Cell Culture: Adherent Cultures

Myoblasts: C2C12 cells, a muscle cell line generated from the hind limb muscle of adult C3H mice (Yaffe and Saxel, 1977; Blau et al, 1983), were obtained from Dr Helen Blau (Stanford University). The cells were maintained in myoblast growth medium (C2GM) containing DMEM with 20% fetal bovine serum (FBS: Gibco) and antibiotics (50 µg/ml Penicillin and 60 µg/ml Streptomycin) in a 5% CO₂ environment. Cells were harvested for passaging or for performing experiments using Trypsin-EDTA (0.1% each, in PBS). Cultures were never allowed to grow beyond 70% confluence except when cultured for differentiation.

Subcloning: Cells were plated at a density of 100 cells per 100 mm diameter tissue culture dish (55 cm² area). After allowing the cells to multiply and form isolated colonies, the clones were picked using cloning rings and plated in 24-well dishes for further expansion. Individual clones were tested in suspension cultures (described below) and frozen for further use.

Cryo-preservation of cells: Cells were trypsinised, resuspended in growth medium and counted. Cells (myoblasts or fibroblasts) were collected by centrifugation and resuspended at a density of 5 X 10⁵ cells/ml in freezing medium (freezing medium: half volume growth medium, half volume FBS with 10% DMSO-final concentration). Cells resuspended in freezing medium were distributed in freezing vials (1 ml aliquots) and stored at -70°C overnight in an insulated container (for slow freezing). Frozen vials were transferred to liquid nitrogen for long-term storage.

Differentiation: Cells were grown to around 90% confluency. The C2GM was removed, cells rinsed with PBS to remove residual growth factors and differentiation medium added (DM: DMEM with 2% horse serum and antibiotics). DM was replaced every 24 hr for 3 to 6 days.
Multinucleated fibres become apparent by 24-48 hr in DM and by 72 hr the fusion index (percentage nuclei in myotubes) was 60-70%.

**Fibroblasts:** C3H 10T1/2 cells, a fibroblast cell line generated from C3H mice, obtained from Dr Helen Blau (Stanford University), were maintained in DMEM with 10% FBS and antibiotics (fibroblast growth medium-FGM). Cultures were expanded upon reaching 70% confluency.

**Serum starvation:** Fibroblasts cultured in FGM were shifted to low-serum medium (2% FBS) after rinsing with PBS to remove growth medium. Growth arrest was induced by maintaining the cultures in low serum (without change of medium) for 30 hr. To stimulate reentry into cell cycle, cells were treated with FGM for various times.

**Cell Culture: Suspension Culture**

*Preparation of 2% Methylcellulose medium:* Methylcellulose powder (Methocel 4000 centipoise; Sigma) was autoclaved in a wide-mouthed media bottle along with a stir-bar. Half of the final volume of DMEM, heated at 56°C for 10 min, was added to the bottle and stirred at room temperature for 30 min. The remaining volume of DMEM with antibiotics was warmed to 37°C and added to the bottle and stirred for 30 min more. Stirring was continued at 4°C overnight until the medium became viscous and clear and then transferred to 50 ml polypropylene centrifuge tubes (Corning). Undissolved fibres and other debris were removed by centrifugation at 2500 rpm (Sorvall H1000B horizontal rotor) for 30 min before storing at 4°C ready for use.

*Suspension culture of myoblasts and fibroblasts:* (A schematic representation of the suspension culture protocol is presented in Fig. 4, Chapter 3). Suspension mix containing the following components was prepared and filter sterilized.
<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (at 37°C)</td>
<td>20% for myoblasts and 10% for fibroblasts</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4 mM</td>
</tr>
<tr>
<td>HEPES pH 7.5</td>
<td>10 mM</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>1X (50µg/ml Penicillin &amp; 60 µg/ml Streptomycin)</td>
</tr>
</tbody>
</table>

Myoblasts or fibroblasts were harvested from adherent cultures using trypsin-EDTA and resuspended in fresh GM by titration to obtain a single cell suspension as ascertained by visual examination under microscope. The cells were added to the pre- aliquoted suspension mix and the required volume of Methocel (at 37°C) in a 250 ml Nalgene centrifuge bottle, to obtain a final density of 10^5 cells/ml. The bottle was swirled vigorously to distribute the cells evenly (checked by visual examination) and then incubated for the required times in 5% CO₂ at 37°C with the cap loosened.

To harvest cells from methocel cultures, the culture was diluted with warm (37°C) PBS to 5 times the volume and mixed well. The diluted suspension was then spun to pellet cells at 2500 rpm (Sorvall H-1000B horizontal rotor) for 20 min at room temperature. The supernatant was drained out gently to prevent cell loss and the pellet resuspended well in PBS by pipetting. After a second wash at 1500 rpm for 10 min the cell pellet was either harvested directly for molecular analysis or resuspended in GM and replated on tissue culture plates for analysis of reactivation.

**Cell Culture: Hybridoma cells**

Hybridoma cells producing monoclonal antibodies F1.652 (specific to fetal myosin heavy chain) or A4.1025 (against all myosin heavy chains), were cultured in DMEM supplemented with 10% FBS and antibiotics (concentrations as described above). In order to collect antibodies secreted into the culture medium the cells were allowed to grow to high confluence. The culture supernatant was collected after the
beginning of cell death in the medium. The cell debris was separated from the supernatant by centrifugation. The antibody solution was further cleared by sterile filtration through 0.2 μ filters and stored frozen in the presence of 0.05% sodium azide.

