

Transcription of the Muscle Regulatory Gene *MYF4* Is Regulated by Serum Components, Peptide Growth Factors and Signaling Pathways Involving G Proteins

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Abstract. The muscle regulatory protein myogenin accumulates in differentiating muscle cells when the culture medium is depleted for serum. To investigate the regulation of myogenin gene expression, we have isolated and characterized the *Myf4* gene which encodes the human homologue of murine myogenin. Serum components, basic FGF (b-FGF), transforming growth factor β (TGF- β), and EGF, agents which suppress differentiation of muscle cells in vitro, down-regulate the activity of the *Myf4* gene, suggesting that it constitutes a nuclear target for the negative control exerted by these factors. The 5' upstream region containing the *Myf4* promoter confers activity to a CAT reporter plasmid in C2C12 myotubes but not in fibroblasts and undifferentiated myoblasts. Unidirectional 5' deletions of the promoter sequence reveal that \sim 200 nucleotides upstream of the transcriptional start site are sufficient for cell type-specific expression. The

forced expression of the muscle determining factors, *MyoD1*, *Myf5*, and *Myf6* and to a lesser degree *Myf4*, results in the transactivation of the *Myf4* promoter in C3H mouse 10T1/2 fibroblasts. Pathways potentially involved in conveying signals from the cell-surface receptors to the *Myf4* gene were probed with pertussis- and cholera toxin, forskolin, and cAMP. Dibutyl-*cAMP* and compounds that stimulate adenylate cyclase inhibit the endogenous *Myf4* gene and the *Myf4* promoter in CAT and LacZ reporter constructs. Conversely, pertussis toxin which modifies G β protein stimulates *Myf4* gene expression. In summary, our data provide evidence that the muscle-specific expression of the *Myf4* gene is subject to negative control by serum components, growth factors and a cAMP-dependent intracellular mechanism. Positive control is exerted by a pertussis toxin-sensitive pathway that presumably involves G proteins.

IN vertebrate organisms skeletal muscle cells develop from multipotential mesodermal stem cells. Following commitment to the myogenic lineage, the cells form the myotomal compartment which gives rise to body musculature. Myogenic differentiation in vivo and in vitro is characterized by the synthesis of muscle-specific structural proteins. A family of myogenic regulatory factors has recently been identified and was suggested to play a crucial role in myogenic lineage determination and during the acquisition of the differentiated muscle phenotype. *MyoD*, the prototype of positive regulators in muscle development, was cloned from 5-azacytidine converted C3H mouse 10T1/2 myoblasts (Davis et al., 1987) and subsequently from human (Braun et al., 1989a) and chicken (Lin et al., 1989), designated as *Myf3* and *cMD1*, respectively. Myogenin, another muscle regulatory protein was identified in rat (Wright et al., 1989) and mouse myogenic cell lines (Edmondson and Olson, 1989), and in human muscle tissue where it was called *Myf4* (Braun et al., 1989a). Two other cDNAs encoding the struc-

turally related proteins *Myf5* and *Myf6* were also isolated from human skeletal muscle (Braun et al., 1989a, 1990). The homologues of *Myf6*, *MRF4* and *herculin* have been identified in rat (Rhodes and Konieczny, 1989) and mouse (Miner and Wold, 1990), respectively. Each of the four known myogenic factor cDNAs when expressed in 10T1/2 fibroblasts or in other nonmuscle cells is sufficient to convert these cells to myoblasts, which under appropriate growth conditions withdraw from the cell cycle and differentiate into myotubes. A similar myogenic activity has also been reported for the human *myd* gene which has not been characterized at the molecular level to date (Pinney et al., 1988).

Given the muscle determining capacity of these factors, it is evident that the regulation of their genes constitutes an important level of control in myogenesis. Expression of *Myf* genes in vivo is restricted to skeletal muscle tissue and its embryonic precursor cells (for review see Olson, 1990; Sassoon et al., 1989; Ott et al., 1991; Bober et al., 1991). The overexpression of transfected myogenic factor cDNAs in nonmuscle tissue culture cells results in autoactivation of the corresponding endogenous gene and the other members of

Antero Salminen and Thomas Braun contributed equally to this work.

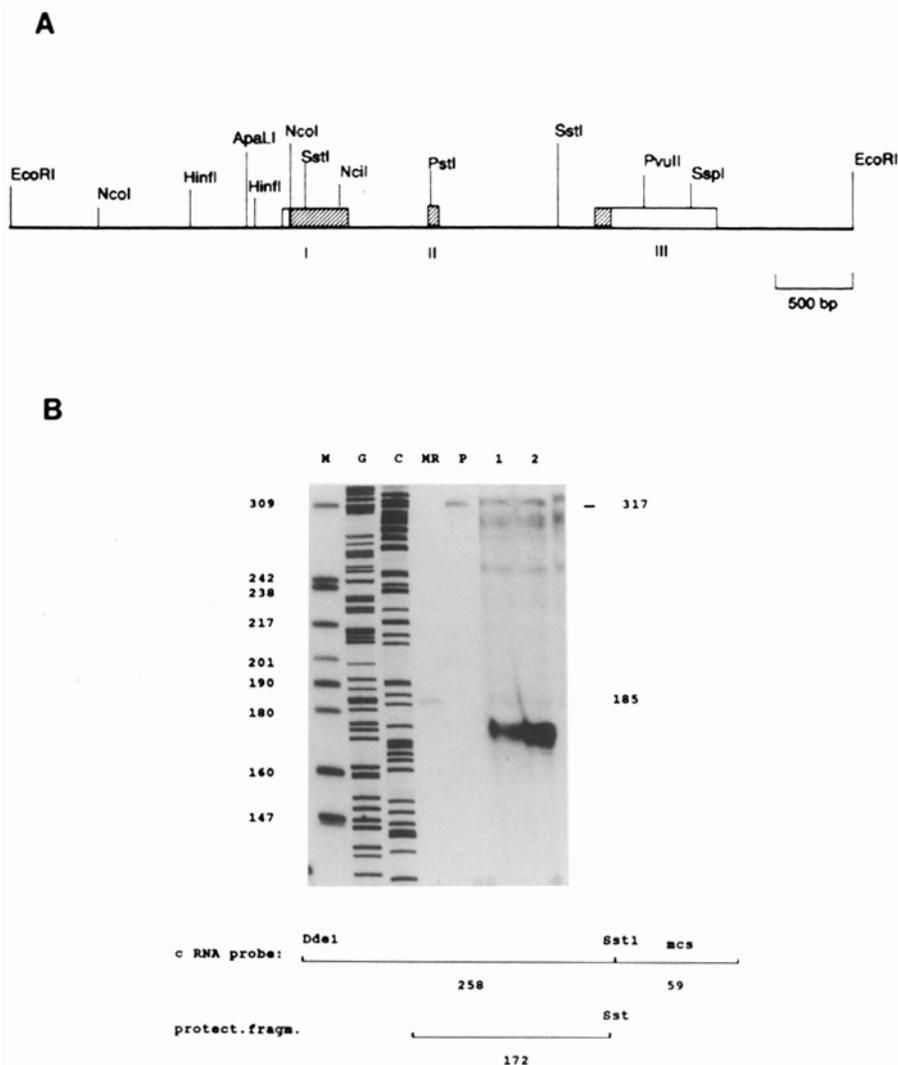


Figure 1. Organization and nucleotide sequence of the human myogenin gene Myf4. (A) Partial restriction map indicating sites used to generate subclones for sequence determination. Exons are shown by numerals. Coding and noncoding sequences are indicated by hatched and open bars, respectively. (B) Determination of the start site for transcription: analysis by RNA protection. The RNA probe was generated from a genomic fragment (*DdeI/SstI*) as described in Materials and Methods. mcs indicates the multiple cloning site of the plasmid pBS which is part of the transcribed probe. Plasmid PBR 322 digested with *HaeIII* was used as size marker (*M*). G and C are sequencing ladders. MR is a RNA size marker of 185 nucleotides. P indicates the RNA probe. Protected RNA fragments obtained with 20 μ g (lane 1) and 40 μ g (lane 2) of human fetal limb RNA are shown. (C) Nucleotide sequence and the predicted amino acid sequence of the Myf4 gene. The transcriptional start site is referred to as +1. The putative TATA box sequence is underlined. Potential MyoD1 binding sites are indicated by boxes.

the MyoD1 gene family (Braun et al. 1989b; Thayer et al., 1989). In established muscle cell lines and during development, however, only subsets of these genes, for example, either MyoD1 or Myf5 but not both, are active (Braun et al., 1989b), indicating that the muscle determination genes are regulated differently. In proliferating myoblasts, myogenin fails to be expressed and its synthesis starts only at the onset of differentiation (Wright et al., 1989; Edmondson and Olson, 1989; Braun et al., 1989b). Together, these observations suggest that positive autoregulation, but also other cellular factors and extrinsic signals may play a role in controlling the activity of the MyoD gene family.

