Genetic Complementation Reveals a Novel Regulatory Role for 3' Untranslated Regions in Growth and Differentiation

Farzan Rastinejad and Helen M. Blau
Department of Pharmacology
Stanford University School of Medicine
Stanford, California 94305-5332

Summary

Differentiated skeletal muscle cells cease dividing and sustain expression of a battery of tissue-specific genes. To identify regulators of growth and differentiation, we used a genetic complementation approach. Following introduction of a cDNA expression library into a differentiation-defective myoblast mutant (NMU2), cDNAs were isolated that activated muscle-specific promoters. The complementing cDNAs were identified as muscle structural genes, troponin I, tropomyosin, and α-cardiac actin, and their activity was mapped to the 3' untranslated region (3'UTR). The 3'UTRs augmented the differentiation of wild-type muscle cells. Upon expression in 10T1/2 fibroblasts, proliferation was suppressed, indicating that the effects of the 3'UTRs are not limited to myogenic cells. These data suggest that 3'UTRs of certain differentiation-specific RNAs are trans-acting regulators in a feedback loop that inhibits cell division and promotes differentiation.

Introduction

A complex regulatory circuitry underlies mammalian growth and differentiation. Most genes with a role in this process have been isolated by biochemical purification of DNA-binding proteins (Courtois et al., 1988; Castrillo et al., 1989; Kieran et al., 1990; Ghosh et al., 1990), by homology with known genes (He et al., 1989; Benezra et al., 1990; Johnson et al., 1990), or by binding to known regulators (Blackwood and Eisenman, 1991; Ron and Habener, 1992). Although many tissue-specific regulators, like MyoD (Davis et al., 1987), are transcription factors that bind DNA, others act indirectly. For example, regulators can sequester other regulators in intracellular compartments (Basuvarlo and Baltimore, 1988), bind cooperatively with ubiquitous transcription factors (Tsai et al., 1987; Diamond et al., 1990), modify those factors by changing their phosphorylation state (Yamamoto et al., 1988; Carrier et al., 1992; Janknecht et al., 1992; Li et al., 1992), or facilitate their dimerization (Mendel et al., 1991). Thus, an elucidation of growth and differentiation control pathways requires a combination of assays based on binding to known molecules with assays based on function.

A genetic complementation approach provides a means of identifying regulators based on function without preconceptions as to their nature. In some cases, complementing genes correct the primary defect induced by mutation (for reviews see Herskowitz, 1985; Nüsslein-Volhard, 1991), whereas in other cases they circumvent or override the primary defect (Surana et al., 1991; Simon et al., 1991). Although genetic complementation has had a major impact on our understanding of regulatory pathways in prokaryotic and lower eukaryotic organisms, it has not been extensively employed in mammals. This approach entails introducing DNA from a donor cell into a recipient cell that is then assayed for a gain of function by the expression of an altered phenotype in tissue culture (for review see Blau, 1988). Numerous genes encoding products such as cytokines and cell surface receptors have been cloned in this manner (Pan et al., 1992; Simmons et al., 1992; Dürkop et al., 1992), but relatively few regulators (Greco et al., 1987; Schatz and Baltimore, 1988; Kitayama et al., 1989; Velazquez et al., 1992). Major obstacles to the application of a genetic complementation approach to mammalian differentiation have included devising a means of identifying appropriate cells to complement and a method for recovering the DNA once complementation is achieved. Moreover, regulators of differentiation may be inhibitory to growth that is essential to isolation of complemented cells by drug selection.

Our interest in characterizing regulators of mammalian growth and differentiation stems from our previous experiments with muscle heterokaryons. These experiments suggested that the differentiated state requires continuous regulation by both positive and negative regulators (Blau et al., 1983, 1985), a finding now supported by cell fusion experiments with a diversity of mammalian cell types (Wright, 1984; Baron and Mariatis, 1986; Spear and Tilghman, 1990; Wu et al., 1991) and by experiments with temperature-sensitive mutants in Caenorhabditis elegans and Drosophila (Way and Chalfie, 1989; Belote and Baker, 1987). This finding suggests that to maintain a stable differentiated state, certain regulators act in a feedback loop to ensure threshold concentrations of other critical regulators (Blau, 1992). We reasoned that a genetic complementation approach would allow isolation of regulators of this type that act indirectly to control the differentiation process.

In tissues such as muscle, there is a dichotomy between growth and differentiation. Growth factors prevent the expression of myogenic genes (Vaidya et al., 1991; Miner and Wold, 1991; Li et al., 1992), whereas inhibitors of cell growth, such as cytosine arabinoside or tumor suppressors, promote differentiation (Silberstein et al., 1986; Gu et al., 1993). Thus, withdrawal from the cell cycle and differentiation are tightly coupled in myogenesis.

Here we report the results of mammalian genetic complementation experiments. Critical to these experiments were the production of a mutant myogenic cell type to complement and the design of procedures for recovering the cDNAs from complemented cells without extensive proliferation. The complementing cDNAs were analyzed for their effects on mutant and wild-type myogenic cells and on a nonmuscle cell type, 10T1/2 fibroblasts. Our findings demonstrate that expression of certain differentiation-specific 3' untranslated regions (3'UTRs) inhibits proliferation and promotes differentiation. Although the 3'UTRs...
Figure 1. Mutagenesis of C2F3 Cells
Aliquots of 1 x 10^6 cells were incubated in different concentrations of NMU for 30 min to induce mutations. Cell survival was assessed based on the cloning efficiency of NMU-treated cells in nonselective liquid media and is presented as percent of mock-treated (PBS) cells. Mutation efficiency was assessed based on cloning efficiency of NMU-treated cells in liquid media containing 6-thioguanine (HPRT mutants) or in soft agar (anchorage-independent mutants).

Results

Isolation of Differentiation-Defective Myogenic Mutants
The genetic approach requires that introduction of a single cDNA leads to a detectable change in differentiation-specific gene expression, a property that is difficult to predict for any given cell type. To overcome this problem, we generated differentiation-defective myogenic mutants, cells that had expressed the genes of interest prior to mutagenesis. For this purpose, a stable subclone of C2C12 wild-type myoblasts was isolated, and mutagenesis was carried out at low dose to minimize the chance that more than one mutation occurred in a myogenic pathway. In addition, a simple selection procedure was used to identify rare mutants, and methods were devised for determining whether a mutant was defective in a regulatory step that could be complemented by regulators present in wild-type myoblasts. This strategy for the production and characterization of mutants is described in detail below.

Prior to mutagenesis, a stable subclone of the C2C12 mouse myogenic cell line was isolated that generated spontaneous differentiation-defective variants at low frequency. This was critical, since such unstable nonfusing myogenic cells have been reported to occur at a frequency as high as 10^{-3} (Wright, 1984; Black and Hall, 1985; Pinset et al., 1988), presumably because they derive from epigenetic rather than stable genetic inactivation events or from mutations. The C2F3 subclone was selected for mutagenesis because it rarely gave rise spontaneously to differentiation-defective variants and had the additional attractive feature that it generated anchorage-independent colonies at remarkably low frequency, <1 x 10^{-9}, as compared with 5 x 10^{-4} for the C2C12 parent, allowing selection of rare mutants by growth in soft agar.

