devoted to primer group A and half to primer group B).

Combinatorial Expression of MRF Regulators during Satellite Cell Activation

Single-cell data for coexpression of c-met, m-cadherin, myf-5, MyoD, myogenin, and MRF4 are summarized in Fig. 7. All cells shown were positive for c-met, and no cells were ever found that were positive for any of the other five genes, but negative for c-met. At time zero following fiber isolation, no cells expressed detectable myogenin or MRF4, and only a few were positive for m-cadherin, myf-5, or MyoD. We do not know whether the infrequent MyoD- or myf5-positive cells at time zero represent a small subset of cells that were activated *in vivo* before myofiber explant or whether these are the very first MRF-positive cells produced in response to activation at the time of explant and fiber preparation. By the 24 hr time point, many cells were singly positive for either MyoD or myf5 (32%), and many others (35%) were positive for both. The double-positive MyoD/myf5 cells might have begun as coexpressors or they may represent a second expression state that follows expression of just one of the pairs, as is currently thought to occur in the embryonic lineages (reviewed in Cossu *et al.*, 1996a). At 0- and 24-hr time points there appeared to be no correlation between MRF expression status and m-cadherin, although the fraction of m-cadherin-positive cells increased with time. At 48 hr we first observed cells that are myogenin positive, and note that these were always also positive for both MyoD and myf5 as well as m-cadherin. At this time MRF4 expression was detected for the first time and it was always expressed in cells that were also positive for myogenin; with only a few rare exceptions, MRF4 expression was restricted to cells scoring positive for all four MRFs. Because MRF4 RNA is expressed at relatively high levels in differentiated muscle, the observation that it is absent from all satellite cells at early time points argues further that the MRF mRNA present in our samples was due to harvested satellite cells and not to contamination from the adjacent fibers. The later time points saw further increase in the proportion of cells that were m-cadherin positive (100% by 96 hr). The only apparent correlation between MRF expression and m-cadherin expression was noted at 48 hr where all cells expressing myogenin were positive for m-cadherin and only half in the nonmyogenin compartments were m-cadherin positive. The timing of individual MRF gene expression summed over the population at each time point here agrees well with that reported previously (Table 1). Minor differences were detection of myf-5 and myogenin slightly earlier than by RT-PCR of rat mass cultures (Smith *et al.*, 1994) and persistence of myogenin expression longer than observed by antibody staining in rat fiber cultures (Yablonka-Reuveni and Rivera, 1994). Both could easily be explained by expected differences in assay sensitivities.

DISCUSSION

Molecular Markers of Satellite Cell Identity

Molecular measurements currently used to identify mouse satellite cells, including DNA synthesis when the adjacent muscle is damaged or removed into culture (Bischoff, 1986b), onset of MRF expression after activation (Grounds *et al.*, 1992), or myogenic differentiation in culture (Rando and Blau, 1994), cannot be used to identify satellite cells prior to activation and they also generally preclude further study of the cells. The observations reported here suggest that expression of the c-met receptor tyrosine kinase can serve as an effective molecular marker for quiescent or activated satellite cells in fiber culture, in preparations of mononucleate muscle-derived cells, and probably in sections of intact healthy muscle when coupled with associated anatomical criteria. Molecular markers in systems such as developing neural crest and the immune system have been critical for much progress, and the proposed use of c-met as a satellite cell molecular marker presents the prospect of a similarly important contribution to the study of adult muscle precursor cells and muscle regeneration. However, care must be taken in the application of c-met for marker purposes because molecular markers are necessarily context dependent. For any new or varied context such as damaged muscle from whole animals, the marker will have to be validated by appropriate independent assays akin to the positional definition of satellite identity used in this study. In particular, we do not currently know if any c-met-positive cells other than activated satellite cells will be present during a regeneration response *in vivo* when other diverse cell types such as immune cells invade the healing tissue. Some of these cells might express c-met, although there is no evidence for this at present. We also note that because c-met is a cell surface protein, it offers the future possibility of nondestructive identification and cell sorting, but the immune reagent used in this study is directed against an epitope located on the cytosolic portion of the receptor.