In proliferating myoblasts in vitro the decision to differentiate depends on the removal of serum factors, usually by replacing high concentrations of FCS by low concentrations of horse serum. Addition of FGF or transforming growth factor β (TGF- β)¹ to BC3H1 mouse muscle cells in culture has been shown to inhibit differentiation and the accumulation of muscle-specific gene products (Clegg et al., 1987; Lathrop et al., 1985; Massagué et al., 1986; Olson et al., 1986; Vaidya et al. 1989). The repression of myogenin expression in growing myoblasts and its rapid up-regulation af-

ter the removal of serum suggests that transcription of the myogenin (Myf4) gene and the induction of terminal differentiation may be correlated.

To begin to study myogenin regulation at the molecular level, we have isolated and sequenced the human Myf4 gene. Here, we present evidence that the Myf4 promoter directs transcription only in differentiated myotubes, but not in fibroblasts and proliferating myoblasts. The activity requires the presence of muscle-specific regulatory helix-loop-helix proteins, and/or factors acting downstream of these since the Myf4 promoter can be transactivated in nonmuscle cells by the expression of MyoD1, Myf5, and Myf6. High concentrations of FCS or growth factors, such as basic FGF, TGF- β , and EGF, suppress Myf4 expression. Similarly, compounds that lead to high intracellular cAMP levels also exert negative control on the Myf4 gene. Stimulation of the Myf4 promoter by a pertussis toxin (PT)-sensitive signal transduction pathway suggests that Gi protein may be involved in the regulation of Myf4.

Materials and Methods

Materials

Bovine recombinant basic FGF (bFGF) and mouse EGF were purchased

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase gene; CT, cholera toxin; HS, horse serum; MCK, muscle creatine kinase; PT, pertussis toxin; TGF- β , transforming growth factor beta.

C CCATGGCCTGAGAGGCTGAAGATTAAGTCCCTGCTCTACCTGACATTTGAGACCTGCATGATACGTG -1054
TAAAATTGATGGCTCAGACAGTAAGAGGCAGAACCAAGCCCGGTTGGCCAAAGGAGAGAGATTAA -982
ACTGAGTTTGAAGGGCTCTGTTTTTCTACTTTTTACCCCCCTCCACATTAATGAGCCAGGAAAATCTGG -910
TGTTGGA AAAAGAGTTGAGGAGGTAACATAGAGGACAGATCAATGGTCCGGAGTGAGACAGGGAGGAGACA -838
GGGAGCTTAGGACATCTTGACCTTCCCGCTCTGGGGAAGGTGGCAAGCTGGAGGCTGTAATTTCTAATCTCT -766
GCTCTGACCACGGCTGCTGTATTAAAGAGGGAATTTCTCTTTACCCCTGCCAAGAAAACAAAACCCAAAT -694
AATTTTCTCTTGCTCAATTTACCCCCAGATGAGACCTAAGAGAACATACAGCTCAAAGCTGCTTGGGAA -622
CCCCAAAAGTGAATCTGTTAGCTGCTCTGAGTCTTGATCCTTCTTATCCCTGGATACTGAGTGCCCATGAA -550
TGCCAGAACTCTGAAGCAGTCCCATTTTCCAGGAAGCTTTAAAGAGTCTCATCGACTGATGTAGTGT -478
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CAGCTAGGACTAATTTGAAAGGAGCAGATGAGAGGGAATGTGTCTCCCCAACGCCCTGCCCCACAGGGG -262
CTGCTGAGAAATGAAAATAATCAAATTAACCCGACGGCTCCCGACCCGTCACAGGAGCCCGCTGGGC -190
CAGGGGCAGGCTGGCAGGTTGGGGTGGGGCCATGCGGGAGAAAGAGGGGAATCACATCTAATCCACTGTA -118
AACGCCCTATGTGACGCAACAGCTTAGAGGGCTCAGTTTCTGTGGCTTGGCTATATTTATCTCTGGTTCC -46
ATGCCAGCGGGGAGGGT⁺¹T¹TAAATGGCACCCAGCAGTTGGCTGAGGGGCTGCTGGAGCTTGGGGGCTGGTGG 27
CAGGAACAAGCCCTTCCGACCCCATGGAGCTGTATGAGACATCCCCCTACTTCTACCAGGAACCCCGCTTC 99
MetGluLeuTyrGluThrSerProTyrPheTyrGlnGluProArgPhe
TATGATGGGAAAACCTACCTGCTTCCACCTCCAGGGCTTCGAACCACCAGGCTACGAGCGGACGGAGCTC 171
TyrAspGlyGluAsnTyrLeuProValHisLeuGlnGlyPheGluProProGlyTyrGluArgThrGluLeu
ACCCTGAGCCCGAGGCCCCAGGGCCCTT²GAGGACAAGGGGCTGGGGACCCCGAGCACTGTCCAGGCCAG 243
ThrLeuSerProGluAlaProGlyProLeuGluAspLysGlyLeuGlyThrProGluHisCysProGlyGln
TGCTGCCGTGGCGTGTAAAGTGTGTAAGAGGAAGTCGGTGTCCGTGGACCGGCGGGCGGCCACACTG 315
CysLeuProTrpAlaCysLysValCysLysArgLysSerValSerValAspArgArgArgAlaAlaThrLeu
AGGGAGAAGCGCAGGCTCAAGAAGGTGAATGAGGCCTTCGAGGCCCTGAAGAGAAGCACCTGCTCAACCCC 387
ArgGluLysArgArgLeuLysLysValAsnGluAlaPheGluAlaLeuLysArgSerThrLeuLeuAsnPro
AACCAGCGGCTGCCAAGGTGGAGATCCTGCGCAGTCCATCCAGTACATCGAGCGCCTCCAGGCCCTGCTC 459
AsnGlnArgLeuProLysValGluIleLeuArgSerAlaIleGlnTyrIleGluArgLeuGlnAlaLeuLeu
AGCTCCCTCAACCAGGAGGAGCTGACCTCCGCTACCGGGCGGGGCGGGCCAGCCAGGGGTAAGTGGC 531
SerSerLeuAsnGlnGluGluArgAspLeuArgTyrArgGlyGlyGlyProGlnProGly
CATCCCATCCCCCTGCCCAAGGG -0.4kb- GGACTAGGCAGAGAGACATCAAAGAATNGCTGTGCC 994
CAGCCTTGCCCTTCGCCCTGTCTTGCAGGTGCCAGGAAATGCAGCTCTCACAGCGCTCTGCAGTCCAGAG 1066
ValProSerGluCysSerSerHisSerAlaSerCysSerProGlu
TGGGGCAGTGCCTGGAGTTCAGGCCAACCCAGGGGTAAGTGAGGCCTGACTGGTAACCTTGCCTCAATC 1138
TrpGlySerAlaLeuGluPheSerAlaAsnProGly
CCTCAGTCCCTCCCTGGGCCAGGTTCTCCCTCTT -0.9kb- GCCTCAAGAACCCCCACTGCTCAGCCAG 2101
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AspHisLeuLeuThrAlaAspProThrAspAlaHisAsnLeuHis
TCCCTCACCTCCATCGTGGACAGCATCACAGTGAAGATGTGTCTGTGGCCTTCCCAGATGAAACCATGCC 2245
SerLeuThrSerIleValAspSerIleThrValGluAspValSerValAlaPheProAspGluThrMetPro
AACTGAGATTGCTTCCAAAGCCGGGCATCTTGCAGCCCCCAAGCTGGCCACAGATGCCACTACTTCTGT 2317
Asn
AGCAGGGGCCCTCCTAAGCCAGGCTGCCCTGATGCTAGGAAGCCAGCTCTGGGGTGCCATAGGCCAGACTATC 2389
CCCTTCTCATCCATGTAAGGTTAACCCACCCCGCAGCAAGGGACTGGACGCCCTCATTAGCTGCCCTCCTT 2461
AGAGGAGAGGGCATCCCTTTCAGGAGGTAAGCAGGGGACCAGAGCGCCCTCGTGTATGCCCCAGCTC 2533
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CAAACCTTTTGCAGTGAATTTGGGAACCTTCCAGTGGTTTTATGTTTTGTTTTGTTTCGTGTGTTGTTGTA 2821
AAGCTGCCATCCGACCAAGGTCTCCTGTGCTGAAGTTGCCGGGACAGGCAGGGAAAAGGGTTGGGGCCTC 2893
TTGGGGTGATTTCTTTGTTAAACAAAGCATCGTGTGGTTTTGCCGGAATT 2944

Figure 1.

from Boehringer-Mannheim Biochemicals (Germany). Human transforming growth factor (hTGF- β 1) was obtained from R&D Systems (England). Cholera toxin (CT), PT, forskolin, dibutyryl cAMP, and heparin-Sepharose, were purchased from Sigma Chemical Co. (St. Louis, MO). (14 C)-L-chloramphenicol was purchased from NEN Products (Boston, MA) and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranosid from Boehringer-Mannheim.

Methods

Isolation of the Human Myf4 Gene and Determination of the Nucleotide Sequence. 1×10^6 plaques of a human genomic DNA library constructed in phage lambda EMBL3 (kind gift of A. M. Frischauf, MRC, London) were screened according to Benton and Davis (1977) using the restriction

fragments of the human Myf4 cDNA as hybridization probes which represent the 5' and the 3' end, respectively. A positive recombinant phage was purified and analyzed with various restriction enzymes according to standard procedures (Maniatis et al., 1982). Subfragments were cloned into M13 mp18 and 19 vectors and used for DNA sequencing (Sanger et al., 1977). In addition, unidirectional nested deletions (Henikoff, 1984) were utilized. All presented sequence data were generated on two strands and by repeated sequencing runs.

