MyoD-Dependent Induction during Myoblast Differentiation of p204, a Protein Also Inducible by Interferon

CHUAN-JU LIU,¹ HONG WANG,¹ ZHIYONG ZHAO,² SHUANG YU,² YUN-BIAO LU,¹
JEFFREY MEYER,¹ GOURI CHATTERJEE,¹ STEPHANE DESCHAMPS,³
BRUCE A. ROE,³ AND PETER LENGYEL^{1*}

*Department of Molecular Biophysics and Biochemistry, Yale University,¹ and Yale Child Health Research Center,
Department of Pediatrics, Yale University School of Medicine,² New Haven, Connecticut 06520, and
Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Oklahoma 73019³*

Received 18 February 2000/Returned for modification 20 April 2000/Accepted 21 June 2000

p204, an interferon-inducible p200 family protein, inhibits rRNA synthesis in fibroblasts by blocking the binding of the upstream binding factor transcription factor to DNA. Here we report that among 10 adult mouse tissues tested, the level of p204 was highest in heart and skeletal muscles. In cultured C2C12 skeletal muscle myoblasts, p204 was nucleoplasmic and its level was low. During myoblast fusion this level strongly increased, p204 became phosphorylated, and the bulk of p204 appeared in the cytoplasm of the myotubes. Leptomycin B, an inhibitor of nuclear export that blocked myoblast fusion, inhibited the nuclear export signal-dependent translocation of p204 to the cytoplasm. The increase in the p204 level during myoblast fusion was a consequence of MyoD transcription factor binding to several MyoD-specific sequences in the gene encoding p204, followed by transcription. Overexpression of p204 (in C2C12 myoblasts carrying an inducible p204 expression plasmid) accelerated the fusion of myoblasts to myotubes in differentiation medium and induced the fusion even in growth medium. The level of p204 in mouse heart muscle strongly increased during differentiation; it was barely detectable in 10.5-day-old embryos, reached the peak level in 16.5-day-old embryos, and remained high thereafter. p204 is the second p200 family protein (after p202a) found to be involved in muscle differentiation. (p202a was formerly designated p202. The new designation is due to the identification of a highly similar protein—p202b [H. Wang, G. Chatterjee, J. J. Meyer, C. J. Liu, N. A. Manjunath, P. Bray-Ward, and P. Lengyel, *Genomics* 60:281–294, 1999].) These results reveal that p204 and p202a function in both muscle differentiation and interferon action.

p204 is a 72-kDa interferon-inducible murine protein (12, 13). The interferons are cytokines with antimicrobial, immunomodulatory, and cell growth-regulatory activities, which also affect differentiation (50, 72, 75). The various activities of the interferons are performed by numerous interferon-inducible proteins. p204 is a member of the interferon-inducible p200 family of proteins which are encoded by genes in the gene 200 cluster. These genes arose from a common ancestor by repeated duplication (12). The p200 family proteins in mice (p202a, p202b, p203, p204, and D3) (12, 13, 17, 30, 34, 46, 51, 76, 79) and in humans (myeloid nuclear differentiation antigen [MNDA], IFI16, and AIM2) (8, 9, 21, 22, 25, 41, 77) share a partially conserved sequence of 200 amino acids adjacent to their C termini. Proteins p204, p202a, p202b, and IFI16 each have two copies of this partially conserved sequence, one of the “a” and one of the “b” type, whereas other p200 proteins have only one copy of either the a or the b type.

p202a is an interferon-inducible protein which inhibits cell growth when only two- to threefold overexpressed, apparently by inhibiting the activities of various transcription factors, e.g., c-Jun, c-Fos, AP2, E2F-1, E2F-4, NF- κ B, MyoD, myogenin, p53, and c-Myc (15, 16, 18, 19, 52, 56, 80). In most of these cases, p202a binds the transcription factor and prevents its sequence-specific binding to DNA. In the case of c-Myc, the mechanism is different: p202a binds to c-Myc, and this inhibits the binding of c-Myc to Max (80). The activity of p202a is

inhibited by the binding of p53 BP1, a protein originally discovered as binding p53 (18).

The level of p202a, which is high in adult mouse skeletal muscle (19), increases more than 10-fold during the fusion of cultured C2C12 myoblasts to myotubes. This increase in the p202a level follows the shift of myoblasts from growth medium (GM) to differentiation medium (DM) after a delay: most of the increase occurs between 48 and 72 h. Overexpression of p202a prior to induction of differentiation inhibits differentiation. p202a inhibits MyoD gene expression and the transcriptional activities as well as the binding of both MyoD and myogenin to DNA. p202a has antiapoptotic activity (44). p202b, which is also inducible by interferon, differs from p202a in only 7 of 445 amino acids (79). The disruption of the gene encoding p202a in mice results in a compensatory increase in the level of the p202b protein.

p204 is primarily nucleolar in AKR-2B, a cloned murine embryo cell line (13). If overexpressed, p204 inhibits cell proliferation (49, 51, 52) and rRNA transcription (52). This inhibition is a consequence of the binding of p204 to the rRNA-specific transcription factor upstream binding factor (UBF), which prevents the specific binding of UBF to the regulatory region of the ribosomal DNA genes. p204 has also been shown to be required for cytomegalovirus (CMV) proliferation in mouse embryo fibroblasts (37). The characteristics of the human and murine p200 family proteins have been reviewed (22, 41, 46, 51).

These studies started by comparing the levels of p204 in various tissues of adult mice. The high levels of p204 observed in heart and skeletal muscles and the increase in the level of p202a, a p204 homolog, during C2C12 myoblast fusion to myo-

* Corresponding author. Mailing address: Yale University, Department of Molecular Biophysics and Biochemistry, P.O. Box 208024, 333 Cedar St., New Haven, CT 06520-8024. Phone: (203) 737-2061. Fax: (203) 785-6404. E-mail: Peter.Lengyel@yale.edu.

tubes (19) prompted the present investigation into the effects of myoblast differentiation on p204 levels.

Myoblast differentiation is coordinated by a family of muscle-specific transcription factors (myogenic factors) that includes MyoD (20), Myf5 (7), myogenin (27, 85), and MRF4 (57, 67). All members of this family share homologous basic helix-loop-helix (bHLH) domains (48, 58, 61, 64) that mediate heterodimerization with the ubiquitous bHLH proteins E₁₂ and E₄₇ (47) and allow binding to E box sequences (CANNTG) in DNA (10, 47). These sequences are functionally important elements in transcriptional enhancers of muscle differentiation genes (e.g., MyoD or muscle creatine kinase). Ectopic expression of any of the myogenic factors in some nonmyogenic cell types (e.g., 10T1/2 fibroblasts) results in increases in the expression of various muscle differentiation genes and possibly in the fusion of the myoblasts to form myotubes (2, 11, 20, 71). Differentiation of skeletal muscle entails transcriptional activation of muscle-specific genes coupled with irreversible cell cycle withdrawal (48, 60, 68). In general, MyoD and Myf5 are expressed in proliferating myoblasts, whereas myogenin and MRF4 are expressed only after the myoblasts exit the cell cycle (54, 61, 68). The negative regulators of muscle differentiation include Id (inhibitor of differentiation) proteins, which form heterodimers with the myogenic factors and inhibit their binding to DNA (5, 40, 53, 55, 87).

MyoD function is regulated in various ways. For example, phosphorylation of MyoD Ser200 (by CDK2) in proliferating myoblasts accelerates its degradation by the ubiquitin pathway (42, 65, 74), CDK4 binding to MyoD in the nucleus inhibits its binding to DNA (88), and acetylation of lysine residues in MyoD by pCAF increases its affinity for DNA, stimulates transcription, and stimulates myogenic conversion of transfected mouse fibroblasts (70).

Here we report that (i) during the fusion of cultured C2C12 myoblasts to myotubes, the level of p204 increases significantly, p204 becomes phosphorylated, and the bulk of p204 appears in the cytoplasm of the myotubes; (ii) this increase in p204 during myoblast fusion is due to transcription by the muscle-specific transcription factor MyoD; (iii) overexpression of p204 in C2C12 myoblasts (carrying an inducible p204 expression plasmid) accelerates the fusion to myotubes in DM and can induce the fusion even in GM; and (iv) the level of p204 also increases significantly during mouse heart muscle differentiation. These results reveal that p204 is involved in two biological processes: muscle differentiation and interferon action.