**Cycloheximide treatment of myoblasts**

Cells were grown in C2GM to approximately 70% confluence. Cycloheximide (Chx) (from a 10 mg/ml stock solution in water, Sigma) was added to the culture medium to a final concentration of 10 μg/ml and the cells maintained in Chx medium for 8 hr. To inhibit translation in suspended cells, Chx was added to the Methocel suspension to a final concentration of 10 μg/ml and mixed vigorously. Translation was inhibited during reattachment of suspended cells by replating for various times in C2GM containing Chx.

**Actinomycin-D treatment of cultured myoblasts**

In order to inhibit transcription in myoblasts, Actinomycin D (Act-D) (Sigma) was added to a final concentration of 5 μg/ml (from a 2 mg/ml stock solution in ethanol) to cultures of growing and suspended or replated myoblasts. The period of treatment varied from 2-8 hr.

**Detection of DNA synthesis in cells by bromodeoxyuridine incorporation**

Cells for bromodeoxyuridine (BrdU) incorporation assays were plated on coverslips at subconfluent densities. BrdU (stock: 100 mM in PBS) was added to the culture medium to a final concentration of 100 μM and incubated for 15 min for pulse labeling or 15 μM for varying periods for cumulative labeling.

Following labeling, the medium was removed, cells washed with PBS and fixed in 70% chilled ethanol at 4°C for 30 min. After two rinses
with PBS, non-specific binding was blocked with 10% horse serum in PTX (PBS with 0.5% TritonX100) overnight at 4°C. The primary antibody (mouse monoclonal anti-BrdU 1:200, Sigma) diluted in the blocking solution was applied to the cells 1 hr. Secondary antibody (1:200, biotinylated horse anti-mouse IgG, Vector Labs) incubations were for 30 min. AB complex (avidin-biotin-Horse radish peroxidase) was prepared by mixing and incubating solutions A and B (Vectastain Elite kit, Vector Labs) for at least 30 min and cells were incubated with ABC for 30 min. All antibody and reagent incubations were interspersed with at least three 1 hr washes in PTX. The presence of the peroxidase was detected using the chromogenic substrate diaminobenzidine (DAB), in the presence of hydrogen peroxide. The frequency of positive nuclei (dark brown in colour) was estimated using a Zeiss Axioscop microscope equipped with DIC optics. At least 3 fields (a total of ~250 cells) were counted for each sample and the percentage of positive cells calculated.

Alternatively, biotinylated horse anti-mouse IgG (1:200, Vector Labs) and avidin-FITC (1:50, Vector Labs) was used for detection of BrdU and positive nuclei were visualized by excitation at 494 nm using a Zeiss microscope equipped with epifluorescence. Nuclei were counterstained with 0.4 μM Hoechst 33342 (Molecular Probes) to aid in counting.

**Immunofluorescence on cultured cells**

*Myosin heavy chain (MHC):* The cells were seeded on coverslips as described for BrdU assays. For differentiation, cells on coverslips were incubated in DM for at least 3 days. Myotubes and myoblasts were fixed in 2% formaldehyde in PBS for 5 min after a wash with PBS. Following permeabilization in cold methanol (-20°C) for 5 min, blocking was performed in 5% horse serum in PTX for 3 hr at room temperature. Anti-MHC mouse monoclonal hybridoma (A4.1025 from H. Blau, Stanford University) supernatant (prepared in-house) was applied to cells at a dilution of 1:3 (in blocking buffer) for 1 hr. Cells were incubated in
secondary antibody, biotinylated horse anti-mouse (1:200, Vector Labs) and AMCA-avidin (1:50, Vector Labs) for 30 min each. All washes were done for at least 1 hr in PTX. Stained cells were visualized after excitation at 349 nm. Nuclei were counterstained with propidium iodide (1 μg/ml).

**Fluorescent detection of actin**

For detection of actin microfilaments, a fluorescent probe Phalloidin-Oregon Green was used. Asynchronous myoblasts were plated on coverslips and allowed to attach for at least 24 hr. Arrested myoblasts (48 hr in suspension) were allowed to attach for 15 min for ease of staining. To detect microfilaments in reactivated cells, suspended myoblasts were replated on coverslips in C2GM for varying periods of time. The coverslips were rinsed twice in PBS and cells fixed with 3.5% formaldehyde in PBS for 5 min. Cells were permeabilized using 0.1% Triton X-100 (in PBS) for 15 min. Actin microfilaments were stained with Phalloidin-Oregon-Green (1:50) in PBS for 1 hour. DNA was counterstained with Hoechst 33342. All intervening washes were in PBS. The coverslips were mounted and viewed using FITC filter (Oregon Green excitation wavelength is 496 nm).

**RNA isolation from cultured cells and tissue samples**

RNA was isolated from cultured cells or tissue using Trizol (Gibco) or Trireagent (Sigma) (monophasic phenol-guanidinium HCl solution) as per the manufacturer’s instructions (same procedure for both).

*For cells in culture:* Adherent cultures were washed three times with cold PBS and drained. The required amount of Trizol (1 ml per 5 X 10^6 cells) was added to the tissue culture flask/plate and the cells were scraped into it using a cell lifter (Fisher). The lysate was vortexed vigorously to disrupt the cells and then stored at ~20°C until isolation. For isolation of RNA, the lysate was thawed at room temperature. Chloroform was added (0.2 ml per ml of Trizol) for phase separation, vortexed and
incubated for 15 min at room temperature before centrifugation at 13,000 rpm (Biofuge-pico, Heraeus Instruments) for 10 min at 4°C. The aqueous phase was collected into a fresh tube and 0.5 ml isopropanol (per ml of Trizol) added for precipitation of RNA. The pellet obtained after centrifugation for 20 min at 4°C was washed in 70% ethanol, air dried briefly and dissolved in autoclaved distilled water. The typical yield was ~18-20 µg/10⁶ cells. The RNA was quantified by spectrophotometry at 260 nm and 280 nm. The quality of the RNA was checked by agarose gel electrophoresis of 1 µl of RNA solution in a 2% formaldehyde gel.