Determination of the Transcriptional Start Site. The DdeI/SstI fragment encompassing the Myf4 promoter region was subcloned into the SmaI/SacI sites of the vector pBS. To generate complementary RNA, the vector was linearized by digestion with HindIII and transcription was started from the T7 promoter in the presence of ³²(P)-UTP (3,000 Ci/mmol). The probe was purified on a polyacrylamide gel and hybridized to 40 µg of total RNA from human fetal limb muscle. Hybridization was performed in 10 µl of buffer containing 80% deionized formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA at 45°C overnight after an initial denaturation step at 85°C for 10 min. 300 µl of a digestion mix containing 0.3 M sodium acetate, pH 7.0, 5 mM EDTA, 8 µg/ml RNase A, 16 µg/ml RNase T1 were added, and the reaction was incubated for 1 h at 37°C. After digestion with proteinase K, phenol/chloroform extraction, and ethanol precipitation, the probe was denatured and resolved on a sequencing gel.

Construction of Myf4 Promoter Driven Reporter Plasmids. To generate the plasmid Myf41, the 1.1-kb NcoI/NcoI fragment encompassing the 5' upstream region of the Myf4 gene up to the translational start codon was blunt ended with T4 polymerase and inserted into the blunt ended HindIII site of the 3N-polylinker CAT vector (kind gift of G. Schütz, German Cancer Research Center, Heidelberg, Germany) in 5'-3' orientation to obtain an in frame fusion. The same NcoI/NcoI fragment was digested with mung bean nuclease to remove the AUG start codon and cloned into the LacZ vector containing a nuclear localization signal (Bonnerot et al., 1987) to obtain Myf4-nls LacZ. The shorter Myf4 promoter fragment NcoI/PfI/M1 was blunt ended and cloned into the SmaI site of the plasmid 3N-polylinker chloramphenicol acetyltransferase gene (CAT) to generate the plasmid Myf4s. The 5' deletion clones were generated in the plasmid pBS by directed ExoIII digestion (Henikoff, 1984) or by using the restriction enzymes ApaI and MboI. The resulting fragments were recloned into the CAT vector and analyzed by nucleotide sequence determination.

Cell Culture and DNA Transfections. Mouse C2C12 muscle cells (Yaffe and Saxel, 1977) were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and grown in DMEM supplemented with 20% FCS, 5 mM glutamine, 50 U/ml of penicillin, and 50 µg/ml of streptomycin. To induce differentiation, cells were shifted to DMEM medium containing 5% horse serum (HS). C3H 10T1/2 fibroblasts were grown in BME medium supplemented with 10% FCS. Culture media and sera were obtained from Gibco Laboratories (Grand Island, NY). C2C12 cells (5 × 10⁵/10-cm dish) were stably transfected with 1 µg of supercoiled pSV2-neo plasmid and 20 µg of Myf41 plasmid or Myf4-LacZ reporter plasmid. Stable C2C12 cell lines harboring the plasmids RSV-CAT or β-actin-CAT (PI-CAT; Lohse and Arnold, 1988) were also generated and used as constitutively expressing reporter control. Cotransfections were performed by the standard calcium phosphate precipitation method described earlier (Braun et al., 1989c). G418-resistant colonies were selected in medium containing 400 µg/ml G418 (Geneticin; Gibco Laboratories) and tested for the expression of CAT or β-galactosidase. All expressing clones showed proper up-regulation of either CAT or nuclear lacZ under differentiation conditions (5% HS). Four randomly selected clones were used in this study. Transactivation of the Myf41 plasmid in 10T1/2 fibroblasts was performed by transient transfections of 10 µg of reporter plasmid together with 10 µg of the expression vectors pEMBL-Myf3, pEMBL-Myf4, pEMBL-Myf5, and pEMBL-Myf6 and 5 µg of βRSV-gal expression plasmid used as internal standard. 20 µg of Myf41 or the various 5' deletion mutants Myf4/883, Myf4/619, Myf4/377, and Myf4/211 were transiently transfected into C2C12 cells and chick primary breast muscle cells or skin fibroblasts together with 5 µg of the internal control plasmid RSV-βgal. CAT activity was determined 3 d later according to standard procedures (Gorman, 1985). Media containing drugs and toxins were changed daily.

CAT Assays and Determination of LacZ-Positive Cells. CAT activity of stably transfected lines was measured in cell extracts normalized to protein concentration which was determined using the BioRad-Kit. In transient transfections, the cotransfected RSV-βgal plasmid was always used to calibrate the CAT assays. The conversion of chloramphenicol substrate was quantitated by scintillation counting of the products separated on thin-layer plates. Each CAT value represents the mean of at least three independent experiments. LacZ (β-galactosidase) activity was determined by cytochemical staining using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid as substrate.

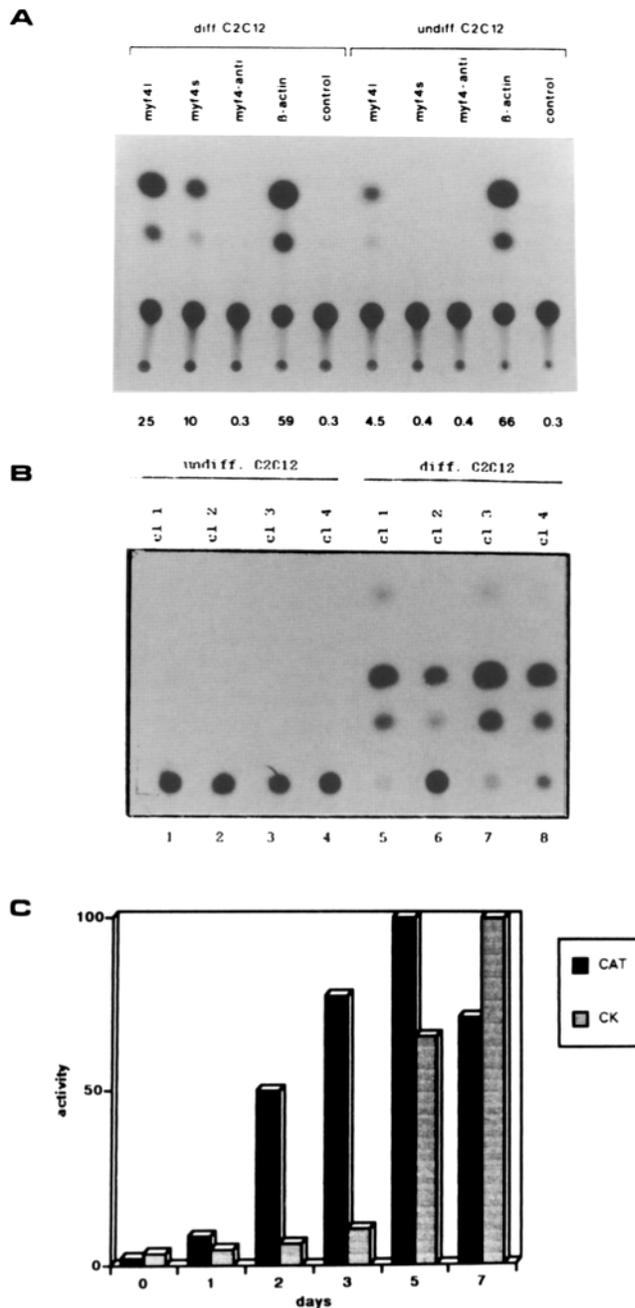


Figure 2. Activity of the Myf4 promoter in transiently and stably transfected C2C12 muscle cells. (A) Transient CAT activity of the plasmids Myf41 and Myf4s in differentiated and undifferentiated C2C12 cells. Numbers indicate the percentage of substrate conversion. The β-actin promoter containing CAT plasmid has been described previously (Lohse and Arnold, 1988). Plasmid SVO-CAT was used as negative control. Myf4-anti contains the promoter fragment in antisense orientation. (B) Myf41 CAT activity in stably transfected C2C12 clones grown in 20% FCS (lanes 1-4) and 5% HS (lanes 5-8) for 5 d. (C) Comparison of activation pattern for the Myf41 transgene and the endogenous creatine kinase (MCK) gene. Specific enzyme activities were determined in duplicates as described under Materials and Methods at the indicated times after serum was shifted to induce differentiation. One representative result from three independent experiments is shown.