MATERIALS AND METHODS

Plasmid constructs. To obtain green fluorescent protein (GFP) fusion protein expression plasmids, full-length 204 cDNA or a mutant 204 cDNA ($\Delta 70-99$) lacking 30 nucleotides (70-TTATTTAAGTCATTGCTGGCC AGAGATTTA-99) from the sequence encoding the nuclear export signal (NES) was inserted into the *EcoRI/BamHI* sites of the pEGFP-N1 expression vector (Clontech).

The Ifi204-specific reporter gene plasmids pGL3MyoD3-luc, pGL3MyoD4-luc, and pGL3MyoD6-luc were constructed from three segments that were obtained as PCR products from the Ifi204 gene 5'-flanking region of BAC clone 225 (nucleotides -1578 to -1324, -1578 to -710, and -1578 to +38 [see Fig. 7A]) by using three sets of primers (5'-AAGCGCTAGCCCTCAGCTGTG-3' and 5'-AAGCAGATCTGTGTATGGCAGC-3'; 5'-AAGCGCTAGCCCTCAGCTGTG-3' and 5'-AGCAGATCTTGAAGCTGGCAGC-3'; and 5'-AAGCGCTAGCCCTCAGCTGTG-3' and 5'-AAGCAGTCTTACGGCTGGTCTC-3'). The PCR products were digested with *BglII* and *NheI* and inserted into a pGL3 vector (Promega) previously cleaved with *BglII* and *NheI*. The wild-type plasmid and three pairs of primers (with mutations or deletions) [5'-AAGCGCTAGCCCTGCGTGTG-3' and 5'-AAGCAGATCTGTGTATGGCAGC-3'; 5'-AAGCGCTAGCCCTCAGCTGTG-3' and 5'-AAGCAGATCT(cac)CTGACAGACCAG-3'; 5'-AAGCGCTAGCCCTGCGTGTG-3' and 5'-AAGCAGATCT(cac)CTGACAGACCAG-3'] were used to generate three mutants of the pGL3MyoD3-luc reporter plasmid by PCR. (The mutated nucleotides in the primers are underlined, and the deleted nucleotides are enclosed in parentheses and lowercased.)

After amplification the PCR products were inserted into the *BglII/NheI* sites of the pGL3 vector.

Generation of stable cell lines in which Muristerone treatment induces p204. Cell lines were derived from C2C12 myoblasts by transfection of constructs encoding Muristerone receptors and selectable markers from the Ecdysone-Inducible Expression Kit (Invitrogen), as well as inducible p204. Briefly, plasmid pVgRXR encoding hormone receptor was introduced into the myoblasts, and the transfectants were selected by using 1 mg of Zeocin/ml. C9, one of the cell lines obtained, was highly inducible in an assay based on β -galactosidase activity. Subsequently, an empty vector (pIND) or a p204 expression plasmid (pIND-204) was transfected into the clone C9 line, transfectants were selected using 1.2 mg of G418/ml, and the levels of p204 induced by Muristerone in the individual transfected lines were compared by Western blotting.

Expression and purification of GST fusion proteins. For expressing glutathione S-transferase (GST) fusion proteins, the appropriate plasmids (pGEX-MyoD [47], pGEX-204 [52], and pGEX-202 [14]) were introduced into *Escherichia coli* DH5 α (GIBCO/BRL). The fusion proteins synthesized were affinity purified on glutathione-agarose beads as previously described (52).

Preparation of immunoaffinity-purified anti-p204 antibodies. The preparation of a rabbit antiserum against a p204 segment linked to GST has been described elsewhere (52). To purify the anti-p204 antibodies, the anti-GST activity in the rabbit serum was depleted by using GST protein immobilized on glutathione-agarose beads. The depleted serum was incubated with Affi-Gel-10 (Bio-Rad) beads to which purified GST-204 (amino acids 129 to 177) was covalently linked. The bound antibodies were eluted from the beads with 0.15 M glycine buffer (pH 2.5) and immediately neutralized with 1.5 M Tris-HCl buffer (pH 8.0). The preparation of rabbit antiserum against p202a has been described elsewhere (17).

Assay of the tissue distribution of p204 in mice by immunoblotting. Representative samples of organs from adult mice (of the C129 and C57BL/6 inbred strains) were dissected and homogenized in a buffer (20 mM Tris · HCl (pH 7.5), 150 mM NaCl, 10 mM dithiothreitol (DTT)) supplemented with 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μ g of aprotinin/ml, 5 μ g of ml pepstatin/ml, and 5 μ g of leupeptin/ml. The protein concentrations of the solutions were determined after the solutions were clarified by centrifugation using the Protein Assay Kit (Bio-Rad). Protein samples (20 μ g) were subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and electroblotted onto Immobilon-P transfer membranes (Millipore). After being blocked with blocking solution (20 mM Tris · HCl [pH 8.0], 150 mM NaCl, 8% [wt/vol] nonfat dry milk, 0.1% Tween 20), the membranes were incubated with anti-p204 antibodies (diluted 1:1,000) in blocking solution, and then goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase was applied and the signal was detected by enhanced chemiluminescence (Amersham).

Immunofluorescent cell staining. Cultures of C2C12 murine thigh muscle myoblasts (ATCC 1172-CRL) (86) and 10T1/2 cloned murine embryo fibroblasts (ATCC 226-CCL) (66) were plated on glass coverslips coated with polylysine and grown in GM (Dulbecco's modified Eagle's medium [DMEM] supplemented with 20% [for C2C12] or 10% [for 10T1/2] fetal bovine serum [FBS; GIBCO/BRL]) under an atmosphere of 10% CO₂ and 90% air at 37°C. Where indicated, after reaching confluency, the cultures were shifted to DM (DMEM-0.5% horse serum) for the times indicated. Where indicated, C2C12 cultures at around 40% confluency were treated with 1,000 U of alpha interferon/ml (81) for 48 h. 10T1/2 cells at 50% confluency were transfected with 3 μ g of pCMV-MyoD by the Lipofectamine procedure (GIBCO/BRL); 24 h after transfection, the cultures were confluent and were shifted to DM for 3 days. For isolating and culturing neonatal murine myocytes (84), hearts from newborn mice were dissected. The cells were dispersed by trypsinization, plated on glass coverslips, and cultured in DMEM-10% FBS in 5% CO₂ and 95% air for 4 days. For staining, the cells were fixed with cold acetone-methanol (1:1) for 20 min and air dried. After rehydration in phosphate-buffered saline (PBS) and blocking with 30% goat serum in PBS for 30 min, the cells were incubated with primary antibodies against p204 (diluted 1:100) at room temperature for 1 h. After being washed with PBS, the coverslips were incubated with secondary antibodies (against rabbit IgG) conjugated with fluorescein isothiocyanate (FITC; diluted 1:400; Santa Cruz) for 45 min. In the case of double immunofluorescence, the neonatal murine myoblasts were fixed, rehydrated, blocked with 10% goat serum in PBS for 30 min, and incubated with primary antibodies against p204 (diluted 1:100) and α -actinin (Sigma; diluted 1:800) at room temperature for 1 h. Secondary antibodies against mouse IgG conjugated with rhodamine (diluted 1:2,000; Sigma) and against rabbit IgG conjugated with biotin (diluted 1:200; Vector) were applied for 30 min, followed by an incubation with streptavidin conjugated with fluorescein (diluted 1:200; Vector) for 30 min. To test whether C2C12 myoblasts incubated in DM in the presence of 2 ng of leptomycin B (LMB)/ml for 3 days become biochemically differentiated, the cultures were incubated first with primary antibodies (i.e., mouse monoclonal anti- α -actinin antibodies [Sigma; diluted 1:800] and rabbit polyclonal anti-MyoD antibodies [Santa Cruz; diluted 1:100]) at room temperature for 1 h and thereafter with secondary antibodies (i.e., anti-mouse IgG conjugated with rhodamine [Santa Cruz; diluted 1:100] and anti-rabbit IgG conjugated with FITC [Santa Cruz; diluted 1:400]) for 45 min. In each case, the nuclei were stained with 0.5 μ g of 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI). The specimens were observed under a fluorescence microscope with appropriate optical filters. Microscopic images were captured using the

Image Pro program (Media Cybernetics) and an Olympus microscope. Pictures were arranged using the Adobe Photoshop program.