Functional Implications of c-met Expression Patterns

The observation that the c-met receptor protein is expressed *in vivo* by quiescent satellite cells and in culture by dividing and differentiating satellite cells has interesting functional implications. The first of these concerns the initial activation of satellite cells. A number of defined growth factors, including PDGF, IGF-2, EGF, and FGF, have been assayed for their effect on satellite cell activation and proliferation in culture systems. While these factors can enhance satellite cell proliferation once it has been initiated, Bischoff observed that only FGF appears capable of affecting initial activation but it is uncertain whether FGF factors are prominently available at the earliest times following damage *in vivo* (Bischoff, 1986b, 1990), and in other systems exogenous FGF was not found to elicit a response from quiescent satel-

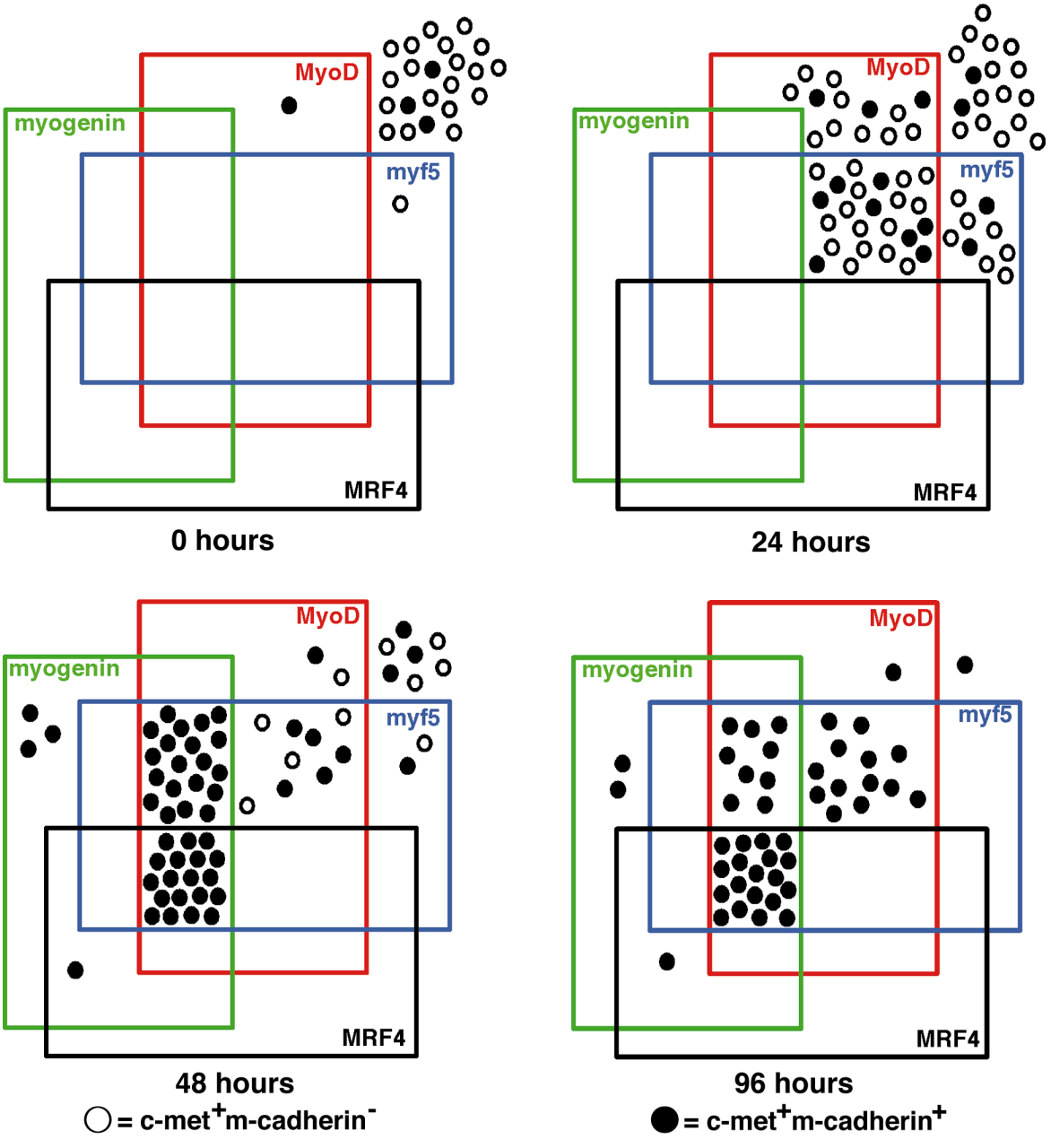

FIG. 7. Representational summary of the combined gene expression status for c-met, m-cadherin, MyoD, myogenin, myf-5, and MRF4 among single satellite cells at different time points. Open circles indicate m-cadherin-negative cells, and filled circles indicate m-cadherin-positive cells. The location of a cell within a colored rectangle indicates that the cell expressed that mRNA; cells in compartments formed by the overlap of two or more rectangles coexpressed those mRNAs.