breast muscle cells

skin fibroblasts

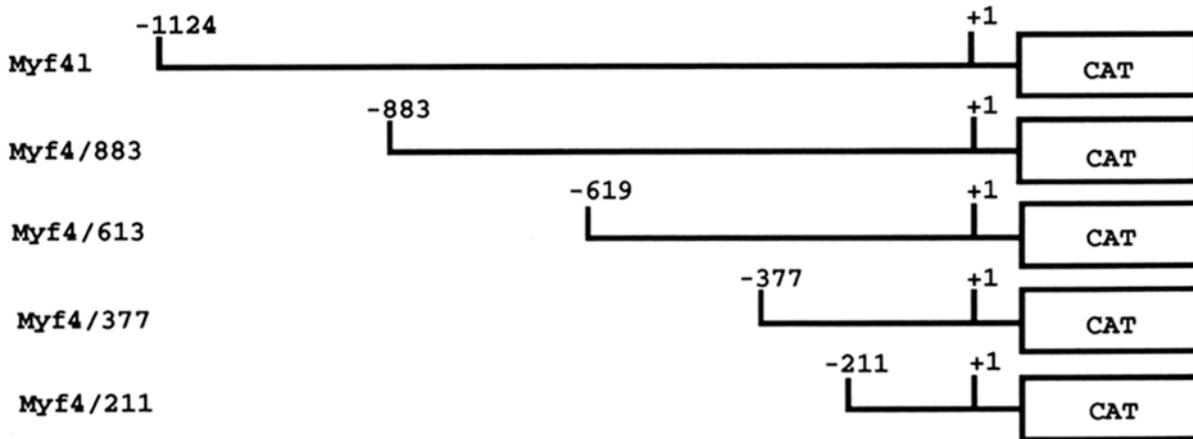
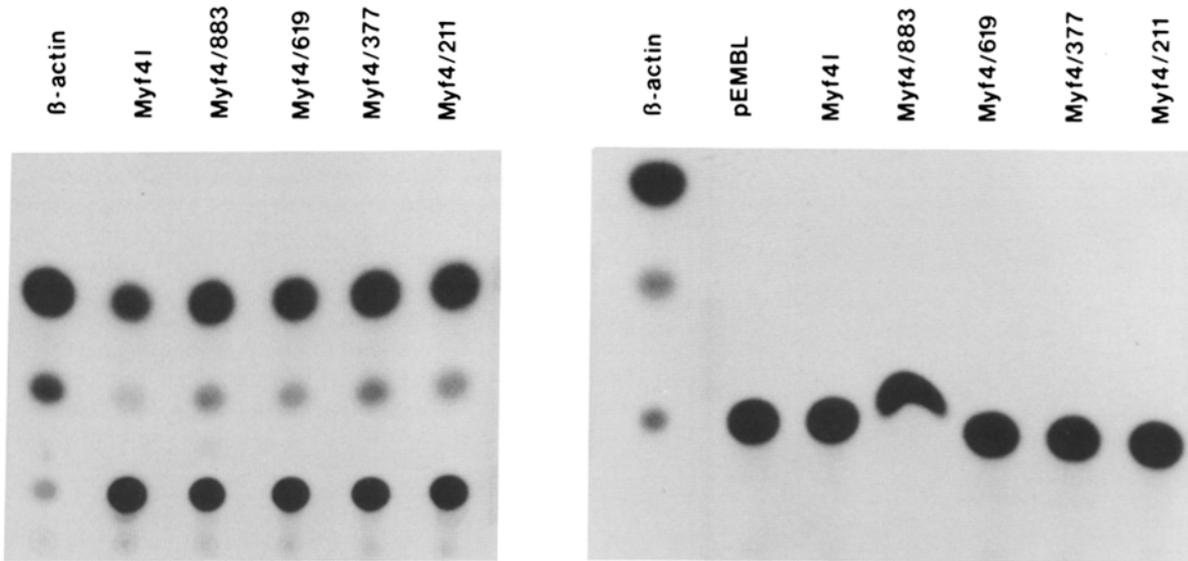


Figure 3. 5' promoter deletions of Myf4-CAT reporter constructs are active in chick primary breast muscle cells but not in fibroblasts. The indicated plasmids were transiently transfected into primary cell cultures of 10-d chick embryos and CAT activity was determined after transfection. The amount of cellular extracts used was standardized according to the β -gal activity from the cotransfected RSV- β gal vector. The structure of the promoter deletion constructs is shown schematically.

Determination of Creatine Kinase and Intracellular cAMP. Specific activity of muscle creatine kinase (MCK) was measured in cellular extracts using the kit from Sigma Chemical Co. according to the procedure given by the supplier. The concentration of cAMP was determined by RIA using the kit from Amersham International (Amersham, UK). Values represent means of three independent measurements.

RNA Analysis from Tissue Culture Cells. Isolation of RNA, gel electrophoresis, RNA transfer, and hybridization conditions have been described previously (Braun et al., 1989b). Myogenin (Myf4) mRNA has been detected using a 700-bp probe from the 3' end of the rat cDNA (Sassoon et al., 1990) labeled with (^{32}P) dCTP by random priming. The GAPDH probe is a 1,100-bp segment of the mouse glyceraldehyde phosphate dehydrogenase cDNA.

Results

Isolation and Structural Analysis of the Human Myogenin Gene MYF-4

To isolate the MYF4 gene, an EMBL-3 phage lambda library

of human genomic DNA was screened with Myf4-specific cDNA probes. Several recombinant phages were purified which hybridized to separate probes of the 5' and the 3' end of the Myf4 cDNA. The coding information for Myf4 is located on an internal 5-kb EcoRI restriction fragment (Fig. 1 A). It is split into three exons, the first of which encodes the complete NH_2 -terminal half of the molecule, including the entire HLH domain. The transcriptional start site has been determined by RNase protection using the complementary RNA transcript of an appropriate genomic DNA fragment and RNA from human fetal limb muscle (Fig. 1 B). According to this analysis, the mRNA starts 52 nucleotides upstream of the AUG initiation codon for translation. The nucleotide sequence encompassing all three exons, part of the introns and 1.1-kb of 5' upstream DNA containing the putative promoter region has been determined (Fig. 1 C). The TATA-box is preceded by a second AT-rich element and several motifs for potential MyoD1 binding sites. The se-

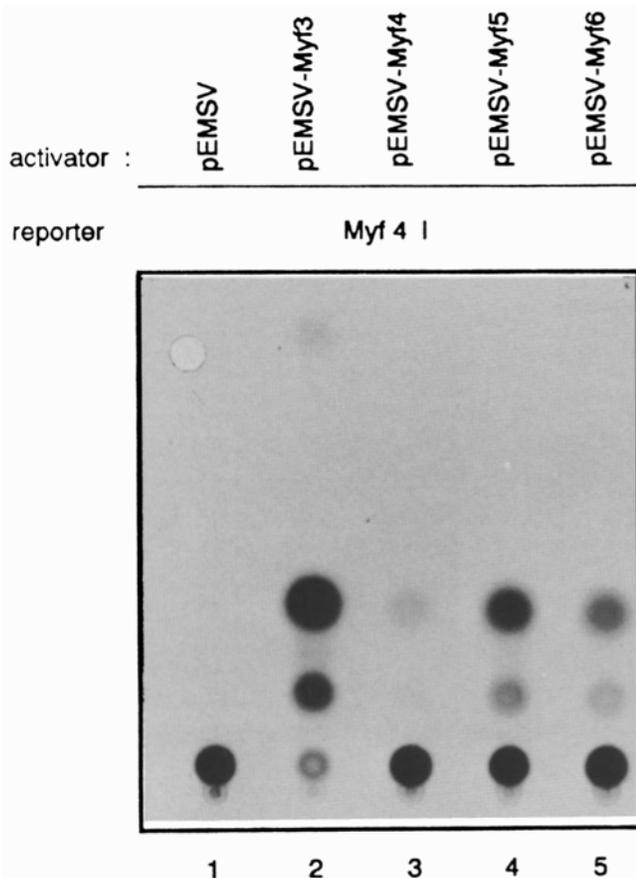


Figure 4. Transactivation of plasmid Myf41 in 10T1/2 fibroblasts. Transient CAT activity of the reporter plasmid (*Myf41*) was determined in extracts from 10T1/2 cells which were cotransfected with vectors expressing the indicated myogenic determination factors.

quence element which most closely resembles the consensus motif for MyoD1 binding in the MCK enhancer is located between nucleotides -14 and -7 , downstream of the TATA box and proximal to the transcriptional start site.