Immunohistochemistry. To obtain timed-pregnant animals, C57BL/6J mice were paired overnight. The next morning was considered embryonic day (E) 0.5 if a vaginal plug was present. The embryos were dissected and frozen in freezing medium on dry ice. Sections (8 μ m) were cut on a cryostat and stained either with preimmune serum or with anti-p204 antibodies using the Histostain-Plus Kit (Zymed Laboratories, Inc.). To quench endogenous peroxidase activity, the slides were submerged in 3% hydrogen peroxide for 10 min. After blocking, the slides were incubated with either preimmune serum or anti-p204 antibodies (diluted 1:50) in TBS (50 mM Tris $\cdot$ HCl (pH 7.8)–0.025% Tween 20) for 45 min, followed by addition of biotinylated secondary antibodies, streptavidin-peroxidase, and, for visualization, DAB substrate-chromogen solution. The nuclei were stained with methyl green, and the slides were observed under a light microscope. The images were processed as described above.

Two-dimensional (2-D) nonequilibrium pH gradient electrophoresis. C2C12 myoblasts, either treated with 1,000 U of interferon/ml in GM for 48 h or maintained in DM for 3 days, were lysed in radioimmunoprecipitation assay buffer (56) supplemented with the following proteinase inhibitors: 1 mM PMSF, 10 μ g of aprotinin/ml, 5 μ g of pepstatin/ml, and 5 μ g of leupeptin/ml. To generate cell lysates for calf intestinal phosphatase (Cip) treatment, the cells were harvested in a buffer (50 mM Tris $\cdot$ HCl [pH 7.5]–1 mM MgCl₂) and briefly sonicated. The supernatant fraction was incubated with Cip (30 U/100 μ g of total protein) at 30°C for 30 min, and the reaction was terminated by addition of an equal volume of 2 $\times$ NEPHGE loading buffer, comprising 8 M urea, 65 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2.5% β -mercaptoethanol, 3.75% Bio-Lyte 3–10 ampholyte (Bio-Rad), and 1.25% Bio-Lyte 7–9 ampholyte (Bio-Rad). To generate cell lysates for fractionation, the cells were lysed in a buffer (50 mM HEPES-NaOH [pH 7.6], 150 mM NaCl, 5 mM NaF, 1 mM Na₂VO₄, 0.5% NP-40) supplemented with the proteinase inhibitors listed above. The lysate was divided into nuclear and cytoplasmic fractions as described previously (17). Each sample was diluted with NEPHGE loading buffer (1:1) and loaded onto a 4.5% PAGE gel containing 8 M urea, 25 mM CHAPS, 3.3% Bio-Lyte 3–10 ampholyte, and 1.6% Bio-Lyte 7–9 ampholyte. The first dimension was electrophoresed at 250 V for 1.5 h. After equilibration, the rod gels were subjected to SDS–10% PAGE. The separated proteins were transferred onto a nitrocellulose membrane and processed for immunodetection with anti-p204 antibodies as described above.

Transient transfection assay. 10T1/2 or C2C12 cells grown to 50% confluency in GM in 6-well plates were transfected with 1 μ g of either one of the p204-specific reporter plasmids (pGL3MyoD3-luc, pGL3MyoD4-luc, or pGL3MyoD6-luc) or one of the mutants of pGL3MyoD3-luc [i.e., pGL3MyoD3(mut1)-luc, pGL3MyoD3(mut2)-luc, or pGL3MyoD3(mut1,2)-luc] using the Lipofectamine procedure (GIBCO/BRL). In the case of 10T1/2 cells, the indicated amounts of pCMV-MyoD were cotransfected, and the pCMV vector was added to bring the total amount of DNA transfected to 5 μ g. In each case, a pSVgal internal control plasmid (1 μ g) was cotransfected. At 48 h after transfection, when the cultures were confluent, they were either harvested or shifted to DM for the times indicated. Luciferase and β -galactosidase activities were analyzed using the Luciferase Reporter Gene Assay (Boehringer Mannheim) and the β -Galactosidase Enzyme Assay System (Promega), respectively.

Gel mobility shift assay (GMSA). A 255-nucleotide DNA segment from the murine Ifi204 gene 5'-flanking region (nucleotides –1578 to –1324 from pGL3MyoD3-luc) was labeled with ³²P using T4 DNA kinase. The binding assay was performed in a 20- μ l volume containing 10 mM Tris $\cdot$ HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μ g of poly(dI-dC), 3 ng of the labeled DNA probe, and various combinations of GST, GST-MyoD, GST-204, GST-202, anti-MyoD IgG, and the wild-type or mutant MEF-1 oligonucleotide (Santa Cruz). After incubation at room temperature for 35 min, the samples were subjected to 6% PAGE in 0.5% TBE (45 mM Tris-borate–1 mM EDTA) at 15 V/cm at room temperature for 3 h. The gel was dried, and autoradiography was performed at –70°C.

Sequence analysis. To search for MyoD recognition sequences in the Ifi204 gene 5'-flanking region (GenBank accession number AC006944), the MatInspector, version 2.1, database (63) was used, and the parameter selected for both core similarity and matrix similarity was 0.8.

RESULTS

Tissue distribution of p204 in adult mice. The distribution of p204 in adult mice was examined by multiple-tissue Western blotting (Fig. 1). Both the C129 and C57BL/6 inbred strains had the highest levels of p204 in the heart, followed by skeletal muscle and kidney (Fig. 1). Since p204 is inducible by interferon in C129 mice, but not in C57BL/6 mice or their tissues (in consequence of a defect in transcription factor activity) (13, 31), these results suggested that the levels of p204 in these tissues were not determined solely by interferon.

FIG. 1. Tissue distribution of p204 in adult mice. A multiple-tissue Western blot with 20- μ g protein samples from the indicated tissues of adult C129 (top) or C57BL/6 (bottom) mice was probed using anti-p204 antibodies. Arrows indicate the positions of p204. For further details, see Materials and Methods.

Increase in the p204 level and appearance of p204 in the cytoplasm during the differentiation of C2C12 myoblasts to myotubes. The high level of p204 in heart and skeletal muscle tissues of adult mice suggested that p204, first identified as an interferon-inducible protein, might also be involved in muscle differentiation. This consideration, together with the fact that p202a, another p200 family member, was found earlier to be strongly induced during the fusion of C2C12 myoblasts to myotubes (19), prompted us to compare the expressions of p204 before and after C2C12 myoblast fusion using immunofluorescence microscopy (Fig. 2, left panels). p204 was undetectable or barely detectable in C2C12 myoblasts proliferating in GM (Fig. 2A, B, and C). Upon treatment with interferon, p204 was induced in the nuclei, but more in the nucleoplasm than in nucleoli (Fig. 2D, E, and F). This clearly is different from the case of AKR-2B fibroblasts, in which the highest concentration of interferon-induced p204 was found in the nucleoli (13). The fusion of myoblasts into myotubes in DM for 3 days resulted in a large increase in the p204 level and the appearance of p204 in the cytoplasm, while it also remained in the nucleus (Fig. 2G, H, and I). This p204 distribution persisted in the cultured myotubes 1 week after their fusion (Fig. 2J, K, and L). p204 was formerly considered a nuclear protein (13). This is the first time that p204 was observed in the cytoplasm.

To establish whether the distribution of p204 between the nucleus and the cytoplasm is correlated with differential phosphorylation (see also reference 13), 2-D gel electrophoresis and immunoblotting were performed (Fig. 3). The protein sample from C2C12 myoblasts in GM treated with alpha interferon for 48 h gave rise to a single spot designated the original spot (Fig. 3, top left). The protein sample from C2C12 cultures fused to myotubes in DM for 72 h showed, in addition to the original spot, another, more acidic spot (Fig. 3, top right). To test whether this additional spot was due to the phosphorylation of p204, the sample was treated with alkaline Cip. As shown, the additional spot disappeared after Cip treatment, indicating that it represented a phosphorylated p204. The Cip treatment did not change the mobility of the original spot, suggesting that p204 induced by interferon was not phosphorylated. Samples from differentiated C2C12 myotubes were fractionated into nuclear and cytoplasmic fractions, and 2-D gel electrophoresis revealed that each fraction produced only one spot. The spot from the nuclear fraction corresponded to the original spot, whereas that from the cytoplasmic fraction corresponded to the more acidic, phosphorylated additional spot. These data reveal that cytoplasmic p204 isolated from C2C12 myotubes was phosphorylated (or at least more phosphorylated than p204 from the nuclei). Although the lack of