For tissue samples: The animal was sacrificed, tibilalis anterior (TA) muscle dissected free of other tissue and collected without delay. The tissue was chopped into small pieces using a sterile scalpel, weighed, placed in polypropylene 5 ml tubes (Falcon) and snap-frozen in liquid nitrogen for storage at −70°C until RNA isolation. For isolation of RNA, the tissue was thawed on ice in the presence of the required amount of Trizol (2.5 ml/ 100 mg tissue). Following homogenization using a Polytron (Kinematica) fitted with a 0.5 cm probe, the tissue slurry was aliquoted into microfuge tubes and the debris removed by centrifugation. The clear supernatant was used for extraction of RNA as described for cells.

Differential Display PCR

RNA sample preparation: Growing myoblasts (Mb) and suspended myoblasts (S) were cultured as previously described. To remove residual growing cells from the myotube (Mt) cultures, the culture was treated with cytosine arabinoside (10⁻⁵ M) for an additional 2 days after a 3-day differentiation period. Total RNA from the cultures was isolated using Trireagent (Sigma) as described above. The quality of the RNA was checked on denaturing formaldehyde-agarose gels and the expression profiles for growth regulatory and differentiation specific genes were characterized (Fig. 11) using Northern blot analysis.
To remove contaminating genomic DNA, 100 μg each of total RNA from Mb, S and Mt samples was subjected to DNase (10 U) treatment according to the MessageClean kit (GenHunter) instructions, for 30 min at 37°C. The RNA was extracted with Tris-saturated phenol, precipitated and dissolved in DEPC-treated water and quantified using spectrophotometry. Equal amounts were loaded on a denaturing agarose gel and the quantification was visually confirmed.

Reverse transcription: Reverse transcription (RT) reactions were performed with 0.2 μg of DNase-treated total RNA from each sample (Mb, S, Mt) in the presence of each of the three anchored primers (HT11G, HT11C, HT11A in separate reactions) supplied with the RNAimage kit (GenHunter) using MMLV Reverse Transcriptase as specified by the manufacturers.

Anchored primers were:

HT11G  5'-AAGCTTTTTTTTTTTG-3'
HT11C  5'-AAGCTTTTTTTTTTTCC-3'
HT11A  5'-AAGCTTTTTTTTTTTTTA-3'

The reaction mix with RNA, primer and the reaction buffer was heated at 65°C for 5 min to denature the RNA and the primers were allowed to anneal to the mRNA at 37°C for 10 min before adding the enzyme. The reaction was incubated at 37°C for 50 min and the enzyme inactivated at 75°C for 5 min. The RT products were stored at −20°C.

Because of the use of 3 different anchored oligo-dT primers, the RT reactions divided the total mRNA population in the cells into 3 sub-populations thus decreasing the complexity of the cDNAs generated (Fig. 10, Chapter 4). A number of arbitrary primers were used for PCR in combination with the same anchored primers as in the RT reactions.
**Polymerase Chain Reactions:** All PCR reactions (20 µl total volume) were performed in duplicate, using 2.0 µl of the RT products using the same anchored primer used for RT and one arbitrary primer in the presence of 0.2 µl of α-33P-dATP (2000 Ci/m mole, NEN). Low concentrations (2µM) of dNTP were used to increase the specific activity of the products. The template was denatured at 94°C for 30 sec, the primers annealed at very low stringency (40°C) for 2 min and extension performed at 72°C for 30 sec for 40 cycles. A final extension was also performed at 72°C for 5 min.

A total of 14 arbitrary primers were used for PCR reactions and those which yielded differential cDNA fragments were

- **H-AP1**: 5’-AAGCTTGATTGCC-3’
- **H-AP12**: 5’-AAGCTTGAGTGCT-3’
- **H-AP15**: 5’-AAGCTTACGCAAC-3’

**Gel separation of PCR products:** The radioactively labeled PCR products were separated on a 6% urea-polyacrylamide gel for 3 hr at 1700 constant voltage. After drying the gel onto Whatman#3 paper in a vacuum gel dryer at 80°C it was stapled to X-ray film (Biomax MR films-Kodak) to allow exact alignment and exposed without an intensifying screen (for better resolution) at room temperature overnight.

**Identification of differentially expressed fragments:** Differentially expressed cDNAs were identified on the autoradiogram as the bands that were expressed in both samples from arrested myoblasts and not in any sample from growing or differentiated cells. The dried gel (with Whatman paper) excised from the corresponding region after alignment with the autoradiogram. The DNA was eluted from the gel by boiling in 100 µl water for 15 min after prior soaking and the eluate was precipitated using ethanol. The precipitate was dissolved in 10 µl of water, of which 4 µl was used for re-amplification with the same set of primers and same
conditions as used before but with 20 \( \mu M \) concentration of dNTPs to increase product yield.

**Validation of differential expression:** To confirm differential expression of the cDNA fragments isolated, Northern analysis was used. The re-amplified fragments were labeled using same PCR conditions as used for the differential display. Low dNTP concentrations (2\( \mu M \)) were used and 2\( \mu l \) of \( \alpha^{32}P \) dATP (\( >3000 \) Ci/mmol: BRIT) was added for labeling. Northern hybridizations were performed with 10 \( \mu g \) RNA at 60\(^\circ\)C in order to confirm the differential expression of the isolated fragments. Post-hybridization washes were performed at 55\(^\circ\)C (2X SSC, 0.1\% SDS and 1X SSC, 0.1\% SDS).