For mutagenesis, we sought a relatively low dose of mutagen that would yield mutants at a detectable frequency, yet relatively few mutations per cell. This would increase the chance that complementation of a myogenic pathway could be achieved by a single gene. To this end, we used nitrosomethylurea (NMU), a point mutagen for which the mutation efficiency at different doses has been.

Figure 2. Comparison of Wild-Type C2F3 and the Differentiation-Defective Myogenic Mutant NMU2
(A) Only the C2F3 cells give rise to multinucleated myotubes (left). NMU2 cells adhere to the dish but remain rounded and refractile (right). Cells are shown in medium containing 10% calf serum at low density (upper panels) and high density (lower panels) photographed with phase-contrast optics.
(B) Total RNA was isolated from cells grown to high density (approximately 3 x 10^6 per 100 mm dish) in medium containing 10% calf serum. RNA (10 μg) from each cell type was electrophoresed, blotted onto a hybridization membrane, and probed sequentially for the HLH regulators, myf-5, myogenin, and MyoD; for sarcomeric myosin heavy chain (MyHC), actins, troponin I, and tropomyosins; and for the muscle-specific isoform of β-enolase.
All of the mutants that grew in soft agar failed to form structural genes and regulatory genes of the myogenic helix-ways could lead to the anchorage-independent growth of selecting for differentiation-defective myogenic mutants. The frequency of generating mutants approximated 10^{-5}. This suggests that more than one mutation can lead to the anchorage-independent phenotype. Based on these data, all myogenic mutants were selected after exposure to 12.5 pgl/ml NMU, a low dose at which mortality was negligible and the second dish was selected in medium containing G418 (neo'). Colonies were counted 14 days later. The number of colonies represents an average of duplicate transfections.

To determine whether the defect in NMU2 involves regulators of myogenic genes, we examined the activity of the α-cardiac actin promoter. Both α-cardiac and α-skeletal actins are expressed in C2F3 cells, but neither one of these two sarcomeric actins accumulates in the NMU2 mutant either at the level of mRNA detected by Northern analysis (Figure 2D) or in protein detected by immunofluorescence (data not shown). To analyze the expression of the α-cardiac actin promoter in NMU2 cells, we used the well-characterized 485 bp promoter fragment of the human α-cardiac actin (HCA) gene (Minty and Kedes, 1986; Gustafson and Kedes, 1989). Stable transfection experiments were performed using a construct, called HCA-gpt, in which the HCA promoter drives the expression of bacterial guanine-xanthine phosphoribosyl transferase (gpt) that confers resistance to mycophenolic acid. The expression of this construct was compared in NMU2 and C2F3 to determine the relative level of promoter activity. As a control for potential differences in reporter gene activity in the two cell types, each was cotransfected with the plasmids SV2-gpt and SV2-neo. Cells from each transfected dish were divided and grown either in mycophenolic acid to select for gpt expression or in G418 to select for neo expression. Since the transfection efficiency of the two cell types differed, SV2-neo was used to correct for transfection efficiency in all cotransfections with SV2-gpt and HCA-gpt. SV2-gpt was expressed equally well in both NMU2 and C2F3 cells, whereas HCA-gpt was expressed efficiently only in C2F3 cells. As shown in Table 1, the markedly reduced activity of the α-cardiac actin promoter in NMU2 as compared with C2F3 cells indicates that NMU2 lacks the trans-acting factors necessary for expression of this promoter.

### Table 1. Relative Activity of HCA Promoter in C2F3 and NMU2 Cells

<table>
<thead>
<tr>
<th>Cotransfected Plasmids</th>
<th>Wild-type (C2F3)</th>
<th>Mutant (NMU2)</th>
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<tbody>
<tr>
<td></td>
<td>gpt+ / neo+</td>
<td>gpt+ / neo+</td>
</tr>
<tr>
<td>SV2-gpt + SV2-neo</td>
<td>21 18</td>
<td>40 52</td>
</tr>
<tr>
<td>HCA-gpt + SV2-neo</td>
<td>29 21</td>
<td>1 38</td>
</tr>
<tr>
<td>Relative HCA activity</td>
<td>1.2</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Cells (1 × 10^5) were cotransfected with 5 μg of each plasmid. Cells from each of two duplicate transfections were plated in two dishes. One dish was selected in medium containing mycophenolic acid (gpt+), and the second dish was selected in medium containing G418 (neo'). Colonies were counted 14 days later. The number of colonies represents an average of duplicate transfections.

### Extensive Documented

Extensive documented (Richardson et al., 1987; Bouck and de Mayorca, 1982). Aliquots of C2F3 cells were incubated in different concentrations of NMU for 30 min. Viability, an indirect measure of the number of mutations, was assessed by comparing the plating efficiency of NMU-treated and mock-treated (phosphate-buffered saline [PBS]) only C2F3 cells. The mutation rate was determined based on the dose–response curve for inactivating a single well-defined gene, the gene encoding hypoxanthine phosphoribosyl transferase (HPRT) on the X chromosome (Figure 2). A similar dose–response curve was generated for the production of anchor-transcendant C2F3 mutants by assaying growth in soft agar. The slope for the production of anchor-transcendant mutants was somewhat greater than that for HPRT mutants. This finding suggests that more than one mutation can lead to the anchor-transcendant phenotype. Based on these data, all myogenic mutants were selected after exposure to 12.5 μg/ml NMU, a low dose at which mortality was negligible and the frequency of generating mutants approximated 10^{-5}.

Growth in soft agar proved to be an effective method of selecting for differentiation-defective myogenic mutants. All of the mutants that grew in soft agar failed to form myotubes in liquid tissue culture media. The mutants expressed different combinations of muscle-specific structural genes and regulatory genes of the myogenic helix-loop-helix (HLH) family (data not shown). This finding further suggests that disruption of several different pathways could lead to the anchor-transcendant growth of myoblasts, in agreement with the dose–response curve.

### Characterization of the NMU2 Mutant

NMU2 was isolated as a clone that grew in soft agar, and it was recloned in liquid medium to ensure its single cell origin. Myotube formation was not detected when cells were grown at high density either in 10% calf serum or in 2% horse serum, conditions under which C2F3 differentiates (Figure 2A). The doubling time for NMU2 in 10% calf serum is 12–14 hr, similar to that of the parental C2F3. NMU2 cells are more refractile and less adherent than C2F3 cells. In addition, the NMU2 mutant fails to express messenger mRNAs (mRNAs) encoding myogenin, myosin heavy chain, sarcomeric α-actins, tropinin I, and at least one isoform of tropomyosin (Figure 2D). However, NMU2 expresses muscle-specific enolase, MyoD, myf-5, and nonmuscle β- and γ-actins. In addition, nuclear MyoD and cell surface neural cell adhesion molecule (N-CAM) proteins are detected in nearly 100% of the cells by immunocytochemistry (data not shown). Thus, it appears that a subset of steps in the myogenic regulatory network have been disrupted in the NMU2 mutant, demonstrating that the control of certain pathways can be dissociated. The finding that multiple muscle pathways cease to be expressed in NMU2 suggested that mutagenesis affected a regulatory step and not the structural genes themselves, a point confirmed by the experiments described below.