FIG. 2. p204 and p202a are barely detectable in C2C12 myoblasts in GM; in GM supplemented with interferon they accumulate in the nuclei. During myoblast fusion in DM, p204 and p202a levels strongly increase, and both proteins become distributed between the nuclei and the cytoplasm of the myotubes. (Left) C2C12 myoblasts were cultured in GM in the absence (GM) or presence (GM+IFN) of 1,000 U of interferon/ml for 48 h. Upon reaching confluency, the cultures were shifted to DM and incubated for 3 days (3d) or 7 days (7d). After fixation and blocking, the cultures were stained with anti-p204 antibodies (A, D, G, and J), and the nuclei were stained with DAPI (B, E, H, and K); the overlapping regions of these two signals are shown as "merge" (C, F, I, and L). (Right) The cultures were processed as for the left panel except that an anti-p202a antiserum was used (M, P, and S), and the cultures were incubated in DM for 3 days. Bars, 6 μ m. The anti-p202a antiserum recognized both p202a and p202b. There is no antiserum available at present to distinguish these two highly similar proteins. Thus, what is designated p202a is likely to consist of a mixture of p202a and p202b (see reference 79). For further details, see Materials and Methods.

change in the mobility of the interferon-induced nuclear p204 spot upon treatment with Cip is consistent with the protein being unphosphorylated, further studies are needed to verify this conclusion.

Earlier we reported that the level of p202a (like that of p204) increases during the differentiation of myoblasts to myotubes (19). An immunocytochemical examination (Fig. 2, right panels) revealed (i) the low level of p202a in C2C12 myoblasts in GM (Fig. 2M); (ii) the induction by interferon, and the primarily nuclear localization of the induced p202a in myoblasts in GM (Fig. 2P); and (iii) the large increase in the p202a level, and the partial translocation of p202a to the cytoplasm, during the fusion of myoblasts to myotubes in DM (Fig. 2S) (see also reference 19). A small portion of p202a was cytoplasmic even in the myoblasts treated with interferon in GM (Fig. 2P). It is conceivable that this is due to the slow movement of the interferon-induced p202a from the cytoplasm to the nucleus (17) and that upon incubation beyond 48 h, the p202a might all become nuclear.

The NES is required for the translocation of p204 to the cytoplasm during myoblast fusion. A typical leucine-rich NES (-Leu-X-X-X-Leu-Leu-X-X-X-Leu-X-Leu- [where X is any amino acid]) (28, 32, 36) occurs in the N-terminal region of p204. To establish whether this NES is required for the appearance of p204 in the cytoplasm, we generated a p204 derivative lacking all but the last two amino acids of the NES. To facilitate the subcellular localization of p204 and its derivative lacking the NES, we generated expression plasmids encoding p204 linked to GFP (p204GFP) and also p204 lacking the NES linked to GFP [p204(-NES)GFP]. Figure 4 reveals that in C2C12 myoblasts in GM, free GFP is distributed throughout the entire cell (Fig. 4A), whereas p204GFP and p204(-NES)GFP are restricted to the nucleus (Fig. 4B and C). In C2C12

cultures in DM, GFP is spread over the myotubes. p204GFP is present both in the nuclei and in the cytoplasm of the myotubes (Fig. 4E), whereas p204(-NES)GFP is restricted to the nuclei (Fig. 4F). These results reveal that the NES is required for the appearance of p204 in the cytoplasm of the myotubes during differentiation. It should be noted that although only two nuclei are shown (in Fig. 4F), p204(-NES)GFP was exclusively nuclear in the whole inspected visual field with numerous myotubes.

LMB inhibits the appearance of p204 in the cytoplasm, the fusion of C2C12 myoblasts into myotubes, and the synthesis of the myotube protein α -actinin. We examined the effect of LMB, an inhibitor of NES-dependent nuclear export (29, 45, 62), on the subcellular localization of p204 in a C2C12 culture induced to undergo myotube formation in DM. As expected, p204 appeared in the cytoplasm of myotubes in the absence of LMB (Fig. 5A and C). However, when LMB at a concentration as low as 2 ng/ml was present in the DM, p204 remained essentially restricted to the nuclei (Fig. 5D and F). The effect of LMB on the differentiation of C2C12 myoblasts into myotubes was also examined (Fig. 5G through I). As expected, shifting of the confluent culture of C2C12 myoblasts from GM (G) to DM (H) resulted in the fusion of the myoblasts to myotubes. Surprisingly, however, in the presence of 2 ng of LMB/ml, a confluent culture of C2C12 myoblasts did not form myotubes (though changed in shape) after being shifted to DM (Fig. 5I). This phenomenon was reproduced using higher concentrations of LMB (20 and 200 ng/ml [data not shown]). The morphology of the cultures remained similar to that in GM, except that more floating cells were observed. We also explored whether LMB blocks the accumulation of α -actinin, a protein present in differentiated myotubes but not in the precursor myoblasts (73). The results (Fig. 5, right panel) reveal

FIG. 3. p204 in the cytoplasm of C2C12 myotubes is phosphorylated, whereas p204 in the nuclei of myoblasts is not. Cell lysates, prepared from C2C12 myoblasts incubated in GM supplemented with 1,000 U of interferon (IFN)/ml for 48 h, or incubated in DM (without interferon) for 3 days, were either left untreated or treated with Cip. The lysate of cells incubated in DM for 3 days was fractionated into nuclear (N) and cytoplasmic (C) fractions. Samples from these were analyzed by 2-D electrophoresis (NEPHGE), electroblotted to membranes, and visualized using anti-p204 antibodies. The first isoelectric focusing (IEF) and second (SDS) dimensions of the analysis are indicated. Large and small arrowheads indicate phosphorylated and nonphosphorylated p204, respectively. For further details, see Materials and Methods.

that it does. α -Actinin was not detected in C2C12 myoblasts in GM (Fig. 5J), whereas it accumulated in the myotubes formed after the myoblast culture was incubated in DM (Fig. 5K), and it was again undetectable in the cultures incubated in DM in the presence of LMB (Fig. 5L). Figure 5 also shows the inhibition of MyoD accumulation by LMB (compare Fig. 5N and O) and the partial overlap of α -actinin with MyoD in the cytoplasm of the myotubes (Fig. 5N and T). These results indicate that LMB also blocks the synthesis of a protein (α -actinin) normally appearing during the fusion of myoblasts to myotubes. It remains to be explored whether and how the inhibition of the appearance of p204 in the cytoplasm contributed to the inhibition of differentiation by LMB.

The level of p204 is similarly low in 10T1/2 fibroblasts cultured in GM or DM. Transfected MyoD increases this level in cells in GM, and results in the fusion of cells in DM to myotubes, as well as a further increase in the p204 level and a shift of the majority of p204 to the cytoplasm.

We have reproduced a finding that the transfection of MyoD into 10T1/2 fibroblasts converts them to myoblasts, which fuse to myotubes in DM (20, 83), as C2C12 myoblasts do. Figure 6 shows the effects of the transfection of MyoD into 10T1/2 fibroblasts on the p204 level as examined by immunofluorescence microscopy. The p204 staining was equally faint in the nuclei and somewhat more pronounced in the nucleoli of

10T1/2 cultures in GM or DM (Fig. 6A, C, G, and I), indicating that shifting of the culture to DM did not induce p204 accumulation. Transfection of MyoD and incubation of the transfected cultures in GM resulted in a pronounced increase in p204 expression in the nuclei, mostly in their nucleolar regions (Fig. 6D and F). Shifting of a confluent culture of 10T1/2 cells which had been transfected with MyoD to DM resulted in the appearance of myotubes, a further strong increase in the p204 level, and the appearance of the majority of p204 in the cytoplasm (Fig. 6J and L). These results indicate a correlation between the differentiation of myoblasts to myotubes and the induction and appearance of p204 in the cytoplasm.