**Preparation of probes for Southern and Northern hybridization**

All probes used in this work were labeled by either of the following two methods: random priming (RP) (Feinberg and Vogelstein, 1984) or PCR labeling (Genhunter instructions).

*Random priming* was performed using an RP kit (BRIT). Heat-denatured template DNA (approx. 100 ng of insert or whole plasmid) was added to a reaction mix containing 0.8 mM of all dNTPs except dATP, random primer and Klenow enzyme (2 U). The reaction was incubated at 37\(^\circ\)C for 1.5 hr in the presence of 50 \( \mu Ci \) (5 \( \mu l \)) of \( \alpha^{32}P \)-dATP (\( >3000 \) Ci/mmol: BRIT).

*PCR labeling* was done in the presence of low amounts of all four dNTPs (2 \( \mu M \)) and 2.0 \( \mu l \) (20\( \mu Ci \)) of \( \alpha^{32}P \) dATP. All other conditions were the same as for cold PCR reaction that had been optimized for that particular probe. 40 cycles were used for labeling probes by PCR.

The labeled probes were purified from the unincorporated nucleotides by size exclusion chromatography: spin-column (Sephadex G-50, Pharmacia) or gravity column (Biogel P-60, Pharmacia). The purified
probes were heat-denatured and added to the blots at a concentration ＞10^6 cpm/ml of hybridization solution.

Note: Although labeling conditions have been kept constant through this study, due to inconsistencies in the quality of the label (α-32P dATP: BRIT) the specific activities and consequently the sensitivity of the probes used vary. Labeling reactions that yielded less than 8 X 10^6 cpm were not used for hybridization.

**Northern and Southern hybridizations**

For Southern hybridizations DNA samples were separated in agarose gels (1-1.5%). Prior to transfer onto Hybond N (Amersham) or Micron Separations Incorporation (MSI) membranes the DNA in the gel was alkali denatured with an alkaline solution (1.5 M NaCl, 0.5 N NaOH) for 20 min. All subsequent steps were the same as used for Northern hybridizations.

For Northern hybridizations 10-20 μg of the total RNA samples were separated in 1% agarose gels containing 2% formaldehyde, transferred to Hybond N membranes by capillary method using 6X SSC and immobilized by UV crosslinking. The crosslinking time was optimized on damp blots and a time of 30 sec at the automatic energy setting was used (Amersham UV-crosslinker). Hybridizations were performed in 7% SDS, 0.5 M sodium phosphate and 1 mM EDTA (Kevil et al, 1997) at 65°C. Post-hybridization, the blots were washed with 1X SSC, 0.1% SDS and 0.1X SSC, 0.1% SDS at 65°C for 20 min each. Hybridization was detected either by autoradiography (exposures ranged from 2 days to 1 week) or on a Fuji BAS-1800 Phosphor Imager (typically overnight- 5 day exposures). The L-Process and Image Gauge programs (Fuji) were used to quantify background-subtracted signals.
Colony hybridizations

Bacterial colonies obtained after transformation of ligation products (different inserts into bacterial vectors) were screened for clones containing inserts by colony hybridizations (Buluwela et al, 1989). The colonies were “patched” on duplicate LB plates, one with Hybond N membrane and one without. Following growth overnight, the master plate was stored at 4°C and the membrane from the duplicate plate was placed on a Whatman #1 paper soaked in 2X SSC with 5% SDS for 2-5 min for lysis of bacteria. The DNA released from the cells was fixed onto the membrane by microwaving at a setting of 650 W for 2.5 min and the cell debris was removed from the membrane by gentle scrubbing with tissue paper soaked in a washing solution (5X SSC and 0.1% SDS). After a final wash in the same solution for 10 min the membranes were patted dry between Whatman paper and used for Southern hybridization with the labeled purified insert as probe. Positive colonies were then identified on the master plate and glycerol stocks prepared for long-term storage.

Northern affinity capture and purification of DD-PCR products

Affinity capture was performed to separate individual fragments from a mixture of fragments of identical size obtained from DD-PCR. These fragments hybridized to different mRNAs on the Northern blot (Li et al, 1994). The autoradiograms generated from the Northern blots were aligned with the blot and the region containing the differentially expressed band was cut out from the blot. The PCR-generated probe (cDNA fragment) that bound to the piece of membrane was eluted out by boiling the membrane in 100 µl water for 5 min. The eluted probe was precipitated and dissolved in 10 µl water and 4 µl of this product was used for re-amplification by PCR using the same conditions as used before. Purified fragments were labeled and re-tested on fresh Northern blots for confirmation.
Nuclear Run-On Assays

Harvesting nuclei: Cultures were prepared to obtain the required numbers of cells (Mb: $2 \times 10^7$, S48 & R2: $3 \times 10^7$, Mt: $2 \times 10^7$) (Note: More cells were allocated for S48 and R2 to compensate for cell loss during harvesting). All subsequent steps were performed in the cold room, at 4°C using pre-chilled buffers and plastic ware. To cells washed with ice-cold PBS was added 2 ml (for every $1-1.5 \times 10^7$ cells) of NP40-lysis buffer (10 mM Tris pH7.5, 5 mM MgCl$_2$, 10 mM NaCl and 1% NP40) and the cell lysates collected into a tube. The lysate was vortexed gently (setting 6: Vortex Genie) and incubated on ice for 5 min while complete lysis of plasma membrane and release of nuclei was ascertained by microscopic examination. The nuclei were collected by centrifugation at 1000 rpm (Sorvall H-1000B horizontal rotor) at 4°C for 5 min and the pellet resuspended in 5 ml of the same lysis buffer by vortexing. After incubation on ice for 5 min the nuclear pellet was collected by centrifugation and resuspended thoroughly in 100 µl (per $1 \times 10^7$ cells) of glycerol storage buffer (50 mM Tris pH 8.3, 40% glycerol, 5 mM MgCl$_2$, 300 mM KCl, 0.1 mM EDTA) by pipetting. The suspended nuclei were frozen and stored in liquid nitrogen until further use.