### NMU2 Lacks Activators of the α-Cardiac Actin Promoter

To determine whether the defect in NMU2 involves regulators of myogenic genes, we examined the activity of the α-cardiac actin promoter. Both α-cardiac and α-skeletal actins are expressed in C2F3 cells, but neither one of these two sarcomeric actins accumulates in the NMU2 mutant either at the level of mRNA detected by Northern analysis (Figure 2) or in protein detected by immunofluorescence (data not shown). To analyze the expression of the α-cardiac actin promoter in NMU2 cells, we used the well-characterized 485 bp promoter fragment of the human α-cardiac actin (HCA) gene (Minty and Kedes, 1986; Gustafson and Kedes, 1989). Stable transfection experiments were performed using a construct, called HCA-gpt, in which the HCA promoter drives the expression of bacterial guanine-xanthine phosphoribosyl transferase (gpt) that confers resistance to mycophenolic acid. The expression of this construct was compared in NMU2 and C2F3 to determine the relative level of promoter activity. As a control for potential differences in reporter gene activity in the two cell types, each was cotransfected with the plasmids SV2-gpt and SV2-neo. Cells from each transfected dish were divided and grown either in mycophenolic acid to select for gpt expression or in G418 to select for neo expression. Since the transfection efficiency of the two cell types differed, SV2-neo was used to correct for transfection efficiency in all cotransfections with SV2-gpt and HCA-gpt. SV2-gpt was expressed equally well in both NMU2 and C2F3 cells, whereas HCA-gpt was expressed efficiently only in C2F3 cells. As shown in Table 1, the markedly reduced activity of the α-cardiac actin promoter in NMU2 as compared with C2F3 cells indicates that NMU2 lacks the trans-acting factors necessary for expression of this promoter.

### Complementation of NMU2 and Activation of the α-Cardiac Actin Promoter in Hybrids

To determine that the defect in NMU2 could be complemented by regulators provided in trans by a wild-type myogenic cell, fusion experiments were performed. To assay for regulators of the α-cardiac actin promoter, we used...
NMU2 cells stably transfected with a reporter gene construct. NMU2 cells were cotransfected with HCA-gpt and SV2-neo and selected for G418 resistance. Suitable test cells for genetic complementation were identified by their ability to express the HCA-gpt construct after fusion with C2F3 cells in hybrids. Less than 3% of the NMU2 transfecteds expressed the HCA-gpt construct constitutively. Five NMU2 transfecteds that did not express HCA-gpt were fused with C2F3, using polyethylene glycol, and were selected in medium containing mycophenolic acid. We produced hybrids, not heterokaryons, since ultimately complementation by cDNAs had to be performed under conditions that allowed for growth and drug selection. Survival of the hybrids depended on the activation of the HCA-gpt construct in NMU2 in response to transacting factors provided by C2F3. Three clones did not express gpt after fusion with C2F3. Two clones gave rise to mycophenolic acid-resistant (gpt') hybrids when fused to C2F3 but not when fused to themselves (homohybrid control). These results demonstrate the necessity of identifying clones for complementation by cell fusion. In addition, they confirm previous findings by us and by others showing that clones differ in their ability to activate stably transfected constructs, owing presumably to a combination of copy number of the construct and the nature of the DNA flanking the site of integration (Hardeman et al., 1988; Spear and Tilghman, 1990; Wu et al., 1991). The clone, NMU2-HCA-gpt.3, which exhibited vigorous growth when fused in a hybrid, was used as a test cell for isolating activators of the silent 485 bp α-cardiac actin promoter.

Complementation of NMU2 by cDNAs
To minimize differences, cDNA libraries were constructed from poly(A)+ RNA isolated from primary myoblasts or fibroblasts derived from the same human muscle tissue. Myoblasts that had just initiated differentiation following a 36 hr exposure to low serum medium were used to enrich for regulators of myogenesis. cDNAs were cloned in random orientation into the pCDM8 vector that utilizes the human cytomegalovirus (CMV) promoter, which is efficiently expressed in mammalian cells (Seed and Aruffo, 1987). Each library contained approximately \(1 \times 10^6\) cDNAs with an average insert size of 1.4 kb. The myoblast library was subtracted with the fibroblast library to enrich for muscle-specific cDNAs. To determine whether intact cDNAs were present in the library and could be expressed in mammalian cells, we isolated actin cDNAs from the library by colony hybridization with an actin probe. Of 16 hybridizing clones, 7 had cDNA inserts that appeared full length (1.6–1.8 kb) on Southern blots, and 4 of these expressed actin protein as determined by immunofluorescence following transfection into COS cells (data not shown).

Both subtracted and unsubtracted libraries were introduced into the NMU2-HCA-gpt.3 test cells and the cells selected in medium containing mycophenolic acid to assay for the activation of the HCA promoter. No colonies were detected in 10 control dishes of cells transfected either with salmon sperm DNA or library vector plasmid. Only three colonies were detected in a total of 20 dishes of cells transfected with the myoblast cDNA library. By contrast, an average of 15 colonies per dish was obtained when cells were transfected with different pools of the subtracted library. Thus, the subtracted library appeared to be enriched 100 fold for regulators capable of activating the HCA promoter and was used in all further experiments. To confirm the presence of library DNA in the activated cells, colonies were picked, expanded to approximately 5 \(\times 10^6\) cells, and DNA isolated for Southern analysis. The majority of the clones demonstrated a retarded growth rate relative to the parental NMU2-HCA-gpt.3 cells, even when selection in mycophenolic acid was eliminated. In addition, Southern analysis revealed that many of the expanded clones contained fewer than one copy per genome of library vector sequences, which could have been due to loss of transfected DNA during growth of the clones. To overcome the problem of loss of differentiation-inducing cDNAs from the activated clones, colonies were individually harvested when only 50–100 cells had accumulated, 2–3 weeks after the initiation of growth in selective media. Each gpt+ clone was lysed, and nested polymerase chain reaction (PCR) was performed with primers to flanking vector sequences to amplify cDNA inserts. Amplified DNA was detected in 94% of gpt+ clones, suggesting that they contained library sequences. No DNA was amplified from mock lysates prepared from regions of the transfected dish that did not contain cells. Between 1 to 11 amplified bands could be detected in the size range of 0.2 to 1.2 kb, although, in general, the products of smaller size appeared to be preferentially amplified (Figure 3).

To determine which of the amplified products corresponded to a cDNA capable of activating the HCA-gpt construct, PCR-amplified DNAs were isolated from agarose gels and tested individually (Figure 4). The DNA from each of 50 bands was directionally cloned into the pCDM8 vector and pools of cDNAs cotransfected together with SV2-hygromycin (SV2-hygro) into the NMU2-HCA-gpt.3 test cell. Stable transfectants were grown in medium containing hygromycin, but without mycophenolic acid. Thus, in this experiment there was no selective pressure to express gpt. Between 50 and 400 stable colonies were obtained for each pool of cDNAs and combined for Northern analysis. cDNAs in positive pools induced significant gpt expression and were retested individually. Transfection of four single cDNAs repeatedly led to activation of the reporter gene. The total amount of hybridizing signal in each lane reflects the extent of gpt RNA induction by each of the four activating cDNAs. These cDNAs were designated D3, F1, G12, and H3 based on the library pool from which they were derived. No gpt mRNA was detected in negative controls transfected with vector or with salmon sperm DNA (mock) or in cells that received cDNA from amplified bands that did not complement the test cell, such as K1.

cDNAs That Activate HCA-gpt Also Activate HCA-puro and MYG-puro
To determine whether the activation of the HCA promoter by the four cDNAs was dependent on the choice of the reporter gene or on the particular site of integration of
the HCA–gpt construct in NMU2-HCAgpt.3, we created a second test cell. The HCA promoter was linked to a different reporter gene, the bacterial puromycin N-acetyl transferase (puro) gene that confers resistance to puromycin. Stable clones were selected by cotransfection with SV2-neo and selection in medium containing G418. To identify a test cell in which expression of the construct could be induced when exposed to regulators provided in trans, six randomly chosen G418-resistant clones were analyzed. These clones were fused to form homohybrids and heterohybrids with C2F3, as in the case of the first test cell, except that they were selected for growth in media containing puromycin. Of the six clones tested, one expressed the construct constitutively, five did not express the construct, and two of the five could be induced to express it only in heterohybrids. A clone that exhibited vigorous growth in the presence of puromycin as a hybrid fused to C2F3, but that failed to grow when fused to itself, was selected as the second test cell and designated as NMU2-HCAPuro.7.