MyoD-specific sequences in the 5'-flanking region of the Ifi204 gene drive the expression of p204 during skeletal muscle differentiation. The availability of the recently completed Ifi204 sequence allowed us to explore the identity of the enhancer(s) driving the expression of p204 during skeletal muscle differentiation. Ifi204 is a part of BAC clone 225, the clone that our laboratories had identified, sequenced, and analyzed (to be reported elsewhere). A 1.6-kb segment from the 5'-flanking region of the Ifi204 gene (Fig. 7A) was found to contain at least six MyoD-specific sequences. This finding, together with the fact that the transfection of MyoD resulted in an increase of the expression of p204 in 10T1/2 fibroblasts even in GM, i.e., without differentiation (compare Fig. 6A and D), prompted us to test for the involvement of MyoD-specific sequences in the induction of p204 during differentiation. For this purpose three reporter gene plasmids (pGL3MyoD3-luc, pGL3MyoD4-luc, and pGL3MyoD6-luc) were generated in which segments with MyoD-specific sequences from the 5'-flanking region of Ifi204 (−1578 to −1324, −1578 to −710, and −1578 to +38) were linked to the upstream end of a region encoding luciferase in the pGL3 vector (Fig. 7B). As shown in Fig. 7B, the numbers after MyoD (i.e., 3, 4, or 6) in the three reporter plasmids indicate the number of MyoD-specific sequences in the plasmids. Transfection of the reporter plasmid into 10T1/2 fibroblasts in GM resulted in luciferase expression, with the extent of expression increasing with the length of the Ifi204 segment in the reporter (Fig. 7C). Cotransfection of the reporter plasmids with a MyoD expression plasmid strongly increased the expression of each of the reporters in a MyoD dosage-dependent manner (Fig. 7C).

FIG. 4. The NES in p204 is required for the cytoplasmic localization of p204 in C2C12 myoblasts. Pairs of C2C12 myoblast cultures transfected with expression plasmids encoding either GFP (A and D), p204-GFP fusion protein (p204GFP) (B and E), or p204-GFP fusion protein lacking NES [p204(−NES)GFP] (C and F) were cultured in GM (A, B, and C). When they reached confluency, one of each pair of cultures was shifted to DM and incubated for 3 days (D, E, and F). The cultures were observed using a fluorescence microscope with the appropriate filter. Bars, 5 μ m. For further details, see Materials and Methods.

FIG. 5. LMB inhibits the appearance of p204 in the cytoplasm, the fusion of C2C12 myoblasts to myotubes, and the synthesis of the muscle protein α -actinin. (A through F) C2C12 myoblasts grown to confluency in GM were shifted to DM for 3 days in the absence (A, B, and C) or presence (D, E, and F) of LMB (2 ng/ml). After fixation and blocking, the cultures were stained with anti-p204 antibodies (A and D) and the nuclei were stained with DAPI (B and E); the overlapping regions of these two signals are shown as "merge" (C and F). Bars, 5.5 μ m. (G through I) C2C12 myoblasts were cultured in GM to confluency (G) and were shifted to DM for 3 days in the absence (H) or presence (I) of LMB (2 ng/ml). The cultures were observed under a phase-contrast microscope. Bars, 150 μ m. (J through U), C2C12 myoblasts grown to confluency in GM (J, M, P, and S) were shifted to DM for 3 days in the absence (K, N, Q, and T), or the presence (L, O, R, and U) of 2 ng of LMB/ml. After fixation and blocking, the cultures were costained with primary antibodies to α -actinin (J, K, and L) and MyoD (M, N, and O). α -Actinin was visualized with secondary antibodies conjugated to rhodamine, and MyoD was visualized with secondary antibodies linked to FITC. The nuclei were stained with DAPI (P, Q, and R). Overlapping regions of the two antibody signals are shown as "merge" (S, T, and U). Bars, 6 μ m. For further details, see Materials and Methods.

Since p204 has been identified as an interferon-inducible protein, the effects of interferon on the expression of the three reporter genes were tested. As shown in Fig. 7D, exposure of the transfected cells to interferon increased the expression of the longest reporter while inhibiting the expression of the two shorter reporters. Since only the longest reporter (but not the two shorter reporters) included interferon-responsive GA boxes (39) (see Fig. 7A), the boost of this reporter's expression by interferon was expected. The extent of the stimulation (3.5-fold) was, however, much lower than that of the expression of p204 (e.g., in AKR-2B cells) by interferon (13). This might be the consequence, at least in part, of the high basal level of expression of this reporter in the absence of interferon, due apparently at least in part to MyoD activity. The observation that the shorter reporters did not contain known interferon-responsive sequences, and that interferon actually decreased their expression, clearly indicates that the MyoD-dependent induction of p204 expression did not depend on interferon.

The inhibition of the expression of the two shorter reporter genes by interferon might be due to either the decrease in activity of transcription factors, or the increased activity of repressors, elicited by interferon. This inhibition of the activity of the two shorter reporters by interferon is not surprising, since interferon was shown to diminish the activity of many genes in addition to boosting the activity of many other genes (23).

As noted, the level of p204 increased strongly in the course of the differentiation of C2C12 myoblasts to myotubes. This prompted us to compare the ability of these cells incubated in GM and DM for various lengths of time to drive the expression of our reporter genes. Figure 7E reveals that shifting the cultures to DM resulted in a pronounced increase in this ability

within 1 day and a further pronounced increase on day 2. By day 3, however, this activity diminished to a level close to that of a culture in GM. To make sure that this decrease on day 3 was not an artifact due to the inactivation of the reporter gene by day 3, we also kept transfected cultures in GM for 3 days after they reached confluency and observed that the ability of the cultures to drive expression of the reporters did not diminish during this time. It remains to be seen whether this decrease on day 3 is related to the reported increase in the level of p202a at this stage of differentiation (19). We suspect that this is the case because p202a was shown earlier to inhibit both the expression of MyoD and the activity of both MyoD and myogenin (19).

The shifting of 10T1/2 cultures from GM (Fig. 7F) to DM for 1 day did not increase the expression of the reporter plasmids unless the cultures had been transfected with MyoD (compare the values for GM plus MyoD and DM plus MyoD). These results are in good agreement with those of the immunofluorescence assay of the p204 level (compare Fig. 6A and G) showing that the shifting of the 10T1/2 culture from GM to DM resulted in an increase in the p204 level only if the cultures had been transfected with a MyoD expression plasmid.

The muscle-specific transcription factor myogenin is expressed after the differentiating myoblasts exit the cell cycle, at a time when the level of MyoD is decreased. Since myogenin can bind the same E box sequences in DNA as MyoD, it was expected that myogenin might also promote the transcription of the reporter plasmids with the Ifi204 5'-flanking segments. The results in Fig. 7G reveal that it does: it enhanced the expression of the shortest and the longest reporter plasmids in a concentration-dependent manner.

FIG. 6. The levels of p204 are similarly low in 10T1/2 fibroblasts cultured in GM or DM. Transfected MyoD increases this level in the nuclei of cells in GM, and in cells in DM, it results in a further increase in the p204 level as well as a shift of the bulk of p204 to the cytoplasm. 10T1/2 fibroblasts grown to 50% confluency were transfected with 2 μ g of either the pCMV control vector (GM) or the pCMV-MyoD expression plasmid (GM+MyoD) and were grown to confluency in GM. Duplicates of these two types of cultures were shifted to DM for 3 days (DM and DM+MyoD, respectively). After fixation and blocking, the cultures were stained with anti-p204 antibodies and visualized with FITC-labeled secondary antibodies (A, D, G, and J); they were also stained with DAPI to visualize DNA (B, E, H, and K); the overlapping regions of these two signals are shown as "merge" (C, F, I, and L). Bar, 5 μ m. For further details, see Materials and Methods.

The MyoD-dependent increase in the expression of the reporter genes driven by segments from the Ifi204 5'-flanking region depends on MyoD-specific sequences. We wished to verify that the increase in the expression of our reporter genes

in 10T1/2 cells that was observed after MyoD transfection was dependent on the MyoD-specific sequences. Thus, one or two of the three MyoD-specific sequences in pGL3MyoD3-luc were altered by either replacing or deleting nucleotides from the sequence (Fig. 8A). The replacement of the two CA nucleotides with GC in the first of the MyoD-specific sequences in the reporter, or the deletion of the three GTG nucleotides from the third, resulted in a strong decrease in the responsiveness to MyoD of the expression of the reporter in 10T1/2 cells (Fig. 8B). When both of these MyoD-specific sequences were altered, there was a complete loss of responsiveness.

These alterations in the MyoD-specific sequences had the same effect on the enhancement of the expression of the reporter in C2C12 cells (Fig. 8C) as that observed for 10T1/2 cells which had been transfected with MyoD (Fig. 8B). In addition, the level of wild-type reporter gene expression was much higher in C2C12 cultures undergoing differentiation for 1 or 2 days than in cultures in GM (Fig. 8C). In agreement with the observations above, the alterations in the MyoD-specific sequences greatly diminished this difference in expression level. Moreover, alteration of the first MyoD-specific sequence and partial deletion of the third resulted in reporter levels that actually were somewhat lower in differentiating C2C12 cells than in C2C12 cells in GM.