Run-on transcription: Run-on reactions were performed according to the protocol of Greenberg and Ziff (1984) with some modifications. Nuclei were thawed in the presence of 120 µl of pre-warmed 2X reaction buffer [0.5 mM each of rATP, rCTP and rGTP, 1.3 µM rUTP, 2.5 mM DTT and 200 µCi $^{32}$P-α-rUTP (3000 Ci/mmol)] and incubated at 30°C for 30 min with mixing every 10 min. After the transcription reaction, the nuclear proteins were digested in 100 µl of pre-warmed proteinase K buffer (0.5 M Tris pH7.4, 0.125 M EDTA, 5% SDS) with 0.2 mg Proteinase K enzyme at 37°C for 30 min. The released genomic DNA was sheared by passing through a 24-gauge needle and removed by Trizol-extraction of the mix. The RNA extracted was precipitated on ice for 30 min with
equal volumes (850 µl) of ice-cold TCA (10% TCA with 60 mM pyrophosphate) in the presence of 7.5 µg of yeast tRNA. The RNA pellet was washed in 5% TCA (with 60 mM pyrophosphate) and resuspended thoroughly in TES (10 mM Tris pH 8.0, 1 mM EDTA, 1% SDS). The dissolved labeled RNA was re-extracted with Trizol as before and dissolved in TES for hybridization.

*Preparation of dot blot:* Plasmid DNA was diluted to a concentration of 5 µg/200 µl. The DNA was denatured at 65°C for 30 min in the presence of NaOH (0.3 N final concentration). After neutralization with NH₄OAc (1.5 M final concentration), the samples were spotted on a Hybond N membrane pre-wetted in 6 X SSC using a 96-well dot-blot apparatus (Schleicher and Schuell Inc.) and the DNA was UV-crosslinked on to the membrane. Four replicates of each dot blot membrane were prepared, one each for each labeled RNA sample (Mb, S48, R2, Mt) derived from the run-on reactions.

*Hybridization and detection:* The membranes were prehybridized and hybridized as described for Northern hybridizations. Equal counts (~2-3x10⁶ cpm) of each run-on sample were used for hybridization to the respective membranes. Hybridizations were performed in microfuge tubes (the membranes rolled for placement) with 1 ml hybridization solution for 36 hr. Initial washes were done with 2 X SSC at 65°C for 1 hr and 15 min each. Membranes were treated with RNase (10 µg/ml) at 37°C for 30 min in 2 X SSC and a final wash was done at 37°C in 1 X SSC for 15 min.
**Primers And PCR Conditions**

**L7: ribosomal protein (202 bp)**  
Rawls et al, 1995

<table>
<thead>
<tr>
<th>L71</th>
<th>GGA GCT CAT CTA TGA GAA GGC</th>
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<tbody>
<tr>
<td>L72</td>
<td>AAG ACG AAG GAG CTG CAG AAC</td>
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</table>

94°C  
62°C  
72°C  

72°C  

5:00 min

**GAPDH (290 bp)**  
Cornelison and Wold, 1997

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<tr>
<th>GAPDH1</th>
<th>GTG GCA AAG TGG AGA TTG TTG CC</th>
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<tr>
<td>GAPDH2</td>
<td>GAT GAT GAC CCG TTT GGC TTC</td>
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94°C  
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72°C  

72°C  

5:00 min

**MCK (160 bp)**  
Rawls et al, 1995

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<tr>
<td>MCK2</td>
<td>GAT GGG ATC AAA CAG GTC CTT G</td>
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94°C  
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72°C  

72°C  

5:00 min

**Myf5 (379 bp)**  
Rawls et al, 1995

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<td>Myf5-2</td>
<td>GGC TGT AAT AGT TCT CCA CCT GTT</td>
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62°C  
72°C  

72°C  

5:00 min

**LIX (1461 bp)**  
this work

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<td>LIX3</td>
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58°C  0:30 min
72°C  2:00 min

TTP (1705 bp) this work

TTP5: AAT ACC GCG GTC TCT TCA CCA AGG CCA TTC
TTP3: CCC CGC GGT AGC AAT ATA TTA ATA TAT TAT AGC

94°C  1:00 min
50°C  1:00 min
72°C  3:00 min

72°C  5:00 min

Znf216 (732 bp) this work

FZCOD AAA ATA TGG CTC AGG AGA C
RZCOD CAA AGG AAA ATG GCC ATG C

94°C  0:30 min
50°C  0:30 min
72°C  1:00 min

72°C  5:00 min

CD34 (442bp) Beauchamp et al, 2000

FCD34 GAGAATTCTGGAATCCGAGAAGTGAGGT
RCD34 ACTCTAGAACCAGCCTTCTCCTGTAG

94°C  0:30 min
55°C  0:30 min
72°C  0:30 min

72°C  5:00 min

To amplify inserts from common bacterial vectors using T7, T3, M13F or M13R primers