To determine whether the activation of the α-cardiac actin promoter observed with the four cDNAs was specific to that promoter or had a more general effect on myogenic genes in NMU2, we created a third test cell. In this test cell, we used a different reporter construct, a 184 bp fragment of the mouse myogenin (MYG) promoter (Edmondson et al., 1992) linked to the gene encoding resistance to puromycin (MYG–puro). Myogenin is a member of the HLH family of myogenic regulators and, like cardiac actin, is not expressed in NMU2 (see Figure 2). Stable transfectants were produced with this construct, and a test cell (NMU2-MYGpuro.1) was identified in which the reporter construct could be activated in hybrids, as described for the other test cells. Homohybrids of the test cell fused to itself did not activate the construct.

All four cDNAs capable of activating the HCA–gpt construct in the first test cell were also capable of activating the HCA–puro and MYG–puro constructs in the two new test cells (Table 2A). Transfectants were selected in medium containing puromycin, at concentrations empirically determined to kill the untransfected test cells. As a positive control, an expression vector encoding the myogenin protein was transfected into each test cell. The HLH protein myogenin is a transcription factor that is absent from NMU2 cells (see Figure 2B) and known to bind directly to consensus elements within both the cardiac actin and myogenin promoters (Sartorelli et al., 1990; Edmondson et al., 1992). As a negative control for nonmuscle-specific gene activation via HLH proteins, an expression vector encoding E47, a ubiquitous HLH (Schlissel et al., 1991) was introduced into each test cell. As a second negative control, one of the PCR-amplified cDNAs that had not activated HCA–gpt when introduced alone (e.g., K1 cDNA in Figure 4) was transfected. Neither E47 nor K1 activated the promoter constructs in test cells. These negative controls provide evidence that the reversion frequency is below the level of detection in this assay.

Figure 3. Ethidium Bromide Visualization of cDNAs Recovered from Activated Cells by PCR
NMU2-HCAgpt.3 test cells were transfected with 10 μg of DNA from independent pools of the cDNA expression library (pools D, F, G, and H). Complemented gpt-positive clones were selected in media containing mycophenolic acid. Individual colonies were trypsinized and harvested in cloning rings, and the cells were rinsed with PBS and lysed in 110 μl of water. A mock lysate was prepared following a mock harvest from a region of the transfected dish that did not contain cells (lane C). Lysates were used as PCR templates to recover cDNAs flanked by library vector sequences. PCR-generated bands recovered from activated clones are visualized by electrophoresis on 2% agarose gels stained with ethidium bromide. Molecular size markers are shown (M), and arrows indicate bands found to have activity in subsequent experiments (left to right: D3, F1, G12, and H3).

Figure 4. Induction of HCA–gpt mRNA in NMU2-HCAgpt.3 Test Cells
NMU2-HCAgpt.3 cells were cotransfected with 2 μg of SV2-hygro and 8 μg of each of the complementing cDNA expression constructs, the negative control (K1), pCDM8 (Vector), or sheared salmon sperm DNA (Mock). Total RNA was isolated following hygromycin selection and analyzed by Northern hybridization with a probe specific to the gpt coding sequence (upper panel). The extent of gpt RNA induction is represented by the total amount of signal in each lane. The hybridization signals of smaller size are likely to be the result of missplicing of the SV40 small t intron that is located between the gpt-coding sequence and the polyadenylation site of the vector, as reported by Yu et al. (1989). Relative amounts of RNA loaded in each lane are visualized by staining with ethidium bromide (lower panel).
Table 2. cDNAs Activate Muscle-Specific Promoters

A. Activation of HCA and MYG Promoters in Two Test Cells

<table>
<thead>
<tr>
<th>Construct in NMU2 Test Cells</th>
<th>Puromycin Colonies per Transfection</th>
</tr>
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<tbody>
<tr>
<td>HCA-puro</td>
<td>12</td>
</tr>
<tr>
<td>MYG-puro</td>
<td>49</td>
</tr>
</tbody>
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B. Activation of Endogenous Myogenin Gene

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Myogenin-Positive Colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cDNAs</td>
<td></td>
</tr>
<tr>
<td>Myogenin</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>E47</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>K1</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>Complementing cDNAs</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>F1</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>G12</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>H3</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

* Test cells (NMU2-HCAPuro.7 and NMU2-MYGpuro.1) were transfected with 10 μg of plasmid. NMU2-HCAPuro.1 cells were selected in media containing 25 μg/ml puromycin, and NMU2-MYGpuro.1 cells were selected in media containing 1 μg/ml puromycin. Colonies were counted following 10 days of selection. Each cDNA was tested in two or more transfection experiments, of which one representative is shown. All cDNAs use the CMV promoter for expression except for the myogenin cDNA, which uses the mouse sarcoma virus promoter.

NMU2-HCApuro.3 cells were cotransfected with 8 μg of cDNA plasmid and 2 μg of SV2-puro plasmid. Puromycin-resistant colonies were grown under selection with 1 μg/ml puromycin for 7 days and then maintained without puromycin in medium containing 0.5% horse serum for an additional 2 days. The frequency of myogenin-positive colonies was determined following immunostaining for myogenin protein and is expressed as plus or minus the standard error of the proportion. For each cDNA tested, a minimum of 150 colonies was scored, the cumulative results of three different transfection experiments with two independent preparations of the plasmids. Colonies that contained more than two cells with definitive nuclear myogenin staining were scored as positive. Positive cells were generally detected in the denser central region of the colonies.

The activation of three different constructs in three different test cells strongly suggests that the observed effect is not dependent on the site of integration of the reporter gene construct. In addition, the results cannot be ascribed to a peculiarity specific to the metabolism of the reporter gene itself, since similar results were obtained for two different bacterial genes that conferred resistance to mycophenolic acid and puromycin. Furthermore, since two different muscle-specific promoters were activated, it appeared possible that the cDNAs could have a broader effect on the differentiated state of NMU2.

cDNAs Activate the Endogenous Myogenin Gene

We examined the potential of the four cDNAs to activate the expression of the endogenous myogenin and sarcomeric actin genes in NMU2 cells. The activation observed in the three test cells described above involved only 184 bp or 485 bp of the myogenin and cardiac actin promoters, respectively. To determine whether the resident endogenous promoters could also be activated, we used the NMU-HCApuro.3 test cell. The cells were cotransfected with the SV2-puro plasmid and expression vectors containing 1 of the 4 activating cDNAs or the K1 or E47 cDNA negative controls. Clones were selected for uptake of DNA, not for expression of the reporter gpt gene, by growth in the presence of puromycin. Expression of the endogenous myogenin gene was assayed in individual colonies by immunocytochemistry using an antibody to myogenin. The frequency of positive colonies observed with the four complementing cDNAs ranged from 11% for H3 to 29% for F1 in this assay, values well above those observed for the negative controls E47 and K1, which both approximated 1% (Table 2B). These results show that the endogenous myogenin gene can be activated upon expression of the four cDNAs in NMU2 cells.