The Myf4 Promoter Directs Expression in Postmitotic Myocytes but Not in Proliferating Myoblasts

It has been reported previously that synthesis of myogenin in muscle cell lines starts shortly after FCS has been reduced or replaced by HS, conditions which are generally applied to induce differentiation and myotube formation (Wright et al., 1989; Braun et al., 1989b; Edmondson and Olson, 1989). To investigate whether the isolated Myf4 promoter contains the control regions which are sufficient to confer muscle-specific and regulated expression, we have constructed reporter plasmids containing the sequence from $-1,124$ to -10 (Myf4s), $-1,124$ to $+54$ (Myf41), and successively shorter 5' deletions of the promoter fragment containing the regions -883 to $+54$ (Myf4/883), -619 to $+54$ (Myf4/619), -377 to $+54$ (Myf4/377), and -211 to $+54$ (Myf4/211). These 5' upstream sequences were joined to the CAT gene. The promoter fragment in Myf4s is truncated at the 3' end and lacks the most proximal consensus element for MyoD1 binding. As shown in Fig. 2, both reporter plasmids Myf4s and Myf41 when transiently transfected into C2C12 muscle cells markedly stimulate CAT expression in differen-

tiated myotubes but show only marginal activity in undifferentiated myoblasts. Myf41 is approximately threefold more active than Myf4s, which may suggest that the most proximal region of the promoter contributes to the activity as a positive *cis*-acting element. Alternatively, the lack of the 5' leader sequence ($+1$ - $+54$) in Myf4s may also be responsible for reduced CAT expression. As expected, transfection of the β -actin promoter construct used as a constitutively expressed positive control results in equal CAT activities in differentiated and undifferentiated cells. Myf4-anti containing the promoter fragment in the opposite orientation fails to support transcription. The myotube-specific activation of the Myf41 construct was further confirmed in four independently isolated C2C12 clones that carry the stably integrated reporter plasmid. As shown in Fig. 2 B, four distinct C2C12 clones promote CAT activity only when the cells are differentiated. The activation of the Myf4 CAT construct

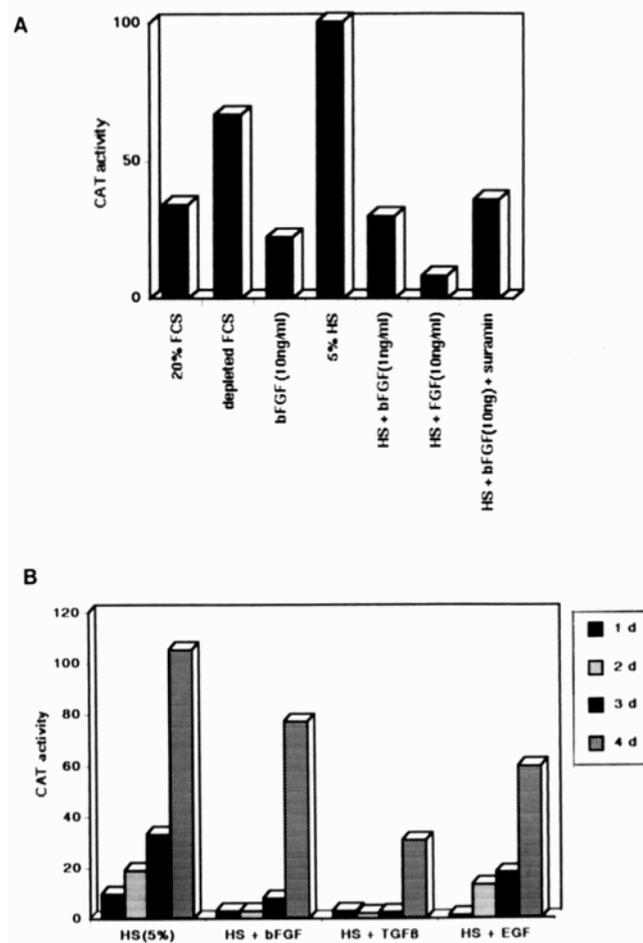


Figure 5. CAT activity of the stably integrated Myf41 gene in C2C12 cells in the presence of serum and purified growth factors. (A) Myf41 containing C2C12 cells (clone 4) were cultured for 2.5 d under the indicated conditions and specific CAT activity was determined by standard procedures. FCS was depleted by two consecutive runs over a Heparin-Sepharose column. Suramin was added at $150 \mu\text{M}$. (B) Four dishes with 2×10^4 cells/5-ml dish were cultured in parallel in the presence of HS alone or in the presence of bFGF (15 ng/ml), TGF- β (5 ng/ml), and EGF (30 ng/ml). The medium was changed daily and specific CAT activity was determined for each day (1-4 d).

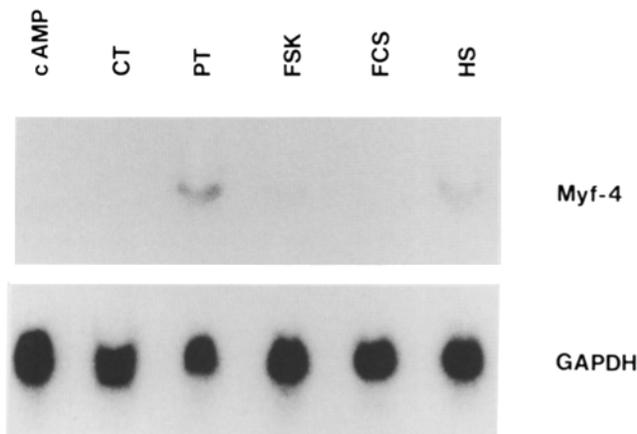


Figure 6. Expression of Myf4 (myogenin) mRNA in C2C12 cells cultured in the presence of bacterial toxins, cAMP, or forskolin. Total cellular RNA (20 μ g) from cells cultured in the presence of the indicated compounds for 2.5 d was analyzed on Northern blot using a Myf4-specific hybridization probe (specific activity 1×10^7 cpm/ μ g) and a GAPDH-specific probe (specific activity 1×10^8 cpm/ μ g) as control. Films were exposed for 18 h.

precedes the upregulation of structural muscle marker genes by several days as shown for MCK activity (Fig. 2 C). Similar results were obtained for the accumulation of MCK mRNA as well as mRNAs for other muscle markers like myosin light and heavy chains (data not shown).

To delimit the sequences involved in cell type-specific transcription of the Myf4 gene with a resolution >1.1 kb, the 5' deletion constructs were transfected into chick primary breast muscle cells, skin fibroblasts, and hepatocytes and assayed for the accumulation of CAT enzyme. The primary muscle cells were chosen because they allowed higher CAT activities in transient transfections than the C2C12 cell line. As shown in Fig. 3, all Myf4 promoter fragments ranging in size from $\sim 1,100$ to 200 bp are active in skeletal muscle cells but not in fibroblasts and hepatocytes (data not shown). Variable efficiencies of DNA transfection in different cell types were corrected according to β -galactosidase enzyme activities obtained from the cotransfected RSV- β gal plasmid.

In summary, these results demonstrate that the Myf4 promoter directs expression in muscle cells which closely mimics the cell type-specific and temporal regulation of the endogenous myogenin gene (Wright et al., 1989; Edmondson and Olson, 1990). Approximately 200 nucleotides upstream of the transcriptional start site are sufficient to maintain this regulation.

The Myf4 Promoter Is Silent in 10T1/2 Fibroblasts but Can Be Transactivated by Muscle-specific Transcription Factors

We and others have previously shown that myogenin mRNA accumulates only in cells that are committed to the myogenic lineage and contain at least one muscle-specific H-L-H protein (Braun et al., 1989a; Edmondson and Olson, 1989; Wright et al., 1989). Here, we demonstrate that the Myf4 promoter can be transactivated in 10T1/2 fibroblasts, when muscle-specific transcription factors such as MyoD or Myf5 are expressed. As illustrated in Fig. 4, the Myf4 plasmid is completely inactive when transiently transfected into 10T1/2

fibroblasts alone or together with the control expression vector pEMSV. Cotransfection of plasmids expressing Myf3, Myf5, or Myf6 leads to activation of the Myf4 promoter. Expression of myogenin (Myf4) stimulates transcription weakly but significantly. Similar results are obtained using the activator plasmids over a tenfold range of different concentrations (data not shown). We conclude from these observations that the muscle regulatory H-L-H proteins themselves or downstream-acting factors that become activated constitute a positive stimulus for Myf4 expression. The same results were obtained with 5' promoter deletions, suggesting that the required target elements to confer this control are contained in the proximal promoter region of Myf4 (data not shown).

Serum Components, bFGF, TGF- β , and EGF Exert Negative Control on the Myf4 Promoter

The downregulation of the myogenin (Myf4) gene by serum components and the purified growth factors bFGF, TGF- β , and EGF have been observed previously (Heino and Masagué, 1990; Brunetti and Goldfine, 1990). Here, we investigate the response of the isolated Myf4 promoter in the Myf4-CAT reporter construct which was stably integrated in C2C12 muscle cells. These cells were grown in the absence and presence of growth factors for 2–3 d and then analyzed for CAT activity. As shown in Fig. 5 A, maximal CAT activity accumulates in cells growing in 5% HS while 20% FCS results in a considerable decrease. When FCS has been depleted for mitogens which bind to heparin-sepharose, some of this repression is released. The activation is counteracted by the addition of bFGF (10 ng/ml), which also represses the activation of the Myf4 promoter in the presence of HS. The inhibition is concentration dependent and can be obliterated at least in part by the addition of suramin, a drug that prevents ligand binding to the FGF receptor (Coffey et al., 1987). Suppression of Myf4 promoter activity is also observed when cells are grown in the presence of TGF- β and EGF. As shown in Fig. 5 B in a time course experiment performed over 4 d, Myf4 promoter-driven CAT activity increases in cells differentiating in 5% HS. In contrast, in the presence of peptide growth factors CAT activity is severely inhibited, particularly 2 to 3 d after medium shift. Inhibition is less pronounced at day 4.

Although cells were generally seeded at low density to avoid precocious differentiation, we always observed this spontaneous activation of Myf4-CAT when cells had grown for more than 3 d in culture and reached confluency. In summary, our results indicate that serum components in FCS and peptide growth factors repress Myf4 promoter activity. This negative control by mitogens is mediated via target elements in the Myf4 promoter located within 1.1 kb of the 5' upstream sequence.