lite cells (Johnson and Allen, 1993) or affect regeneration *in vivo* (Mitchell *et al.*, 1996). Saline extracts of crushed muscle (CME) were found to be able to initiate a response, but the CME active factor has not yet been purified to homogeneity (Bischoff, 1986b). HGF/SF is a candidate for the activating factor in CME because it shares several biochemical and biological properties with the mitogenic activity in CME (Chen *et al.*, 1994) including the ability to stimulate cultured rat satellite cells to divide precociously (Allen *et al.*, 1995). If HGF/SF is an activating factor *in vivo*, a key requirement is that its receptor be expressed on quiescent satellite cells in undisturbed muscle. Our observations show that this requirement is fulfilled for c-met. The proposed initiator role for the HGF/c-met signaling system in muscle regeneration is notably similar to HGF/c-met functions in several other tissues where it is also believed to

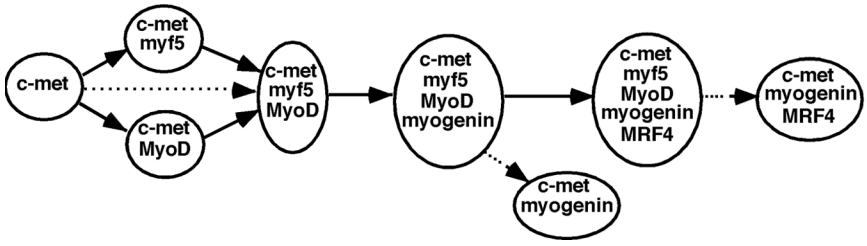

FIG. 8. Model of MRF coexpression status in satellite cells during the course of a regeneration response in fiber culture.

stimulate proliferative/regenerative programs following injury (reviewed in Matsumoto and Nakamura, 1996). Finally, the continued expression of the c-met receptor after activation suggests that it may also mediate one or more functions later in the regeneration response such as cell migration or morphogenesis as well as proliferation.

m-Cadherin Expression in Satellite Cells

We directly compared the expression of c-met with m-cadherin and found that m-cadherin-positive cells comprised only a small fraction of muscle satellite cells at the zero time point following fiber isolation, a time at which all satellite cells express c-met. From this result we conclude that it is very likely that m-cadherin mRNA is expressed by a subset of quiescent satellite cells in intact muscle, although we could not make the direct confirming measurements in intact muscle using this technique nor was double immunostaining possible using the current reagents. Our data support prior observations that m-cadherin is expressed on quiescent satellite cells (Irintchev *et al.,* 1994) but also emphasize that less than 20% of quiescent satellite cells are detectably m-cadherin positive at the earliest time, when all cells are c-met positive. The fact that m-cadherin-positive cells comprise a minor subset of an already rare cell population may account for other reports in which m-cadherin expression could not be detected in intact muscle (Moore and Walsh, 1993; Cifuentes-Diaz *et al.,* 1995). At later times following activation in culture, an increasingly large fraction of satellite cells expressed m-cadherin, and this is consistent with studies reporting robust m-cadherin expression during some *in vivo* regeneration paradigms (Moore and Walsh, 1993; Irintchev *et al.,* 1994; Cifuentes-Diaz *et al.,* 1995).

The significance of the c-met/m-cadherin double-positive cells at the zero time point compared to their more prevalent c-met-positive/m-cadherin-negative counterparts is uncertain. It is tempting to speculate that the early m-cadherin-positive cells represent a functionally distinct subclass, perhaps satellite cells programmed to differentiate quickly upon stimulation. This notion holds some attraction because m-cadherin mediates adhesion with other m-cadherin-expressing cells which could be useful for assembling clusters of early differentiating cells into the earliest myofiber framework.