![Figure 5. Induction of Endogenous Myogenin mRNA by F1 cDNA](image-url)
Table 3. Identification of cDNAs

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Size</th>
<th>Identity</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>D3</td>
<td>0.7</td>
<td>Human α-cardiac actin</td>
<td>Hamada et al., 1982</td>
</tr>
<tr>
<td>F1</td>
<td>0.3</td>
<td>Human tropomyosin</td>
<td>Lin and Leavitt, 1988</td>
</tr>
<tr>
<td>G12</td>
<td>1.0</td>
<td>Human muscle troponin I</td>
<td>Wade et al., 1990</td>
</tr>
<tr>
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<td>Human mitochondrial ATPase 6</td>
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<tr>
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<td>Human octamer-binding protein</td>
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<td>Human histone H3.3</td>
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Activity of cDNAs Maps to Sequences within the 3'UTR

We tested whether the 3'UTR sequences alone, the feature common to all of the complementing cDNAs, was sufficient to induce expression of myogenic genes in NMU2. For this purpose, the troponin I and tropomyosin cDNAs were analyzed as two parts, sequences 3' and 5' to the translation termination codon, respectively. These cDNA segments were generated by PCR, ligated into the pCDM8 expression vector, and tested independently for their ability to activate the reporter gene in the NMU2-MYGpuro test cell. The activity of both of these structural genes mapped to sequences within the 3'UTR (Table 4A). Similarly, when segments of troponin I were assayed for their ability to activate the endogenous myogenin gene, the 3' segment alone exhibited activity, whereas the 5' segment did not (Table 4B). The 2-fold increase in efficiency of the 3' segment alone relative to the entire G12 (troponin I) cDNA sequences may be due to enhanced expression of the shorter cDNA (0.3 kb versus 1.0 kb). These results were confirmed by Northern analysis. In four independent clones expressing the 5' segment of tropomyo...
Table 4. The Activity of cDNAs Maps to the 3'UTRs

<table>
<thead>
<tr>
<th>Transfected cDNA*</th>
<th>Puromycin Colonies per Transfection</th>
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<td><strong>Experiment 1</strong></td>
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<td>5'</td>
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</tr>
<tr>
<td>3'</td>
<td>70</td>
</tr>
<tr>
<td>5'</td>
<td>3</td>
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</table>

* Duplicate dishes containing 1 x 10⁶ NMU2-MYGpuro.1 cells were transfected with 10 µg of each plasmid and selected in media containing 0.8 µg/ml puromycin (Experiment 1) or 1.0 µg/ml puromycin (Experiment 2). Shown is the total number of puromycin-resistant colonies obtained after 10 days of selection. All cDNAs use the CMV promoter for expression except for the puro cDNA (puromycin N-acetyltransferase), which uses the SV2 promoter. 3' and 5' designate sequences downstream and upstream of the translation termination codon, respectively. Full length is the G12 cDNA in its entirety.

To determine whether the activation of myogenic genes is specific to certain 3'UTRs, we tested the activity of additional 3'UTRs in NMU2 cells. For this purpose, the 3'UTRs of jun B, oct I, and histone H3.3 were selected because they encode transcripts of similar size to those of the complementing cDNAs, yet these transcripts are not differentiation specific and are widely expressed in a number of cell types (see Table 3). To facilitate a comparison of the activity of the different 3'UTRs, they were all cloned into the pCDM8 vector, which uses the CMV promoter to drive expression. Thus, the rate of transcription of each cDNA should be comparable. Unlike the 3'UTRs of complementing cDNAs (Figure 6, top panel), the control 3'UTRs did not enhance myogenin expression significantly. Northern analysis revealed that the maximum levels of tropomyosin, troponin I, and α-cardiac actin 3'UTR RNA accumulation in stably transfected NMU2 cells were comparable with those of the 3'UTRs expressed in primary human muscle cells (data not shown). Thus, the levels of 3'UTR RNAs expressed in the stably transfected cells are achieved by human muscle cells in the course of normal myogenesis. Similarly, Northern analyses of the four control 3'UTRs in stable transfectants showed levels of accumulation that were comparable with the levels of accumulation of the muscle 3'UTR RNAs, although this quantitation is not exact owing to differences among the probes (data not shown). These results indicate that the observed effects are not due to toxicity resulting from overexpression of a single 3'UTR and that the effects are specific to a subgroup of 3'UTRs.

Figure 6. Induction of Myogenin Protein by 3'UTR Sequences

NMU2-HCAgpt.3 cells (upper panel) or C2F3 cells (lower panel) were cotransfected with 2 µg of SV2-puro and 8 µg of each cDNA expression construct and were selected in 1 µg/ml puromycin for 7 days in medium containing 10% calf serum. The resulting NMU2-HCAgpt.3 colonies were incubated for an additional 2 days in medium containing 0.5% horse serum. Both sets of stable transfectants were fixed and then stained with antibody specific to the myogenin protein. The frequency of colonies of average size (25-250 cells) that contained greater than 5 cells with definitive nuclear staining was determined by microscopic analysis. Many of the colonies transfected with troponin I (Tn I) or tropomyosin (Tm) 3'UTRs had >25 positive nuclei, usually located in the central dense region of the colony. Each panel shows the cumulative results for two separate experiments with the standard error of the proportion. An average of 230 colonies was analyzed for each 3'UTR transfection.

osin, myogenin mRNA expression was not induced (for example, Figure 5, lane 2).

To determine whether the activation of myogenic genes is specific to certain 3'UTRs, we tested the activity of additional 3'UTRs in NMU2 cells. For this purpose, the 3'UTRs of junB, oct1, and histone H3.3 were selected because they encode transcripts of similar size to those of the complementing cDNAs, yet these transcripts are not differentiation specific and are widely expressed in a number of cell types (see Table 3). To facilitate a comparison of the activity of the different 3'UTRs, they were all cloned into the pCDM8 vector, which uses the CMV promoter to drive expression. Thus, the rate of transcription of each cDNA should be comparable. Unlike the 3'UTRs of complementing cDNAs (Figure 6, top panel), the control 3'UTRs did not enhance myogenin expression significantly. Northern analysis revealed that the maximum levels of tropomyosin, troponin I, and α-cardiac actin 3'UTR RNA accumulation in stably transfected NMU2 cells were comparable with those of the 3'UTRs expressed in primary human muscle cells (data not shown). Thus, the levels of 3'UTR RNAs expressed in the stably transfected cells are achieved by human muscle cells in the course of normal myogenesis. Similarly, Northern analyses of the four control 3'UTRs in stable transfectants showed levels of accumulation that were comparable with the levels of accumulation of the muscle 3'UTR RNAs, although this quantitation is not exact owing to differences among the probes (data not shown). These results indicate that the observed effects are not due to toxicity resulting from overexpression of a single 3'UTR and that the effects are specific to a subgroup of 3'UTRs.
Differentiation and Growth Control by 3'UTRs

A. Clone 7 Clone 1
Zn - + - +

Figure 7. Suppression of 10T1/2 Fibroblast Proliferation by 3'UTR Sequences
F1 cDNA expression profiles and growth characteristics of two representative clones, clone 7 (noninducible) and clone 1 (inducible), are shown. 10T1/2 fibroblasts were cotransfected with 2 µg of SV2-neo and 8 µg of a construct containing the tropomyosin 3'UTR (F1 cDNA) under control of a metallothionein promoter. Stable transfecteds were selected in medium containing G418.