Purified MyoD binds to MyoD-specific sequences in the Ifi204 gene regulatory region in vitro. The binding of MyoD to MyoD-specific sequences in the 5'-flanking region of Ifi204 was tested by electrophoretic mobility shift assays (EMSA). Here we used as a probe the same 255-nucleotide segment from this region that was inserted into the pGL3MyoD3-luc reporter (Fig. 7B). The EMSA revealed that GST did not bind, and GST-MyoD did bind, to the 255-nucleotide segment with the three MyoD-specific sequences (Fig. 9, lanes 1 and 2). This binding was not affected by excess mutant MyoD-specific sequences but was inhibited by excess wild-type MyoD-specific sequences (Fig. 9, lanes 3 and 4). As expected, antibodies to MyoD supershifted the complex (Fig. 9, lane 5). GST-204 and GST-202 did not bind to the segment (Fig. 9, lanes 8 and 9). In agreement with earlier findings, GST-202 inhibited the binding of GST-MyoD to DNA (19), whereas GST-204 did not (Fig. 9, lanes 11 and 10). These results clearly establish the sequence-specific binding of purified MyoD to the MyoD-specific sequences in the 5'-flanking region of Ifi204.

Overexpression of p204 accelerates the fusion of C2C12 myoblasts to myotubes in DM and induces the fusion even in

FIG. 7. The expression of p204 during skeletal muscle differentiation can be driven by MyoD-specific sequences in the 5'-flanking region of the Ifi204 gene. (A) Nucleotide sequence of an approximately 1.6-kb segment from the 5'-flanking region of Ifi204 (GenBank accession number AC006944). MyoD-responsive elements are underlined, and their core sequences are in capital letters. An interferon-responsive "GA box" sequence is displayed in italicized, underlined capital letters. The border between exon 1 and intron 1 is indicated by "e1*↔*i1." As indicated, the last nucleotide of exon 1 was taken as nucleotide -1, and first nucleotide of intron 1 was taken as nucleotide +1. Numbers to the left of the sequence indicate distances in nucleotides from the first nucleotide of intron 1. (B) Schematic structures of three 204-specific reporter genes. The indicated segments from the 5'-flanking region of the Ifi204 gene were linked to simian virus 40 promoter (boxes with checkerboard patterns) and a DNA segment encoding luciferase (open boxes). Solid boxes, MyoD-specific sequences. Numbers indicate distances in nucleotides from the first nucleotide of intron 1 (see panel A). (C through G) Reporter gene assays. (C) MyoD can drive the expression of 204-specific reporter genes in 10T1/2 cells. The indicated reporter gene was transfected into 10T1/2 cells together with the indicated amount of a pCMV-MyoD expression plasmid, as well as a pSVgal internal control plasmid. At 48 h after transfection the cultures were harvested and lysed, and the β -galactosidase and luciferase activities were determined. The luciferase activities were normalized to the β -galactosidase activities. The numeral 1 over the leftmost bar indicates a relative luciferase activity of 1. (D) Treatment with interferon decreases the MyoD-dependent expression of the two shorter reporter genes but increases the expression of the longest reporter gene. After transfection with the reporter genes specified and pSVgal, 10T1/2 cells were incubated with or without 3,000 U of interferon/ml for 48 h. The procedure described for panel C was followed. (E) Expression of the reporter genes increases during the 1st 2 days of the fusion of C2C12 myoblasts to myotubes in DM but diminishes by day 3. C2C12 myoblasts grown to 50% confluency in GM were transfected with the reporter genes specified as well as pSVgal. At 48 h after transfection, the confluent cultures were either kept in GM for another 1 or 3 days (GM1 and GM3, respectively) or shifted to DM for 1 day (DM1), 2 days (DM2), or 3 days (DM3) before harvesting and processing. (F) The shifting of 10T1/2 cells to DM does not increase the expression of the reporter genes unless the cells have been transfected with a MyoD expression plasmid. Four 10T1/2 cultures grown to 50% confluency were transfected with the reporter genes specified and pSVgal. Pair 1 (serving as a control) of the four cultures was also transfected with 3 μ g of pCMV, whereas pair 2 was also transfected with 3 μ g of pCMV-MyoD. One culture from each pair was further incubated for 2 days in GM (GM and GM+MyoD, respectively). After reaching confluency, the second culture from each pair was shifted to DM for 1 day (DM and DM+MyoD, respectively). (G) Myogenin can drive the expression of 204-specific reporter genes in 10T1/2 cells. The indicated reporter gene was transfected into 10T1/2 cells together with the indicated amount of the pMSV-myogenin expression plasmid, as well as pSVgal. The procedure described for panel C was followed. For further details, see Materials and Methods.

FIG. 8. Alteration of one or two of the three MyoD-specific sequences results in a strong reduction or a complete loss of the MyoD-dependent expression of the reporter genes. (A) Diagrams show the alterations in the first and/or the third of the MyoD-specific sequences in the pGL3MyoD3-luc reporter gene. The middle MyoD-specific sequence, between nucleotides -1507 and -1502, was left unaltered. Mutant MyoD binding sites are indicated by stars, mutant nucleotides by arrows, and deleted nucleotides by minus signs. (B) Transient transfection assays in 10T1/2 cells. The reporter gene specified and the pSVgal internal control plasmid were transfected into 10T1/2 cells together with 3 μ g of pCMV (control) or the pCMV-MyoD expression plasmid. At 48 h after transfection, the cultures were harvested and the luciferase and β -galactosidase activities were determined. The numeral 1 over the leftmost bar indicates a relative luciferase activity of 1. (C) Transient transfection assays in C2C12 cells. C2C12 myoblasts grown to 50% confluency were transfected with the reporter genes specified and the pSVgal internal control plasmid. At 24 h after transfection, the confluent cultures were either incubated in GM for 1 further day (GM) or shifted to DM for 1 day (DM1) or 2 days (DM2). Thereafter, the cells were harvested and processed. For further details, see Materials and Methods.

GM. By transfection of appropriate constructs into C2C12 cells, we generated several cell lines in which the level of p204 could be increased by incubation with the ecdysone analog Muristerone.

Incubation of cells of such a cloned line in confluent culture in GM with Muristerone for 2 days had no effect on the morphology of the myoblasts (Fig. 10B). They looked the same as control cells (Fig. 10A). Shifting the cultures to DM for 2 further days, however, resulted in the fusion of the large majority of the myoblasts to myotubes in the culture with induced p204 (Fig. 10D), whereas the control culture remained primarily as myoblasts with only a very few myotubes detectable (Fig. 10C). Extending the time of incubation of the cultures in GM (Fig. 10A and B) to 6 days altogether also resulted in the fusion of the cultures with induced p204 to myotubes (Fig. 10F), whereas the control culture remained as myoblasts, at a higher cell density (Fig. 10E).

These results reveal that an increase in the p204 level can accelerate fusion to myotubes in DM and makes this fusion possible even in GM. It should be noted that the Muristerone-induced level of p204 in the cell line shown in Fig. 10 was two- to threefold higher than in the control line. Other Muris-

terone-inducible C2C12 lines, in which the increase in the p204 level was eight- to ninefold, did not fuse to myotubes, though they seemed to stop dividing and included rounded floating cells (data not shown).

The level of p204 in mouse heart muscle increases strongly during differentiation. Cytoplasmic p204 does not colocalize with the contractile apparatus in the cytoplasm. The observed regulation of p204 expression during skeletal muscle differentiation, together with the high level of p204 in the adult mouse heart, prompted us to examine the expression of p204 during embryonic mouse heart differentiation.

An immunohistochemical assay using antibodies to p204 revealed that the p204 level was very low in a 10.5-day-old mouse embryo heart (Fig. 11B), increased by day 13.5 (Fig. 11C), and by day 16.5 (Fig. 11D) reached a level close to that in a newborn mouse heart (Fig. 11F) and an adult mouse heart (data not shown). The control assays with preimmune serum resulted in only background staining (Fig. 11A and E).

As in the case of differentiated skeletal muscle myotubes, p204 appeared in the cytoplasm of cardiac myocytes isolated from a newborn mouse heart (Fig. 11G).