Pertussis- and Cholera Toxin-sensitive Signaling Pathways Affect Accumulation of Myf4 mRNA

As an attempt to investigate signal transduction pathways which may be involved in the regulation of the Myf4 gene, we have determined the effects of bacterial toxins known to modulate G proteins. CT and PT catalyze the ADP ribosylation of Gs and Gi proteins which respectively stimulate or inhibit the activity of adenylate cyclase and probably that of other cellular functions (for review see Ui, 1990; Fishman,

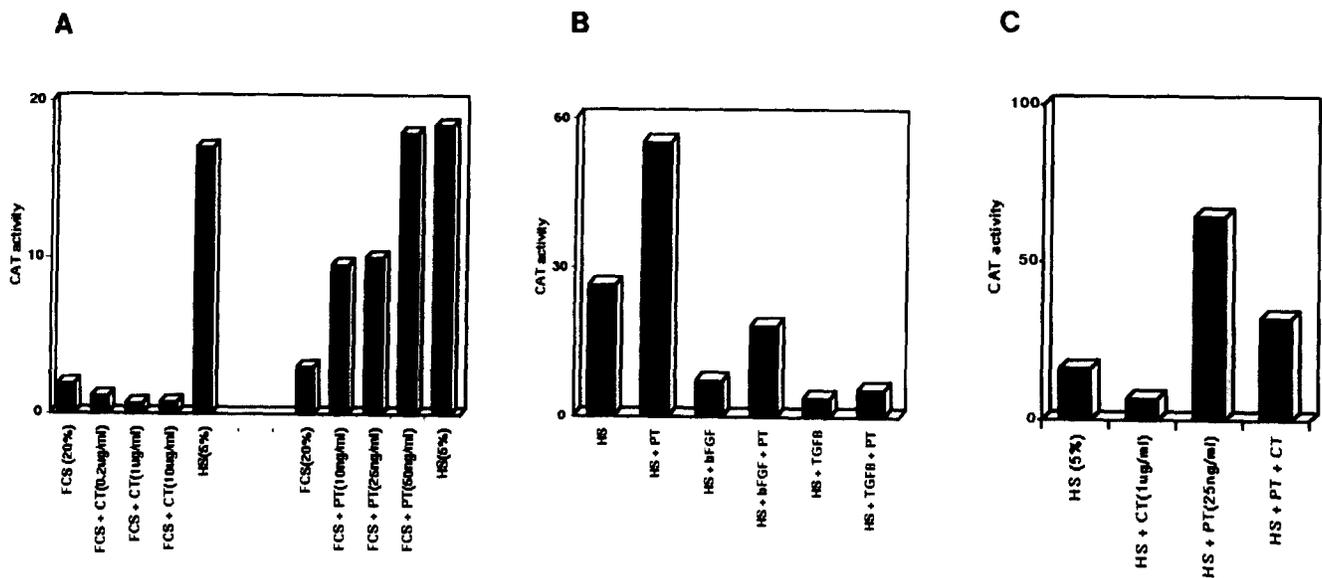


Figure 7. Effects of bacterial toxins PT and CT on the expression of Myf41 in stably transfected C2C12 cells. Cells (2×10^4 /5-ml dish) were cultured for 2.5 d under conditions as indicated. Myf41 CAT activity was determined as described under Materials and Methods.

1990). The toxin concentrations that we used did not affect the viability of the cells as judged by their tight attachment to the culture dish, their general morphological appearance, and the exclusion of trypan blue. RNAs from C2C12 muscle cells growing in the presence of PT, CT, or other agents known to affect intracellular cAMP concentrations, were analyzed on a Northern blot using a Myf4-specific hybridization probe and a probe for GAPDH to control the RNA loading on the blot. As shown in Fig. 6, in cells cultured in HS or in FCS plus PT for 2.5 d, Myf4 mRNA accumulates to readily detectable levels. In cells cultured in FCS or in HS plus db-cAMP, CT or forskolin accumulation of Myf4 mRNA is severely diminished. This result indicates that CT suppresses Myf4 expression probably through increased cAMP levels, whereas PT appears to stimulate Myf4 synthesis.

To test whether the observed effects could be mediated via the 1.1-kb promoter fragment of the Myf4 gene, the same experiments were performed with C2C12 cells containing the Myf41-CAT reporter gene. As shown in Fig. 7, the basal promoter activity of the Myf41 plasmid in cells growing in 20% FCS (Fig. 7 A) and the elevated Myf4 promoter activity in cells cultured in HS (Fig. 7 C) are inhibited by CT in a concentration-dependent fashion. In contrast, PT strongly stimulates the Myf4 promoter from basal activity to a level that approaches that in differentiating cells cultured in the presence of HS (Fig. 7 A). PT plus HS elevates Myf4 promoter activity two- to threefold above the level obtained in HS alone (Fig. 7 B). The stimulatory effect by PT is almost completely abolished by basic FGF or TGF- β when added in concentrations that prevent cellular differentiation (Fig. 7 B). The simultaneous administration of CT and PT results in an intermediate Myf4 activity level which confirms their antagonistic effects (Fig. 7 C). Taken together, the results show that the stimulation of the Myf4 promoter by PT appears to be independent of the serum conditions, since both the basal level in FCS and the elevated level in HS can be further increased which argues for a positive transcription

signal and not for just the counteraction of the negative effects by serum components. Interestingly, PT fails to abolish the repression of the Myf4 promoter exerted by the peptide growth factors bFGF and TGF- β . To test whether the stimulation of Myf4 gene activity by PT may involve changes in cAMP levels, we have analyzed the intracellular concentration of cAMP. In contrast to CT which leads to elevation of cAMP, PT does not noticeably alter cAMP levels in C2C12 cells (data not shown). We therefore conclude that PT activates the Myf4 promoter by a pathway that antagonizes the effect of high concentrations of cAMP but most likely is not mediated via cAMP.

It has been demonstrated previously that treating cells with CT increases intracellular levels of cAMP (Gill, 1977). Consequently, if inhibition of Myf4 expression by CT involves cAMP, other compounds that lead to elevated cAMP levels should also reduce the activity of Myf41-CAT. As shown in Fig. 8 A, C2C12 myoblasts cultured in HS in the presence of CT (2.5 μ g/ml), forskolin (34 μ M) or db-cAMP (3 mM) exhibit reduced CAT activities, particularly during the early phase of the 4-d culturing period. The repression becomes less pronounced at later stages when the cells reach confluency. In C2C12 cell lines carrying stably transfected β -actin CAT or RSV-CAT plasmids, treatment with CT, forskolin, db-cAMP, and PT has no effect on CAT activities, which formally shows that these agents do not alter the stability or activity of CAT mRNA or protein (Fig. 8 B). Taken together, these results suggest that elevated levels of cAMP reduce Myf4 promoter activity, particularly in cells that have not yet initiated morphological differentiation. At later stages, a compensatory mechanism may counteract the negative control exerted by cAMP.

The Response of the Myf4 Promoter Is an Early Event and Does Not Depend on Morphological Differentiation of Muscle Cells

To examine the activation of the Myf4 promoter in single myocytes under the influence of various extrinsic factors, we