94°C  1:00 min
94°C  0:30 min
50°C  0:45 min
72°C  1:00 min

72°C  5:00 min
RT PCR of CD34

To remove genomic DNA contamination, RNA from different samples were treated with RQ1 RNase-free DNase (1 U, Promega) for 30 min at 37°C and the enzyme inactivated at 75°C for 15 min. Samples were checked for absence of DNA by PCR with L7 primers. RT reactions were performed (with approx. 2.5 μg of RNA) using the Advantage RT-for-PCR kit (Clontech) according to the manufacturer’s instructions. The volumes of RT products were normalized so as to obtain equal amount of L7 PCR product. The number of cycles at which the RT-PCR products became detectable by ethidium bromide staining were determined; 24 cycles for L7 and 29 cycles for CD34. All RNA samples from time course experiments were analyzed in duplicate for L7 and CD34 and the products were separated on agarose gels. Products were quantified after performing Southern hybridization (with the respective labeled probes i.e. L7 and CD34) and phosphor imager analysis. For each sample, the CD34 signal was normalized with respect to the L7 signal.

Freeze injury of skeletal muscle in vivo

All experiments on animals were performed in accordance with the CCMB Institutional Animals Ethics Committee. C57BL/6 or Balb/c adult mice (approx. 3 months old) were obtained from the CCMB Animal House. Animals were anesthetized by intraperitoneal injection of 2.5% Avertin (100% contains 1 g tribromoethylalcohol per ml of tertiary amyl alcohol) at a dose of 375 μg/g body weight. Anesthesia was deemed complete when a tail pinch elicited no response. The anesthetized animal was laid on its back on the dissection stage, and the limbs stretched and secured with adhesive tape. The tibialis anterior (TA) muscle was made accessible for manipulation by crossing one leg over other and taping these down. A very small nick was made in the skin of the top leg, just above the surface of the TA, the incision held open with a pair of forceps and a sharp piece of dry ice applied directly to the belly of the muscle for
15 sec (Dhawan et al, 1996) (initial experiments established the length of dry ice exposure required for reproducible injury and regeneration). The nick was sutured using a curved sterile suture needle with silk braid (Surgical Specialties Corporation, USA) and the animal was allowed to recover in a warm chamber. Incisions on animals used for recovery of tissue at the early time points after injury (1-6 hr post injury) were left unsutured.

**Cryosections of muscle tissue**

Animals were sacrificed by CO₂ inhalation. The TA muscle (used for all studies) was dissected from the animal immediately after sacrifice. The muscle was laid in a bed of Histoprep (Fisher) placed in a 1 cm² cryomould (Fisher) and the mould was floated on an isopentane-bath pre-chilled in liquid nitrogen for 15 sec. Frozen tissue was stored at -70°C until use. Cryosections (20 μ) were cut using a Leica Cryocut 1500 cryostat, and collected on gelatin-coated slides for histochemistry and immunofluorescence, or on Probe-On Plus (Fisher) positively charged slides for RNA in situ hybridization (ISH). The sections for histology were stored at -70°C until use but used within a week of cutting for ISH.

**Gelatin coating of slides**

1 g of gelatin was dissolved in 200 ml of water by heating and 0.1 g of CrK(SO₄)₂.12H₂O (potassium chromium alum) and 0.5% (final concentration) of sodium azide were added to prevent bacterial growth. The slides arranged in a slide holder were dipped in the gelatin solution, the excess solution drained and the slides dried overnight in dust free environment.

**Hematoxylin and Eosin staining**

*Preparation of Harris hematoxylin solution:* 25 g of potassium alum was dissolved in 250 ml water by warming and 1.25 g of hematoxylin
(Sigma) dissolved in 12.5 ml of 100% ethanol was added to this solution. After bringing the mix rapidly to boil, 0.625 g of mercuric oxide was added and the whole solution cooled before 10 ml of glacial acetic acid was added. The solution was filtered through crude filter paper before use.

Staining of frozen sections: The sections were thawed at room temperature for 15 min and were stained with Harris hematoxylin solution for 1 min and with 1% eosin (Sigma) solution for 2 min. Washes were done between every two steps in running tap water. Before mounting in Permount (Fisher), the sections were dehydrated through a series of ethanol, ethanol:xylene (1:1) and finally xylene for 2 min each.

Immunofluorescence for fetal myosin isoform

Sections were thawed and the moisture dried before they were ringed with a hydrophobic PAP pen. Blocking to prevent non-specific binding was done with 10% horse serum in PBS for at least 1 hr. Sections were incubated in F1.652 hybridoma supernatant raised against the fetal myosin heavy chain. Tween 20 (0.1% final) was added to reduce non-specific binding. The F1.652 hybridoma is a gift from H. Blau, Stanford University (supernatant was prepared by culturing the hybridoma, described above). Goat anti-mouse secondary antibody conjugated to Alexa 594 (1:500 in blocking solution), was applied to the sections for 30 min. Following antibody binding the sections were fixed with 1% paraformaldehyde for 5 min. DNA was counterstained with Hoechst 33342. All washes were performed in PBS with 0.1% Tween 20. The myosin staining was visualized after excitation at 594 nm.

Implantation of C2C12 cells in TA muscle in vivo

For standardization of RNA ISH protocols, implanted myoblasts were used to provide high intensity local signals within the muscle tissue. C2C12 cells were trypsinised, washed twice in PBS and resuspended in
PBS at a density of $10^4/\mu l$. Approximately 4 month old nude mice were anesthetized with avertin as described above. Five microlitres of the cell suspension ($5 \times 10^4$ cells) was injected into the TA muscle of each leg. The animals were allowed to recover and sacrificed 10 days after implantation. The TA muscle was collected and frozen for sectioning.