Finding that p204 can associate with α -tropomyosin in a

FIG. 9. MyoD (purified GST-MyoD) binds to the MyoD-specific sequences in the pGL3MyoD3-luc reporter gene, as shown by EMSA. A 32 P-labeled segment from the 5'-flanking region of Ifi204 (–1578 to –1324 [see Fig. 7A]) and one or more of the proteins indicated, i.e., GST (0.5 μ g), GST-MyoD (0.5 μ g), GST-204 (1 μ g), and GST-202a (shown as GST-202) (1 μ g), were incubated in the reaction mixtures for 35 min and then analyzed by gel electrophoresis and autoradiography. For competition experiments, a 50-fold excess of the wild-type or mutant MEF-1 oligonucleotide (oligos), as indicated, was added to the reaction mixture at time zero. Where indicated, anti-MyoD IgG (0.5 μ g) was added to the reaction mixture after a 20-min incubation, and the incubation was continued for a further 15 min prior to gel electrophoresis and autoradiography. The positions of the free DNA probe (arrow 3), the MyoD-DNA complex (arrow 2), and the MyoD-DNA complex supershifted by anti-MyoD IgG (arrow 1) are indicated. For further details, see Materials and Methods.

yeast two-hybrid assay (data not shown) prompted us to examine whether cardiac myocyte p204 is associated with the contractile apparatus which can be visualized by the staining of α -actinin (4) (Fig. 11H). A subsequent test for colocalization revealed that there was no significant overlap between p204 and α -actinin (Fig. 11G, H, and J).

DISCUSSION

The high levels of p204 in adult mouse heart and skeletal muscle (Fig. 1), the increase in the p204 level during the differentiation of myoblasts to myotubes, and the appearance of p204 (a nuclear protein in myoblasts) in the cytoplasm of the myotubes (Fig. 2, left panels) resemble findings for p202a, another p200 family protein (19) (Fig. 2, right panels). The similarity between the patterns of expression of the two proteins is not surprising, since the similarity between the 5'-flanking sequences of their genes over a region of at least 3 kb is approximately 97% (data not shown).

p204 contains a leucine-rich NES which is essential for its translocation from the nucleus to the cytoplasm in the course of the fusion of the myoblasts to myotubes: a p204-GFP fusion protein is translocated, whereas an otherwise identical fusion protein lacking part of the NES remains all nuclear (Fig. 4). p202a, which is also translocated to the cytoplasm during skeletal muscle differentiation, is devoid of an obvious NES. Thus, its mode of translocation (possibly attached to a protein with an NES) remains to be explored.

The difference in phosphorylation between cytoplasmic and nuclear p204 (Fig. 3) is similar to that observed for several

yeast and mammalian proteins, including transcription factors and repressors (3, 33, 38), some of which carry an NES (28, 59), as p204 does. It was proposed that phosphorylation of these proteins in the nucleus can convert them into targets for export receptors (3, 38). Binding to such receptors results in the passage of these proteins from the nucleus to the cytoplasm, thereby separating the proteins from the nuclear genes they regulate. Dephosphorylation of these proteins in the cytoplasm may allow their reentry into the nucleus and resumption of the regulation of the expression of their target genes. It remains to be seen whether the reason for the nuclear export of p204 during myoblast differentiation is as discussed above, or whether p204 might have other functions, e.g., serving as a component of a nuclear export complex removing proteins that inhibit differentiation from the nucleus.

The kinase(s) responsible for the phosphorylation of p204, and the amino acid residue(s) phosphorylated, remains to be identified. However, antibodies to phosphotyrosine did not stain the phosphorylated cytoplasmic p204 (data not shown), making it likely that serine or threonine residues are phosphorylated. This would be consistent with the observation that other proteins exported from the nucleus also contained phosphorylated serine or threonine residues (24, 38, 43). An inhibitor of NES-dependent nuclear export, LMB, even when used at a concentration as low as 2 ng/ml, blocked myoblast fusion, the export of p204 to the cytoplasm, and the accumulation of the myotube protein α -actinin (Fig. 5). The extent to which the

FIG. 10. Overexpression of p204 accelerates the fusion of C2C12 myoblasts to myotubes in DM and induces the fusion even in GM. Cultures from the cloned C9 line (derived from C2C12 myoblasts by transfection of a construct encoding Muristerone receptors) were transfected with either the pIND expression vector (control) (A, C, and E), or pIND-204, a Muristerone-inducible p204 expression plasmid (induced p204) (B, D, and F). Transfectants were selected and cloned. The cultures were incubated in GM in the presence of 2.5 μ M Muristerone for 2 days [GM(2d)]. This increased the level of p204 two- to threefold in the experimental culture (induced p204) over that in the control culture (control). Subsequently, the cultures either continued to be incubated in GM for 4 more days [GM(6d)] (E and F) or were shifted to DM for 2 days [DM(2d)] (C and D). The cultures were observed under a phase-contrast microscope. Bars, 150 μ m. For further details, see Materials and Methods.

FIG. 11. (A through F) Expression of p204 in the developing mouse heart. Frozen sections from hearts of C57BL/6 mouse embryos or newborn mice were fixed in acetone, stained with preimmune serum (A and E) or with anti-p204 antibodies (B, C, D, and F), and visualized with horseradish peroxidase-labeled secondary antibodies. Counterstaining was done with methyl green. Heart sections from 10.5-, 13.5-, and 16.5-day-old embryos or a newborn mouse are indicated as E10.5, E13.5, E16.5, and P1, respectively. ra, right atrium; rv, right ventricle; lv, left ventricle; avc, atrioventricular cushion. Bars, 200 μ m. (G through J) Primarily cytoplasmic localization of p204 in isolated murine cardiac myocytes. After fixation and blocking, neonatal mouse myocytes were incubated with primary rabbit antibodies against p204 and secondary antibodies against rabbit IgG conjugated with biotin, followed by incubation with streptavidin conjugated to fluorescein (G), or with murine monoclonal antibodies against α -actinin and secondary antibodies against mouse IgG conjugated with rhodamine (H). (I) Nuclei were stained with DAPI. (J) The overlapping signals are indicated as G+H+I. Bars, 5 μ m. For further details, see Materials and Methods.

inhibition of p204's nuclear export by LMB contributed to the inhibition of myotube formation remains to be explored.

Two findings prompted us to test for the involvement of MyoD in the transcription of the *Ifi204* gene: (i) the fact that transfection of MyoD expression plasmids into 10T1/2 cells resulted in an increase in the level of endogenous p204 (Fig. 6) and (ii) the identification of several MyoD-specific E boxes in the 5'-flanking region of the *Ifi204* gene (Fig. 7A). Our experiments revealed that segments containing the MyoD-specific sequences in the 5'-flanking region of the *Ifi204* gene could drive the MyoD-dependent expression of reporter genes (Fig. 7B and C). The MyoD-dependent transcription of the *Ifi204* gene required the presence of at least two MyoD-responsive E box sequences, in agreement with published data concerning genes other than *Ifi204* (1, 82) (Fig. 8). Purified MyoD bound to the MyoD-specific sequences in the *Ifi204* gene (Fig. 9), and alteration of these MyoD-specific sequences inhibited the activity of the reporters (Fig. 8B). The induction of myoblast differentiation also increased the MyoD-dependent transcription of reporter genes (Fig. 7E and 8C). These and other results established a role for MyoD in the expression of p204 in proliferating as well as in differentiating myoblasts. Not surprisingly, the transfection of a myogenin expression plasmid into 10T1/2 cells also enhanced the expression of reporter genes with the E box sequences in a dosage-dependent manner (Fig. 7G). The extent of the myogenin-induced increase in reporter gene expression was less than that induced by MyoD (compare Fig. 7C and G). The significance of this difference is unclear, however, since the expression of myogenin was driven by a weaker enhancer (LTR) (27) than that of MyoD (CMV) (19). It remains to be determined whether myogenic proteins other than MyoD (20) and myogenin (27, 85) (e.g., Myf5 [7] or MRF4 [57, 67]) can also induce the expression of p204.

The high level of p204 in the adult mouse heart prompted us to examine the p204 levels during embryonic heart development. Immunohistochemical examination revealed very low levels of p204 in an embryonic heart on day 10.5, medium levels on day 13.5, and close to peak levels by day 16.5, which were maintained until birth and beyond (Fig. 11A through F). These findings indicate that p204 also is involved in heart muscle differentiation. As in the case of skeletal muscle myotubes, much of the p204 in isolated heart myocytes occurs in the cytoplasm (Fig. 11G through J). The identity of the transcription factor(s) mediating the expression of p204 in heart muscle remains to be established.