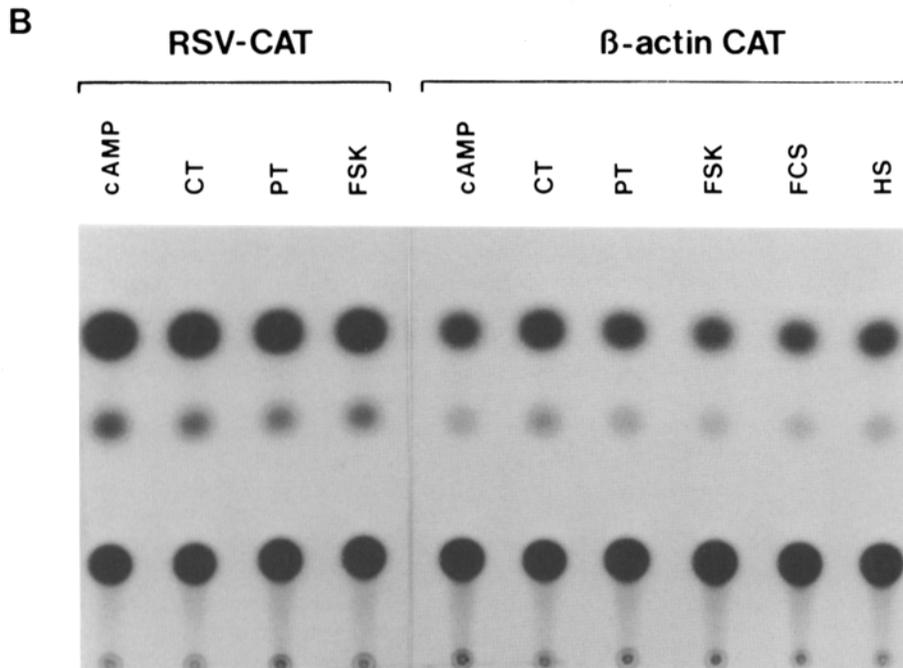
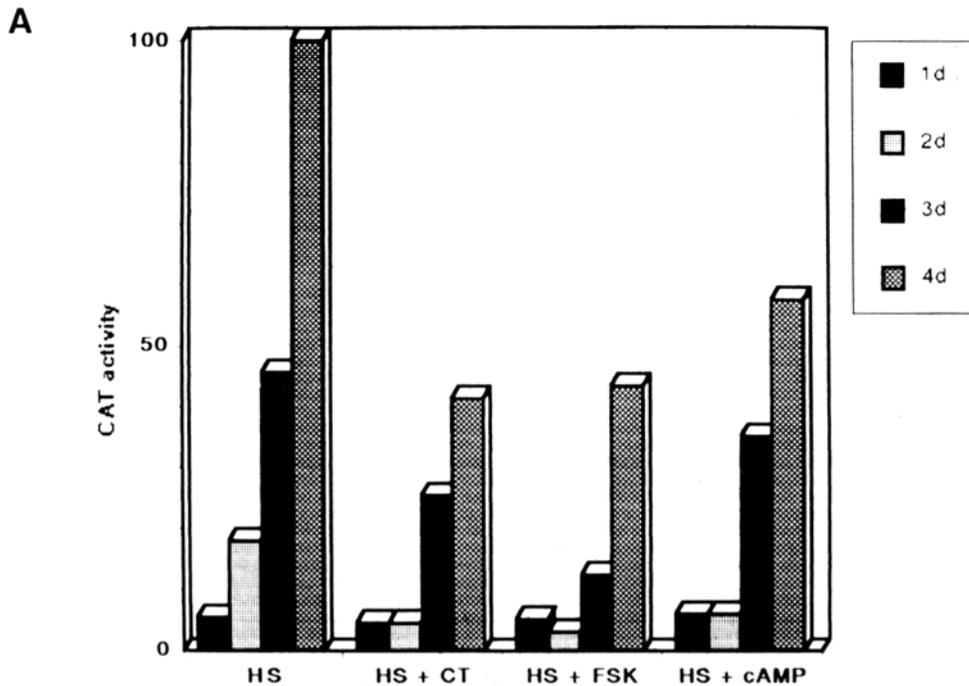


Figure 8. (A) Inhibition of the Myf41 promoter in C2C12 cells in the presence of CT (2.5 μ g/ml), forskolin (34 μ M), and dibutyryl-cAMP (3 mM). Specific CAT activity was determined over 4 d. Media were changed daily. (B) Bacterial toxins and cAMP have no effect on the constitutively expressed β -actin-CAT and RSV-CAT reporter plasmids in stably transfected C2C12 cell lines. These control experiments were performed as described above and under Materials and Methods.

produced a stable C2C12 muscle cell line that carries the Myf4-nls LacZ reporter plasmid. This construct contains the lacZ gene modified with a nuclear localization signal (Bonnerot et al., 1987) under the control of the human Myf4 gene promoter. Nuclear staining for β -galactosidase activity is expected to be seen in differentiating but not in proliferating muscle cells. Indeed, in C2C12 cells growing in the presence of 20% FCS, little or no β -galactosidase staining is observed (data not shown), whereas in C2C12 cells growing in the presence of 5% HS, numerous nuclei appear stained (Fig. 9, A and B). As shown for cells 2-3 d after the shift to differentiation medium (Fig. 9 B), the Myf4 promoter is

activated in the majority of mononucleated cells before any morphological alterations. Interestingly, there is significant variability among the cells in numbers and intensity of stained nuclei, both in mononucleated myoblasts and in multinucleated myotubes. Even after culturing cells in HS for 5 d some myocytes do not express β -galactosidase (Fig. 9 A). This variation within the cell population has been seen in the parental clone as well as in several serial subclones, which excludes the possibility of a heterogeneous cell population. Similar variability of gene expression among muscle cells in culture has been observed previously for the accumulation of MyoD in differentiated C2C12 muscle cells (Tap-

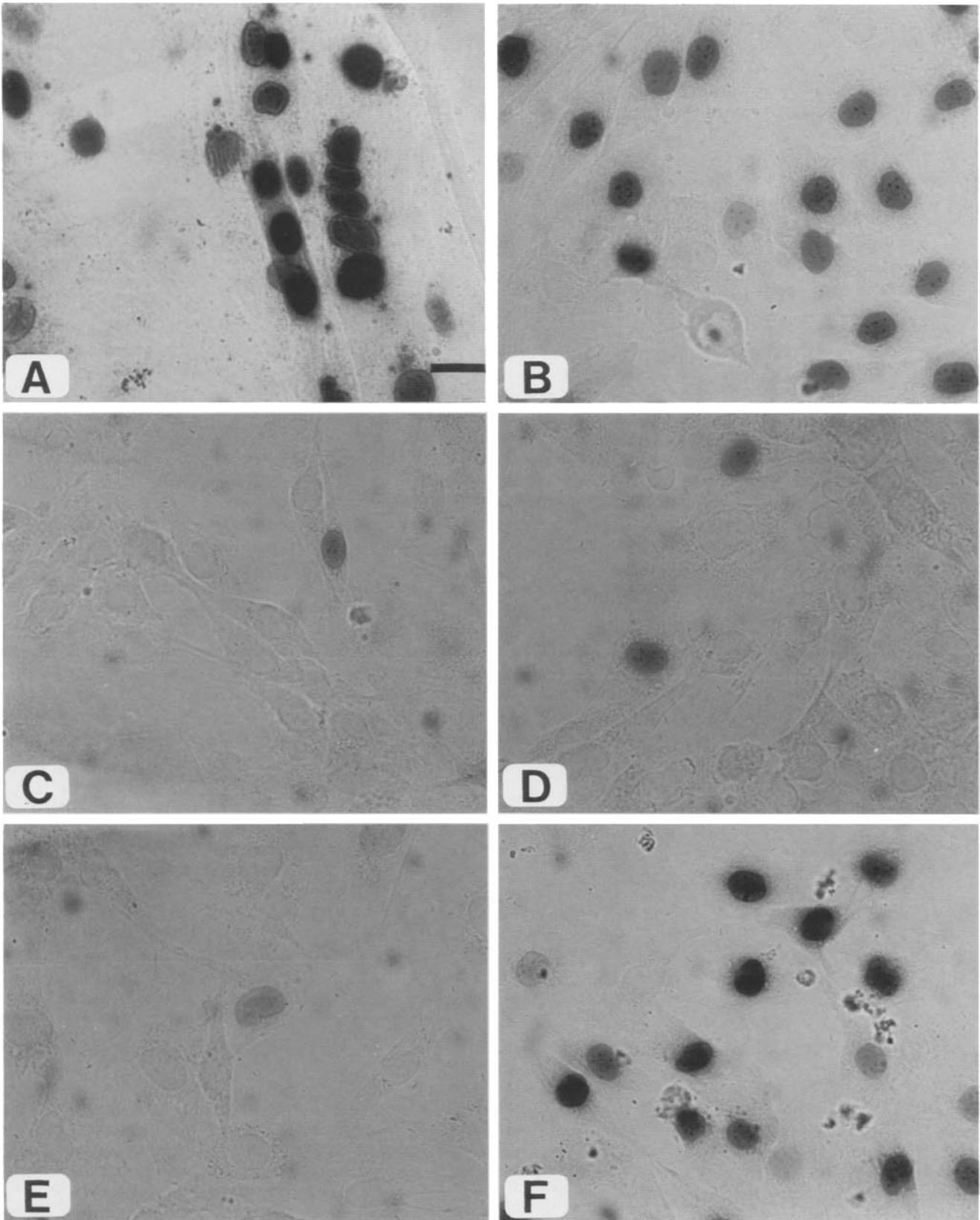


Figure 9. Myf4 promoter activity in C2C12 cells stably transfected with the Myf4-nls lacZ gene. Gene activation is indicated by the nuclear β -galactosidase activity visualized by cytochemical staining. Cells were cultured in HS for 5 d (A) or 3 d (B) and in the presence of b-FGF (15 ng/ml) (C), TGF- β (5 ng/ml) (D), cholera toxin (2.5 μ g/ml) (E), and pertussis toxin (50 ng/ml) (F). Bar, 45 μ m.

scott et al., 1988). In C2C12 cells treated with bFGF (Fig. 9 C), TGF β (Fig. 9 D), or CT (Fig. 9 E) general suppression of the Myf4 promoter occurs even in the presence of HS confirming our previous results obtained with the Myf4-CAT reporter construct. The negative control exerted by these agents appears to be more homogenous since almost all cells lose the LacZ staining. In contrast, treatment with PT strongly stimulates the Myf4 promoter as seen by the very intense staining of most nuclei, exceeding that in control myoblasts grown in the presence of HS alone (Fig. 9 B). Although PT activates the Myf4 promoter, it does not lead to fusion of myoblasts, indicating once more that transcriptional activation of the Myf4 gene is an early event during the differentiation process. The experiment shows furthermore that cell proliferation and the regulation of the Myf4 promoter are uncoupled since CT which inhibits the Myf4 promoter and PT which stimulates Myf4 transcription, both inhibit proliferation (Kelvin et al., 1990 a,b).