MRF Expression-Type Progression

When single-cell expression typing for the four MRF regulators was pooled at each time point to recreate the entire cell population, the picture was very similar to prior studies of satellite cell mass cultures (Table 1). Our MRF expression results also agreed well with single MRF factor determinations made previously for satellite cells from fiber cultures that were similar to, though not identical with, ours. As in all culture preparations, it is important to recognize that the stimulus and course of satellite cell activation may be different from that in the intact animal, though the general course of MRF expression following injury is similar (Grounds *et al.,* 1992). The multiplex single-cell measurements also gave new insights not possible in prior studies of either satellite cells or embryo myoblasts and myocytes. For example, it has never been certain whether all four MRFs are ever coexpressed in individual cells or whether their joint presence within a tissue instead reflected multiple two- or three-member combinations. Data from our 48- and 96-hr time points clearly showed that simultaneous expression of all four MRFs is a preferred expression state concurrent with differentiation in this system. Cells positive for myf-5/MyoD/myogenin were also prominent at these times, and it is not clear whether all cells in our system will at some time express the full MRF set.

From the data in Fig. 7, we propose a model for the use of MRF regulators in the progression of fiber-associated satellite cells from their initial MRF-negative quiescent state through activation and into differentiation (Fig. 8). The model is based on the frequency of individual expression states as a function of time following activation. The resulting progression is quite simple because, with respect to the MRFs, only 6 of the possible 16 expression states were observed at significant frequencies at any time point (two other expression states, myogenin only and myogenin/MRF4, were represented by just 5 and 2 of 201 cells, respectively, and their significance therefore remains uncertain). In this model, cells enter the MRF-positive compartment by expressing either MyoD or myf5 alone. These cells are prominent by 24 hr, but became rare by 48 hr. It appears that these cells become positive for both MyoD and myf5 rather quickly, and it is possible that some cells enter the MRF-positive state by simultaneously expressing both determination class MRFs from the outset, as indicated. The

entry of cells into the MRF-positive pool by either MyoD-first or myf5-first pathways is reminiscent of determination class MRF expression in the developing embryo. In the embryo, different inductive signals are thought to initiate the expression of either myf5 (in the early dorsomedial lineage) or MyoD (in a later ventrolateral lineage) in two distinct sublineages of the myotome (Cossu *et al.*, 1996a,b; Yun and Wold, 1996). It will be interesting in future studies to explore the signaling pathways active in satellite cells to see if there is more than one and to examine possible relationships to MyoD- or myf5-mediated initiation. In the fiber-associated satellite cells, the myf5/MyoD double-positive state appears to be required for later myogenin expression which is absent at 24 hr but prominent by 48 hr. This differs from the earliest myotomal lineage in the embryo, where cells of the myf5-initiated dorsomedial domain apparently remain MyoD negative while they begin to express myogenin and to differentiate (Smith *et al.*, 1994; Yoon *et al.*, unpublished). This myf5/myogenin-positive, MyoD-negative expression state common in the embryo was never observed in activated satellite cells. MRF4 expression also becomes prominent at 48 hr in satellite cells, and it was detected exclusively in cells that also express myogenin. The vast majority of MRF4-positive cells (95%) expressed all four MRFs. We do not know the experimental or biological significance of two rare expression types, myogenin alone and myogenin/MRF4, but a simple possibility indicated in the model is that they arise by the downregulation of determination class MRFs. We cannot, however, rule out the less interesting possibility that they are simply low-frequency false negatives for both MyoD and myf5. Finally, it is noteworthy that even at late time points, there are a few cells that are c-met positive and negative for all four MRFs. It is uncertain if such cells expressed MRFs at any earlier time, but whatever their expression history, they may represent cells that possess muscle progenitor status at the time of the assay.

By coupling the use of c-met as a marker of satellite identity with the use of multiplex RT-PCR, as initially demonstrated here for the four MRFs and m-cadherin, it should be possible to characterize coordinate expression states for other genes involved in controlling and executing activation, proliferation, differentiation, and, perhaps, the quality of stem-cell-like renewal.

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Note added in proof. Allen *et al.* have detected c-met protein on rat satellite cells in culture (*Meth. Cell Biol.* **52,** in press) as well as on satellite cells in sections of intact rat muscle (personal communication and Tatsumi *et al.*, submitted.)

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