(A) Total RNA from the two clones, each growing in 5% dialyzed calf serum with or without 20 µM zinc, was analyzed by Northern blot hybridization with the F1 probe (upper panel). Relative amounts of RNA loaded in each lane are visualized by ethidium bromide staining of the gel (lower panel). (B) Two hundred cells from each of two clones were seeded onto two dishes and grown in medium containing 10% calf serum for 3 days. Medium was replaced with 2% dialyzed calf serum with or without 20 µM zinc for an additional 4 days of incubation, after which colonies were fixed and stained with 2% methylene blue in 50% ethanol and photographed.

3'UTRs Increase Myogenin Expression in Wild-Type Muscle Cells
To determine whether expression of the tropomyosin and troponin I 3'UTRs could affect differentiation of muscle cells other than mutant NMU2 cells, they were transfected into the wild-type C2F3 muscle cells from which the mutant was derived. Each cDNA was transfected in duplicate in two separate experiments, and at least 240 colonies of 25-200 cells were scored for myogenin expression. After 7 days of growth at clonal density in high serum medium, approximately 20% of C2F3 colonies expressed myogenin owing to spontaneous differentiation (Figure 6, vector lane). The transfection of 3'UTR sequences derived from junB, oct1, or histone H3.3 did not alter the frequency of myogenin expression. By contrast, sequences derived from the 3'UTRs of tropomyosin and of troponin I increased the frequency of positive colonies to 50%-60%, 2-3-fold above background. The lack of augmentation of myogenin expression by the control 3'UTRs provides further evidence for the specificity of the effect seen with the differentiation-specific 3'UTRs. In addition, the augmentation of myogenin expression observed in C2F3 cells upon expression of the troponin I and tropomyosin 3'UTR sequences demonstrates that the pathway targeted by 3'UTRs operates in a nonmutant myoblast cell. Thus, this pathway is likely to play a regulatory role in normal myogenic differentiation.

3'UTRs Inhibit Growth of Fibroblasts
To determine whether the 3'UTR sequences could induce muscle gene expression in a nonmyogenic cell type, fibroblasts were transfected. 1OT1/2 fibroblasts were selected for this purpose because they are not myogenic but have the potential to display a myoblast phenotype upon expression of myogenic HLH transcription factors (Davis et al., 1987; Edmondson and Olson, 1989). Although neither myogenin nor myosin heavy chain was detected, there was a significant reduction in the frequency of stably transfected 1OT1/2 clones upon expression of the tropomyosin 3'UTR sequences. To assess the growth effect in established colonies of proliferating cells, the complementing 3'UTR sequences of tropomyosin were cloned into a plasmid in which expression was directed by the zinc-inducible metallothionein promoter. Following cotransfection of the inducible constructs with SV2-neo, ten stable 1OT1/2 transfecteds were isolated by selection in G418. Each stable clone was grown at clonal density for 3 days and then exposed to the inducer for an additional 4 days. Figure 7 depicts two representative clones, one that was inducible by zinc (clone 1) and the other that was not (clone 7). Northern analysis revealed that clone 7 did not accumulate tropomyosin 3'UTR RNA and that its growth was not affected by zinc induction. By contrast, clone 1 accumulated detectable levels of 3'UTR RNA in the presence of zinc, and this level could be induced approximately 3-fold. At clonal density, even the basal level of expression of the 3'UTR in clone 1 led to a detectable impairment of growth relative to the clone 7 control, but growth inhibition was pronounced following exposure to zinc. Although the number of colonies appeared similar on each dish, colony size differed markedly. These experiments demonstrate that the effect of the 3'UTR on growth correlates with the level of accumulated RNA and may be dose dependent.

To analyze the effect of the inducible constructs on 1OT1/2 cell growth in a quantitative manner, an assay of cell number based on metallothionein metabolism was performed (Mosmann, 1983). In this experiment, 500 cells were plated in replicates of four with or without zinc-supplemented medium for a period of 4 days. Cell proliferation was inhibited by 42% upon induction with zinc. These results show that expression of the 3'UTR in fibroblasts suppresses growth.
Discussion

Growth Control and Differentiation

Using a genetic complementation assay that is based on function, we have revealed a novel regulatory role in growth and differentiation for the 3'UTRs of three muscle structural genes, tropomyosin, troponin I, and α-cardiac actin. Our findings were made possible by the isolation of a mutant myogenic cell type that could be partially complemented by single cDNAs. In addition, since growth and differentiation are often antagonistic in skeletal muscle, we used reporter genes that are expressed at detectable levels in proliferating myoblasts and methods that allowed recovery of the complementing DNA from small colonies prior to extensive cell division. The effect of the 3'UTRs was specific: 3'UTRs of similar size derived from nonstructural genes expressed in a wide range of cell types (oct1, junB, and histone H3.3) had no effect. By contrast, expression of the complementing 3'UTRs not only enhanced muscle-specific gene expression in the NMU2 mutant, but also in the parental myoblast cell line. These findings demonstrate that the regulatory pathway affected by the 3'UTRs is not limited to the NMU2 mutant. To show definitively that the 3'UTRs play a role in controlling growth and differentiation in normal development, experiments in which their expression is eliminated are required. However, since there appear to be at least four different sequences with redundant effects, these experiments are not presently feasible.

An essential component of differentiation in certain cell types, such as skeletal muscle, is relief from regulators that promote cell division. It is at this step that the 3'UTRs may play a critical role. In fibroblasts, 3'UTR expression inhibited cell proliferation without inducing myogenic gene expression. These experiments raise the possibility that the expression of myogenic genes observed in NMU2 and C2F3 is a consequence of a primary effect on growth. Inhibitors of DNA synthesis are known to enhance myogenic differentiation (Silberstein et al., 1986). Moreover, the myogenic regulator, MyoD, both activates muscle-specific genes and inhibits cell division (Davis et al., 1987). Growth factors and their receptors, which comprise two classes of proto-oncogenes, suppress the induction of myogenic differentiation (Vaidya et al., 1991; Miner and Wold, 1991; Li et al., 1992). Conversely, tumor suppressor genes appear to play a role in promoting muscle differentiation, as evidenced by molecular interactions between Rb and MyoD proteins (Gu et al., 1993). Thus, withdrawal from the cell cycle and differentiation are normally tightly coupled in myogenes. Taken together, these findings suggest that the primary effect of the 3'UTR transcripts may be to disrupt the cell cycle, which then secondarily leads to an induction of muscle-specific gene expression. Experiments to test this possibility are currently under way.