Discussion

The identification of a family of muscle regulatory genes provides a genetic framework in which molecular mechanisms involved in the development of skeletal muscle cells can be studied. While it is evident that the capacity of the myogenic factors to bind to DNA (Buskin and Hauschka, 1989; Lassar et al., 1989a; Braun et al., 1990), to interact with other regulatory proteins (Benezra et al., 1990; Brennan and Olson, 1990; Braun et al., 1990; Murre et al., 1990a,b; Davis et al. 1990), and to transactivate muscle promoters (Braun et al., 1990a,b; Rosenthal et al., 1990) may all constitute important levels of control, the most basic regulation of myogenesis concerns the expression of the muscle determination genes themselves.

Besides the fact that Myf genes are only expressed in skeletal muscle cells, little is known about their regulation. Among the MyoD gene family, Myf4 (myogenin) seems particularly interesting since it is the only gene that is expressed in every known muscle cell line and therefore may be the most critical factor for the maintenance of the differentiated phenotype. This idea is further supported by the observation that Myf4 expression is strictly initiated at the onset of terminal differentiation, unlike the other myogenic factors which are already expressed in determined myoblasts before differentiation. Furthermore, Brunetti and Goldfine (1990) have shown that repression of myogenin synthesis by antisense oligonucleotides results in down-regulation of the muscle-type acetylcholine receptor in BC3H1 cells, suggesting that myogenin is crucial for at least some aspects of terminal differentiation.

To begin to investigate signals and mechanisms that are important for the regulation of the myogenin gene, we have isolated and sequenced the human Myf4 gene and its promoter. The gene is organized in three exons, very similar to the mouse herculin (Miner and Wold, 1990) and the human and mouse Myf5 genes (our unpublished results), suggesting a common ancestor although their nucleotide sequences outside of the highly conserved helix-loop-helix domain have considerably diverged. The construction of reporter plasmids which are controlled by the Myf4 promoter allows the analysis of the cellular requirements for its activation. As our results demonstrate, a 1.1-kb promoter fragment allows

efficient and regulated activity in C2C12 muscle cells and ~200 nucleotides are sufficient to confer cell type-specific expression in chicken primary breast muscle cultures. In contrast, nonmuscle cells like C3H mouse 10T1/2 fibroblasts, or chicken primary skin fibroblasts and hepatocytes do not support transcription of the Myf4 promoter, which suggests that these cells either contain negative control factors or that they miss crucial positive transcription factors, or both. The failure to express the Myf4 promoter in 10T1/2 fibroblasts could be caused by the absence of positive transcription factors since constitutive expression of the muscle-specific H-L-H proteins Myf3 (MyoD1), Myf5 and Myf6, results in marked stimulation of the Myf4 promoter. This result provides confirmation for the earlier observations that the forced expression of MyoD or Myf5 in 10T1/2 fibroblasts leads to the activation of the endogenous myogenin gene (Thayer et al., 1989; Braun et al., 1989b). A survey of the Myf4 promoter sequence used in our transactivation experiments indicates the existence of several consensus motifs (E-boxes) which resemble the MyoD binding site, previously identified in the muscle-specific enhancer of the MCK gene (Buskin and Hauschka, 1989) and in other muscle-specific genes (Yutzey et al., 1990; Rosenthal et al., 1990). Although we have not investigated the functional significance of these sequence elements in detail, it is reasonable to assume that at least some of them are involved in Myf4 promoter regulation as they have been conserved in evolution from mouse to man (E. Olson, personal communication). Further studies will be performed to dissect the functional sites for protein interactions which may be required for the activation of the Myf4 promoter.

In skeletal muscle cells a general antagonism between proliferation and the acquisition of the differentiated phenotype exists. Consequently, when muscle cells differentiate, they irreversibly withdraw from the cell cycle before they start to synthesize muscle-specific proteins. It has been demonstrated for several muscle cell lines that serum components and the addition of the growth factors bFGF, TGF- β , or EGF will prevent differentiation and shut down expression of muscle marker genes like MCK and actin (Spizz et al., 1986; Clegg et al., 1987; Olson et al., 1986; Florini and Ewton, 1988; Lathrop et al., 1985; Wice et al., 1987; Munson et al., 1982; Wang and Rubenstein, 1988). More recently, it was shown for the BC3H1 muscle cell line that proliferation and differentiation may be regulated by cAMP-dependent and cAMP-independent pathways (Hu and Olson, 1988), as well as by signal transduction cascades which involve G proteins (Kelvin et al., 1989a,b). We have addressed the question of how these signals will affect the activity of the Myf4 gene, as one of the crucial muscle determining factors. As we demonstrate here, the Myf4 gene and its isolated promoter in C2C12 cells are subject to negative control by serum mitogens and peptide growth factors. The DNA target elements for this effect are located within 1.1 kb of the 5' upstream sequence. This result confirms and extends the observations on the endogenous myogenin genes in BC3H1 cells and L6E9 cells which are also repressed by serum, bFGF, and TGF β (Brunetti and Goldfine, 1989; Heino and Massagué, 1990). Similar repression has also been shown for the MyoD gene (Vaidya et al., 1989). It is presently unclear whether the inhibition exerted by FCS, bFGF, TGF β , and EGF involves identical target sequences on the Myf4 pro-

motor and whether the same or overlapping signaling pathways are part of this control.

For some of the growth factor receptors it has been shown that they contain an intracellular tyrosine kinase domain (Westermarck and Heldin, 1989; Yarden and Ullrich, 1988). The cellular substrates and phosphorylation events, which probably involve a number of protein kinases including cAMP-dependent protein kinase A and protein kinase C, and the potential signaling cascades between membrane receptors and the nucleus (Edelmann et al., 1987; Kikkawa et al., 1989) have not been elucidated. In preliminary experiments involving compounds that modulate protein kinase C activity, we have obtained no major effects on Myf4 promoter activity (data not shown). Employing reagents known to modulate cAMP-dependent events, we demonstrate here that elevation of intracellular cAMP levels by forskolin, CT, or dibutyryl cAMP severely represses expression of the endogenous Myf4 gene and its isolated promoter similar to the action of serum mitogens and growth factors. It is unlikely, however, that the mitogens act via cAMP since our results (data not shown), as well as those reported by others (Hu and Olson, 1988; Kelvin et al., 1989a), show that growth factors do not significantly increase intracellular cAMP concentrations. We therefore conclude that repression of the Myf4 promoter can be conveyed by cAMP-dependent and cAMP-independent mechanisms. The possible involvement of protein kinase A in the event of negative regulation is under investigation.

Myogenin and MyoD are known to be phosphorylated proteins (Brennan and Olson, 1990; Tapscott et al., 1988). It is tempting to speculate that the way through which cAMP may suppress Myf4 expression involves changes in the phosphorylation of MyoD, thereby modulating the activity of MyoD as a positive transcription factor of the Myf4 promoter. Alternatively, cAMP may exert its negative control through the proto-oncogene product c-fos. It has been reported that elevated expression of fos protein inhibits transcription of the MyoD1 and myogenin genes and prevents terminal differentiation of myoblasts (Lassar et al., 1989b; Rahm and Sejersen, 1989). Increase in intracellular levels of cAMP stimulates the transcription of the immediate early response gene c-fos. Sassone-Corsi et al. (1988) have shown that the c-fos promoter contains a cAMP response element (CRE) that mediates regulation by the CRE binding protein. Transcription of the c-fos gene is also stimulated by serum (Treisman, 1986), EGF (Prywes and Roeder, 1986), and FGF (Sheng et al., 1988). Thus, both the effect of growth factors and cAMP on the activity of the Myf4 promoter could be mediated by c-fos.

Administration of PT to C2C12 muscle cells activates the Myf4 gene in a dose-dependent fashion both in the presence of FCS (serum mitogens) and HS. G proteins, a family of membrane-associated GTP binding proteins, are believed to transduce stimulatory and inhibitory signals from receptors to intracellular effector molecules (for review see Gilman, 1984, 1987; Lefkowitz and Caron, 1988). As muscle differentiation is regulated by multiple growth factors, G proteins are obvious candidates to be involved in signal transduction. Kelvin et al. (1989a) have recently shown that PT, a specific probe for inhibitory G proteins (Ui, 1990), inhibits proliferation and stimulates differentiation of BC3H1 cells. They furthermore demonstrated that the PT-sensitive pathway

affects signals from the thrombin receptor, but not from FGF and EGF receptors or inhibitory signals exerted by the Harvey ras G protein homologue (Kelvin et al., 1989b). These observations indicate that muscle cell differentiation can be regulated by PT-sensitive and PT-insensitive pathways. In agreement with these findings, we observe strong stimulation of the Myf4 promoter by PT in FCS and HS, but only weak activation in the presence of bFGF and TGF- β , suggesting that components present in FCS prevent Myf4 expression through a mechanism that is different from that exerted by bFGF and TGF- β . Moreover, we believe that PT should affect a pathway that operates in FCS and HS since the Myf4 promoter is activated in both media. As multiple intracellular effectors are regulated via pertussis toxin substrate G proteins, for example, cation channels and processes of calcium homeostasis (for review see Ui 1990), further studies will be required to elucidate the mechanisms involved in PT-sensitive activation of Myf4 gene expression.

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