**RNA in situ hybridization on tissue sections**

The method used in this study is a modification of the method described by Smerdu et al, 1994.

*Preparation of riboprobe:* The plasmid containing the template DNA was linearized at the 3'end (of the prospective transcript) with a restriction enzyme before it was processed for transcription with the appropriate RNA Polymerase (T7, T3, SP6). After complete digestion of the plasmid the restriction enzyme and other protein impurities were digested with Proteinase K (1 mg; from a 20mg/ml stock) in the presence of 0.5% SDS for 15 min at 37°C. The DNA was precipitated after two phenol-chloroform extractions, dissolved in a minimum volume of water and quantified by gel electrophoresis. 0.5 to 1 µg of linearized DNA was used for the transcription reaction performed (according to the manufacturer’s instructions: MAXIscript kit, Ambion). 0.2 mM of digoxigenin-11-UTP (dig-UTP) was used to label the transcript in the presence of 0.5 mM of ATP, CTP and GTP and 0.3 mM UTP with 20 U of T3 or T7 polymerase. The reaction was carried out at 37°C for 2 hr to 6 hr and 1µl was checked in a 1 X TBE gel for product formation. The unlabeled template DNA was removed using 2 U of RNase-free DNase at 37°C for 30 min and the transcript was alkali-hydrolysed to a size of 100 to 200 nucleotides for better penetration into the tissue during hybridization. The time (t) for which the hydrolysis was carried out at 60°C was calculated according to the following formula

$$t = (L - L_i) / k \times L_i \times L_f$$
t = time of hydrolysis in minutes, \( L_i \) = initial length, \( L_f \) = final or desired length (nucleotides); \( k = 0.11 \) cuts per minute

The hydrolysed products were precipitated using ethanol in the presence of 0.5 M ammonium acetate, resuspended in 50 \( \mu \)l of water and an aliquot checked on 1X TBE gel for quantity and approximate size.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe size</th>
<th>Orientation</th>
<th>Restriction enzyme</th>
<th>RNA Polymerase</th>
</tr>
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<tr>
<td>pBS-MyoD</td>
<td>~1800 bp</td>
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<td>Bam HI</td>
<td>T7</td>
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<td></td>
<td>Sense</td>
<td>Not I</td>
<td>T3</td>
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<td>Xba I</td>
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<tr>
<td></td>
<td></td>
<td>Sense</td>
<td>Eco RV</td>
<td>T7</td>
</tr>
</tbody>
</table>

*Pre-hybridization:* Muscle cryo-sections (20 \( \mu \)l) collected on Probe-On Plus slides, stored at -70°C were thawed, dried at room temperature for 15 min to remove excess moisture and ringed with a hydrophobic PAP pen. The sections were fixed in paraformaldehyde (PFA) (4% in PBS-freshly thawed from a frozen stock) for 20 min followed by Proteinase K (20 \( \mu \)g/ml) digestion for 10 min at room temperature. The enzyme was immediately quenched with glycine (0.2% in PBS) and the sections re-fixed in 4% PFA for 20 min. To prevent non-specific binding of probes the sections were acetylated with 0.25% (v/v) acetic anhydride drizzled over slides dipped in 0.1 M triethanolamine for 10 min. Each step was interspersed with 5 min washes in PBS. Sections were dehydrated through an ethanol series from 30% to 50%, 70% and 100%, air-dried in a dust free environment (at least 1 hr) before using for hybridization.

*Hybridization:* The quantity of the probe to be used on sections to achieve a detectable signal was determined for each batch of probe. The probe was added to 50 \( \mu \)l of hybridization solution, heat denatured at
95°C for 5 min and added to the sections. 'Hybrislips' (plastic coverslips, Fisher) were used to cover the solution and spread it on the sections before the whole set-up was sealed in air tight plastic boxes (humidified with 2X SSC and 50% formamide) and incubated at 55°C overnight in a water bath.

Post-hybridization: The slides were washed in 2X SSC with 50% formamide for 20 min at 65°C. Following two rinses in NTE (10 mM Tris pH 7.5, 5 mM EDTA pH 8.0 500 mM NaCl) at 37°C for 5 min each, excess free riboprobe was digested with RNase (20µg/ml) at 37°C for 30 min. Subsequently, slides were washed in NTE (5 min), 1XSSC and 0.1XSSC (15 min each) at 37°C.

After a rinse in TBS (25mM Tris pH 7.5, 136mM NaCl, 2.6mM KCl) sections were blocked in 0.5% BSA in TBS for at least 1 hr at room temperature to prevent non-specific binding. Anti-digoxigenin antibody (1:5000 dilution, alkaline phosphatase conjugated) (Roche) was applied to the sections for 1 hr in a humidified chamber. Following 4 washes in TBS (at least 1 hr each) and one in the detection buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl₂) the colour reagent (NBT + BCIP (Roche) at 1:50 in detection buffer) was added to the sections and incubated in a humidified chamber for 16 to 36 hr for colour development. The sections were observed using a Zeiss Axioskop microscope equipped with DIC optics (and a blue filter).