Mechanism of Action

It is unlikely that the 3'UTR sequences complement the primary defect in the NMU2 mutant. The mutant phenotype is only partially corrected: although some muscle-specific promoters are induced, the cells remain unaltered morphologically and do not fuse. However, the elucidation of the primary defect is not essential to the use of such mutants in complementation studies. Indeed, the experiments reported here show that in mammalian cultured cells, as in lower eukaryotes (Surana et al., 1991; Simon et al., 1991), complementation can suppress or circumvent the primary defect leading to the isolation of novel components in a regulatory pathway.

Previous studies indicated that the 3'UTRs of sarcomeric actin genes play a regulatory role in muscle development; however, it was thought that they acted only in cis. 3'UTRs of genes encoding specific actin isoforms are highly conserved across species, suggesting that they may be important to the expression of these genes (Hamed et al., 1982; Gunning et al., 1984). Moreover, expression of a stably transfected chimeric gene of the 5' regulatory region of β-actin linked to the 3'UTR of α-skeletal actin does not decline during differentiation, but increases like the endogenous α-skeletal actin gene (Sharp et al., 1989). Conversely, when the 3'UTR of β-actin is linked to heterologous promoters, expression of these promoters, like the endogenous β-actin gene, declines during differentiation (DePonti-Zilli et al., 1989). These results established that the expression state of actin genes is strongly influenced by their 3'UTRs. However, in contrast with previous reports, our results demonstrate that regulation by the 3'UTRs is not restricted to a cis-acting mechanism, but can operate in trans as well.

The 3'UTRs appear to complement the NMU2 mutant via the RNAs they encode. The DNA sequence itself is unlikely to mediate the activation of muscle promoters, since expression of the 3'UTRs appears to be required for their function. When expression of the endogenous myogenin gene was disrupted by Northern analysis, 3'UTR RNA was also detected (Figure 5). Stable transfection of promoterless constructs led either to no myogenin accumulation or, if myogenin was detected, to large transcripts containing the 3'UTR that presumably initiated from alternative start sites. Northern analysis revealed that the maximum levels of tropomyosin, troponin, and α-cardiac actin 3'UTR RNAs that accumulated in stably transfected NMU2 cells were comparable with the levels of the 3'UTR RNAs of the endogenous genes in differentiating primary human muscle cells (data not shown). The growth suppression of 10T1/2 fibroblasts was also correlated with the levels of accumulated 3'UTR RNA (Figure 7). In the absence of RNA expression, the 3'UTR had no effect. Low level RNA expression had a moderate effect on the growth of established colonies, but a 3-fold increase in RNA by zinc induction led to significant growth inhibition. Thus, it appears that the 3'UTRs must be expressed to exert their effects.

The RNAs encoded by the 3'UTRs could affect growth and differentiation by several mechanisms. Genes within UTRs of other genes have been reported (Henikoff et al., 1986), suggesting that the 3'UTRs may encode peptides initiated from cryptic start sites. Alternatively, the 3'UTRs could act as antisense to block the function of other RNAs, as in the case of basic fibroblast growth factor (Kimelman and Kirschner, 1989). Possibly the RNAs encoded by the 3'UTRs act as ribozymes (Latham and Cech, 1989). On
the other hand, the RNAs may sequester proteins that promote growth or inhibit differentiation, hindering access to their targets. For example, the RNAs may increase the transcription of muscle-specific genes by competing for negative regulators that bind to single-stranded DNA of promoters (Santoro et al., 1991). The RNAs encoded by the 3'UTRs could also titrate factors that alter the stability of other RNAs, leading to increased stability of differentiation-specific RNAs or to lability of RNAs with a role in growth. Precedents for such a mechanism are provided by the AU-rich elements within the 3'UTRs of several labile transcripts, including those of oncogenes (Gillis and Maiter, 1991) and the iron-repressive element sequence within the 3'UTR of the transferrin receptor transcript (Koeller et al., 1991). These or other mechanisms as yet unknown may be employed by the 3'UTRs to perturb the balance of regulators of proliferation and differentiation.

It is likely that one or more domains within the 3'UTRs are responsible for their activity. The four complementing cDNAs are clearly distinct, and sequence analysis has not revealed obvious homologies. Each 3'UTR has the potential for extensive secondary structure with multiple stem-loop structures. Mutagenesis will be required to identify the regions that are critical for activity and should reveal whether the four cDNAs have overlapping or redundant functions in growth and differentiation. Once the minimal sequences required for activity are defined, it will be possible to determine whether similar domains are present within 3'UTRs of genes of other cell types and whether they play a similar role in controlling growth and differentiation in these cells.

Maintenance of the Differentiated State

Much is known regarding the initiation of the differentiated state, but little is understood regarding its maintenance. Several lines of evidence suggest that differentiation requires continuous regulation (for reviews see Blau and Baltimore, 1991; Blau, 1992). A hierarchy of regulators leads to each distinct differentiated state (Blau, 1988). To maintain that stable state, feedback mechanisms may be required to circumvent the regulatory hierarchy and sustain a threshold concentration of critical regulators. One such mechanism in muscle may be provided by the MyoD family of myogenic regulators, which appears to maintain its own threshold level through autoregulation of transcription (Thayer et al., 1989). We suggest that a second mechanism by which feedback control could be exerted involves 3'UTRs: upon initiation of differentiation, the 3'UTRs of certain structural genes are transcribed and accumulate, resulting in growth inhibition and further differentiation. This mechanism could constitute a feedback loop that acts continuously to control progression through the cell cycle and to stabilize the differentiated state.

Experimental Procedures

Cell Lines, Maintenance, Fusion, and Selection Conditions

The C2C12 mouse myoblast line (Blau et al., 1983) derived from C2 (Yaffe and Saxel, 1977) was used because it is remarkably similar to primary myoblasts. It is diploid (U. J. Francke and H. M. S., unpublished data) and capable of expressing all four of the myogenic HLH regulators (Peterson et al., 1990). Ten subclones of C2C12 cells were plated at low density in high serum medium, and ~200 clones of each subclone were tested for differentiation potential in low serum medium. From among these, the clone C2F3 was selected for mutagenesis because 100% of clones contained myotubes. C2F3 cells differentiate at low density in low serum medium (2% horse serum), and they can be maintained as proliferating cells in high serum medium (10% calf serum). Cells were mutagenized by a 30 min incubation at 37°C with NMU (Sigma) at doses ranging from 1.25–100 μg/ml in PBS. To assess the efficiency of mutagenesis, we monitored the number of HPRT-negative mutant clones that grew after exposure to NMU by plating one aliquot of 5 × 10⁴ mutated cells in 10 μg/ml 6-thioguanine (Sigma). To assess the frequency of anchorage-independent clones, a similar dose-response curve was generated by plating another aliquot of 5 × 10⁴ NMU-treated cells in 0.34% agar (Difco Laboratories) according to the procedure described by Bouch and di Mayorca (1992). Colonies were counted 3 weeks later. The NMU2 mutant was cloned from soft agar following treatment of C2F3 cells with 12.5 μg/ml NMU and recloned from liquid media to ensure its single cell origin.