RNA in situ hybridization on whole embryos

Prehybridization: Mouse embryos (E12.5) were collected in PBS and fixed overnight in 4% paraformaldehyde in PBS overnight at 4°C. After washes in PTw (PBS with 1% Tween 20) embryos were dehydrated by passing through a methanol series (in PTw) of 25%, 50%, 70% and 100% for 5 min each. Rehydration was performed after an overnight incubation in 100% methanol. Embryos were bleached in a 6% solution of
hydrogen peroxide in PTw for 1 hr at room temperature and permeabilized in a detergent mix (1% NP40, 1% SDS, 0.5% Deoxycholate, 50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl) for 1.5 hr. Post-permeabilization fixation was done in 0.2% glutaraldehyde and 4% paraformaldehyde (in PBS) for 20 min at room temperature. After washes the embryos were transferred to solution X [2X SSC (pH = 4.5), 50% formamide, 1% SDS] for 30 min for equilibration.

Hybridization: The solution X was replaced with hybridization solution (50% formamide, 5X SSC, 1% SDS) containing appropriate amounts of the riboprobe and incubated at 70°C overnight.

Post-hybridization: Two washes with solution X was done at 70°C for 30 min and for 1.5 hr each followed by washes with TBST (TBS with 1% Tween 20). Non-specific hybridization was blocked with 10% lamb serum in TBST for 2 hr at room temperature. The embryos were then incubated in pre-adsorbed alkaline phosphatase-anti-dig antibody (raised in sheep) at 4°C overnight with constant shaking. Washes with TBST were continued for the next 24 hr at room temperature and 4°C to be replaced with NTMT (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl₂, 1% Tween 20). After equilibration in NTMT colour reagent (NBT-BCIP) was added and colour development carried out for overnight to 2 days.

*Pre-adsorbed anti-dig antibody (a gift from Dr Shubha Tole, TIFR, Mumbai): 3-5 mg of mouse embryo powder was dissolved in 0.5 ml of TBST by heating at 70°C for 30 min. The solution was chilled on ice for 10-15 min and 5 µl of lamb serum and 1 µl of anti-dig antibody (Roche) added to the solution. The mixture was incubated at 4°C for 1 hr with shaking and the supernatant collected by centrifugation for use at a final dilution of 1:5000 of the antibody. (Note: These experiments were conducted in Dr Shubha Tole's lab at TIFR, Mumbai.)
Preparation of ultra-competent cells

Ultra-competent cells were prepared according to the method described by Inoue et al (1990). A single colony of *E. coli* DH5α freshly streaked on an LB plate from the glycerol stock was inoculated into 5 ml of SOB medium and grown at 37°C overnight. One ml of this overnight culture was used to inoculate 100 ml SOB and incubated at 18°C at 200 rpm till the culture reached log phase (OD₆₀₀ = 0.6; this takes approximately 24 hr).

The log-phase culture was chilled on ice and cells were collected by centrifugation for 15 min at 4°C. The cell pellet was resuspended in 32 ml of ice cold Buffer I (10 mM PIPES pH6.7, 15 mM CaCl₂, 250 mM KCl and 55 mM MnCl₂), incubated on ice for 10 min and centrifuged to pellet the cells. The cells, now competent, were resuspended in 8 ml of ice cold Buffer I containing 7% DMSO, distributed into 100 µl aliquots and snap-frozen in liquid nitrogen for storage at −70°C. The efficiency of the competent cells was checked by transforming with a known amount of pBS (KS) plasmid. Transformations were performed in DH5α as mentioned in Sambrook et al (1989).

Other methods

For all common molecular biology methods not mentioned in this chapter but used during this study, the conventional protocols, detailed in Sambrook et al (1989) were used.

Probes used in this study

Plasmids used for random primed probes and riboprobes are reported as follows:

Histone 2B: DeLisle et al, 1983  
MyoD: Davis et al, 1987
Myf5: Braun et al, 1989
p21: Huppi et al, 1994
PEA3: Taylor et al, 1997
Fibronectin: Schwarzbauer et al, 1983

Analysis of DNA sequences

**BLAST:** Used for comparing DNA sequences to DNA databases (Altschul et al, 1990)

**Amplify 1.2:** Used for designing PCR primers (William Engels, Genetics Department, University of Wisconsin)

**DNA Strider 1.2:** Used for restriction analysis of DNA sequences (Ch Marck and CEA, 1991)

### List of constructs generated in this study

<table>
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<tr>
<th>#</th>
<th>Name</th>
<th>Vector</th>
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<th>Length (bp)</th>
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<td>CF3b</td>
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<td>PCR product from DD-PCR</td>
</tr>
</tbody>
</table>
**Commonly used solutions**

- **Antibiotic stock (100X):**
  - Penicillin (Sigma) 5 mg/ml
  - Streptomycin (Sigma) 6 mg/ml

- **Trypsin (working stock):**
  - Trypsin (Sigma) 0.1%
  - EDTA (Sigma) 0.1%

- **Phosphate buffer saline (PBS):**
  - KCl (0.2 g), KH$_2$PO$_4$ (0.2 g), NaCl (8 g), Na$_2$HPO$_4$ (1.1 g) per litre
  - pH = 4.0 for whole mount embryo ISH and pH = 7.4 for all other protocols

- **SSC (1X):**
  - NaCl (8.8 g), Sodium citrate (4.4 g) per litre, pH = 7.5

- **Hybridization solution for section RNA ISH:**
  - 50% formamide, NTE (0.3 M NaCl, 20 mM Tris pH 7.5, 5 mM EDTA pH 8.0), 10 mM Na$_2$HPO$_4$ pH 8.0, 10% dextran sulphate, 0.5 mg/ml yeast tRNA, 1X Denhardt’s solution

- **Denhardt’s solution (50X):**
  - 1% Ficoll400, 1% Polyvinylpyrrolidone, 1% BSA fraction V