All cells were routinely grown in Dulbecco's modified Eagle's medium with high serum (10% calf serum) in 8% CO₂ at 38°C. The medium was changed every 2 days. For selection of drug-resistant stable transformants with SV2 plasmids, the medium was supplemented with G418 (400 μg/ml; GIBCO), hygromycin (200 μg/ml; Calbiochem-Behring), or puromycin (1 μg/ml; Sigma). For assaying the activation of the muscle promoter constructs in NMU2 cells, the concentration of the appropriate drug was empirically determined for each test cell such that uninduced cells would not survive. NMU2-MYGPuro.7 cells were selected in 5.5 μg/ml mycophenolic acid in media also containing 50 μg/ml xanthine, 7 μg/ml hypoxanthine, 2.5 μg/ml thymidine, and 2.2 μg/ml aminopterin (Sigma). The activation of the HCA-puro reporter construct in NMU2-HCApuro.7 cells was determined by selection in 25 μg/ml pyrouracil. The activation of the MYG-puro reporter construct in NMU2-MYGPuro.7 cells was assessed by selection in 1 μg/ml puromycin.

Fusion of cells to obtain hybrids was performed using a modification of previously reported procedures (Blau et al., 1983; Chiu and Blau, 1984). Approximately 1 × 10⁵ cells of each cell type were mixed, rinsed in PBS, and lightly pelleted by centrifugation. The cell pellet was gently resuspended in 250 μl of 50% polyethylene glycol fusion media for 1 min, then diluted with Dulbecco's modified Eagle's medium and rinsed three times before plating.

Northern Hybridization and Probes

Total RNA was isolated by centrifugation of the cell lysates over a cesium chloride cushion (Chirgwin et al., 1979). RNA samples were electrophoresed through 1% agarose-formaldehyde gels for 40 Vh and transferred to Hybond-N membranes (Amersham). The RNA was cross-linked to the membrane by exposure to ultraviolet light (Stratalinker, Stratagene) and hybridized to the probes in a phosphate buffer as previously described (Peteron et al., 1990). Some membranes were reprobed following stripping of the previous probe on 50% formamide, 5 x SSC for 30 min at 65°C. Probes for actin, myosin, (Lanocasa, MyoD), myogenin, and myf-5 have been previously described (Peterson et al., 1992). The gap coding region probe was isolated as a 0.6 kb HindIII to EcoRV restriction fragment from the SV2-puro plasmid. cDNA probes from the clones described here, including troponin I and tropomyosin, were isolated as the Hox fragment from the pCDM8 vector (Seed and Aruffo, 1987). Each DNA (25 ng) was labeled with [α-32P]dCTP (Amersham) using random hexamers as primer (Random Prime Kit, Amersham).

cDNA Library Construction and Transfections

Cells from the muscle tissue of a human fetus at week 12 of gestation were separated into myoblast and nonmyoblast (fibroblast) populations based on differential staining with 5.1H11 antibody and fluorescence-activated cell sorting (Webster et al., 1988). They were cultured as previously reported (Blau and Welsh, 1991). The identity of the two populations was confirmed by observing that only the 5.1H11-positive myoblasts were able to fuse and express myosin heavy chain following 3 days in low serum medium. Poly(A) RNA was isolated from the two cell types after culturing 70% confluent dishes in low serum
for 36 hr. The mRNAs were reverse transcribed to cDNA and inserted in random orientation into the pCDM8 mammalian expression vector that utilizes the human CMV promoter (Klambt et al., 1987). Each library contained approximately 1 x 10^8 independent colonies with an average insert size of 1.4 kb.

To enrich for muscle regulators, single-stranded myoblast cDNAs that hybridized to the biotinylated single-stranded fibroblast cDNA were removed. Single-stranded phagemid DNA was prepared from both libraries (random-oriented cDNA inserts) using the M13 origin of pCDM8. The fibroblast phagemid library was photobiolytized and hybridized to the myoblast phagemid library (Subtractor Kit, Invitrogen). Myoblast phagemids that hybridized to fibroblast phagemids were removed by phenol extraction, and the remainder (subtracted library) was recovered. The subtracted library was transferred into host bacteria MC1061-P3 and plated on agar plates containing tetracycline. Plates were individually harvested after 16 hr and then in 40 pools of 500-1000 colonies.

All constructs were performed using the calcium phosphate method of Graham and Van der Eb (1973). Cells were plated at approximately 1 x 10^4 per 60 mm dish. The supernatant was recovered. Each supernatant (25 μl) was combined, and seven pools of 4000 cDNAs were individually transfectants.

**Recovery of cDNA from Transfectants by PCR**

Colonies were isolated using cloning cylinders (Belco) and rinsed three times with PBS. The pellets were then lysed by boiling in 100 μl of water, vortexed, and centrifuged at 13,000 x g for 5 min, and the supernatant was recovered. Each supernatant (PS, μl) was used as template in PCR. Nested PCR was performed using two sets of primers that flank the cDNA insertion site of the vector. Primers used in the first amplification reaction were Fl (5′-GGAGACCG-GAACTTTAGAGACATCGTACG) and L1 (5′-TCTAGAG-TGCGGCGCGAGCACGCGCAGA). The PCR was performed in 100 μl for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, with a final extension of 10 min at 72°C using Taq polymerase and conditions recommended by the manufacturer (Perkin-Elmer). Reaction conditions for the second round were the same as for the first round.

**Test Constructs and Subcloning of cDNAs**

To construct the HCA-gpt reporter plasmid, the 485 bp HCA promoter (Edmondson et al., 1992), the 0.9 kb tropomyosin 5′ coding sequence, small t splice, and the SV40 polyadenylation signals (Fiers et al., 1979) were combined, and seven pools of 4000 cDNAs were individually transfectants.

**Immunostaining**

Myogenin expression was assayed using monoclonal supernate from hybridoma line 1F5D7 (gift from W. Wright) (Lessard et al., 1991). Colonies were chosen by visual inspection, circled, and analyzed at the single cell level for nuclear myogenin expression by immunocytochemistry. Cells were rinsed with PBS and fixed with 2% formaldehyde for 10 min at room temperature and permeabilized with 1% Nonidet P-40 (BDH Chemicals) for 10 min on ice. Incubation solutions used thereafter contained 2% horse serum and 0.1% Nonidet P-40. Permeabilized cells were incubated in rinsing solution for 2 min, incubated in the monoclonal supernatant for 1 hr, rinsed twice, and incubated with biotinylated anti-mouse immunoglobulin G secondary antibody (1:200; Vector Laboratories) for 30 min. The staining was developed using Vectastain horseradish peroxidase kit (Vector Laboratories) according to the conditions recommended by the manufacturer. Colonies were examined and counted using bright field optics at 100 x magnification on a Leitz Labovert microscope, and the frequency of the colonies containing myogenin-positive nuclei was determined. The sensitivity of this assay was confirmed by introducing a myogenin cDNA expression vector into the test cell; greater than 99% of stably transfected clones expressed detectable myogenin protein in their nuclei (data not shown). For each cDNA tested, a minimum of 100 colonies was scored, the cumulative results of at least two independent transfection experiments with two independent preparations of the plasmids. As is common in the case for myogenic colonies, positive cells were generally detected in the denser central region of the colonies: only rare positive cells were found at the edges.

**Statistical Analysis**

Statistics were calculated from the standard normal equation: \( \frac{\mu - \mu_0}{\sigma} \). Where error bars do not overlap, differences are significant at the 0.05 level, using a two-tailed Student's t test.

**Acknowledgments**

We are grateful to our colleagues in the laboratory and to Drs. David Anderson, Gerald Crabtree, and David Spiegel for discussion and critical review of the manuscript. We especially thank Michael Conboy for expert assistance with DNA sequencing and cloning of control 3'UTRs.


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