Starting to explore the functions of p204 in skeletal muscle differentiation, we generated C2C12 lines, in which p204 can be induced by Muristerone. The induction of p204 in such a line accelerated the fusion of myoblasts to myotubes in DM and unexpectedly also resulted in myoblast fusion to myotubes in GM (Fig. 10). It remains to be established whether this finding indicates (i) that the shifting of the culture to (growth factor-poor) DM is required primarily for allowing the accumulation of p204 or (ii) only that p204, which is known to inhibit cell proliferation when overexpressed (by Muristerone induction), can overcome the effect of growth factors (in the GM), thus allowing myoblasts to exit the cell cycle, as well as to fuse to myotubes. Whatever the case may be, the results warrant the exploration of the use of p204 in cases in which muscle differentiation is impaired (e.g., malignancy), as well as in cases in which muscle regeneration is beneficial.

The results presented reveal that p202a and p204, though structurally related, have distinct roles in skeletal muscle differentiation: p204, if overexpressed in proliferating C2C12 myoblasts, can facilitate myoblast fusion (in both DM and

GM) (this study), whereas p202a, if overexpressed in proliferating C2C12 myoblasts, inhibits differentiation (in DM) (19).

Although the various roles and modes of action of p204 and p202a (and possibly p202b) in muscle differentiation remain to be determined, these proteins have characteristics such as antiproliferative, transcription-modulatory, and antiapoptotic activities that might be relevant to their roles in differentiation (15, 16, 19, 44, 49, 52, 56, 80). Furthermore, p202a (whose level increases during myoblast differentiation after a delay) was found earlier to inhibit the expression of MyoD, as well as the transcriptional activities of MyoD and myogenin (19). Thus, it is conceivable that p202a might function in muscle differentiation, among other functions, as a feedback inhibitor that limits the accumulation of p204.

Originally p204 was discovered as an interferon-inducible protein (12). The data presented here establish that its expression can also be triggered by muscle differentiation, which, in the case of skeletal muscle, results in the activation of MyoD and myogenin. Remarkably, this differentiation-dependent expression of p204 does not depend on interferon.

It is of interest that several interferon-inducible proteins (in addition to p204 and p202a) have been reported to be induced during myoblast differentiation (6, 69). These interferon-inducible proteins include 2'-5' oligoadenylate synthetase and RNA-activatable protein kinase (PKR). Similarly to p204 and p202a, 2'-5' oligoadenylate synthetase and PKR may contribute to the inhibition of cell proliferation (72, 75), which is a prerequisite for differentiation.

ACKNOWLEDGMENTS

We thank M. Yoshida and C. Brennan for leptomycin B, C. Weissmann and H. Weber for human α_2/α_1 interferon (1-83), A. Lassar for the pCMV-MyoD and GST-MyoD plasmids, T. Koleske for instruction in obtaining timed-pregnant mice, S. Wolin for the use of a 2-D gel electrophoresis system, and L. Vellali for preparing the manuscript for publication.

These studies were supported by NIH research grants R37AI12320 to P.L. and HG02153 to B.A.R. and by a postdoctoral fellowship to H.W. from the Cancer Research Foundation.

REFERENCES

1. Abu Hatoum, O., S. Gross-Meslaly, K. Breitschopf, A. Hoffman, H. Gonen, A. Ciechanover, and E. Bengal. 1998. Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: regulation by specific DNA binding. *Mol. Cell. Biol.* **18**:5670-5677.
2. Aurade, F., C. Pinset, P. Chafey, F. Gros, and D. Montarras. 1994. Myf5, MyoD, myogenin and MRF4 myogenic derivatives of the embryonic mesenchymal cell line C3H10T1/2 exhibit the same adult muscle phenotype. *Differentiation* **55**:185-192.
3. Beck, T., and M. N. Hall. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **402**:689-692.
4. Bellin, R. M., S. W. Sernett, B. Becker, W. Ip, T. W. Huiatt, and R. M. Robson. 1999. Molecular characteristics and interactions of the intermediate filament protein synemin. Interactions with alpha-actinin may anchor synemin-containing heterofilaments. *J. Biol. Chem.* **274**:29493-29499.
5. Benezra, R., R. I. Davis, D. Lockshon, D. I. Turner, and H. Weintraub. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61**:49-59.
6. Birnbaum, M., B. Trink, A. Shainberg, and S. Salzberg. 1990. Activation of the interferon system during myogenesis in vivo. *Differentiation* **45**:138-145.
7. Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* **8**:701-709.
8. Briggs, J. A., G. R. Burrus, B. D. Stickney, and R. C. Briggs. 1992. Cloning and expression of the human myeloid cell nuclear differentiation antigen: regulation by interferon alpha. *J. Cell. Biochem.* **49**:82-92.
9. Briggs, R. C., W. Y. Kao, L. I. Dworkin, J. A. Briggs, E. N. Dessypris, and J. Clark. 1994. Regulation and specificity of MNDA expression in monocytes, macrophages, and leukemia/B lymphoma cell lines. *J. Cell. Biochem.* **56**:559-567.
10. Buskin, J. N., and S. H. Hauschka. 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9**:2627-2640.
11. Choi, J., M. I. Costa, C. S. Mermelstein, C. Chagas, S. Holtzer, and H. Holtzer. 1990. MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. *Proc. Natl. Acad. Sci. USA* **87**:7988-7992.
12. Choubey, D., J. Snoddy, V. Chaturvedi, E. Toniato, G. Opdenakker, A. Thakur, H. Samanta, D. A. Engel, and P. Lengyel. 1989. Interferons as gene activators. Indications for repeated gene duplication during the evolution of a cluster of interferon-activatable genes on murine chromosome 1. *J. Biol. Chem.* **264**:17182-17189.
13. Choubey, D., and P. Lengyel. 1992. Interferon action: nucleolar and nucleoplasmic localization of the interferon-inducible 72-kD protein that is encoded by the Ifi 204 gene from the gene 200 cluster. *J. Cell Biol.* **116**:1333-1341.
14. Choubey, D., and P. Lengyel. 1995. Binding of an interferon-inducible protein (p202) to the retinoblastoma protein. *J. Biol. Chem.* **270**:6134-6140.
15. Choubey, D., S.-J. Li, B. Datta, J. U. Gutterman, and P. Lengyel. 1996. Inhibition of E2F-mediated transcription by p202. *EMBO J.* **15**:5668-5678.
16. Choubey, D., and J. U. Gutterman. 1997. Inhibition of E2F-4/DP-1-stimulated transcription by p202. *Oncogene* **15**:291-301.
17. Choubey, D., and P. Lengyel. 1993. Interferon action: cytoplasmic and nuclear localization of the interferon-inducible 52-kD protein that is encoded by the Ifi 202 gene from the gene 200 cluster. *J. Interferon Res.* **13**:43-52.
18. Datta, B., B. Li, D. Choubey, G. Nallur, and P. Lengyel. 1996. p202, an interferon-inducible modulator of transcription, inhibits transcriptional activation by the p53 tumor suppressor protein, and a segment from the p53-binding protein 1 that binds to p202 overcomes this inhibition. *J. Biol. Chem.* **271**:27544-27555.
19. Datta, B., W. Min, S. Burma, and P. Lengyel. 1998. Increase in p202 expression during skeletal muscle differentiation: inhibition of MyoD protein expression and activity by p202. *Mol. Cell. Biol.* **18**:1074-1083.
20. Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**:987-1000.
21. Dawson, M. J., J. A. Trapani, R. C. Briggs, J. K. Nicholl, G. R. Sutherland, and E. Baker. 1995. The closely linked genes encoding the myeloid nuclear differentiation antigen (MNDA) and IFI16 exhibit contrasting haemopoietic expression. *Immunogenetics* **41**:40-43.
22. Dawson, M. J., and J. A. Trapani. 1996. HIN-200: a novel family of IFN-inducible nuclear proteins expressed in leukocytes. *J. Leukoc. Biol.* **60**:310-316.
23. Der, S. D., A. Zhou, B. R. G. Williams, and R. H. Silverman. 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* **95**:15623-15628.
24. DeVit, M. J., and M. Johnston. 1999. The nuclear export of Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*. *Curr. Biol.* **9**:1231-1241.
25. DeYoung, K. L., M. E. Ray, Y. A. Su, S. L. Anzick, R. W. Johnstone, J. A. Trapani, P. S. Meltzer, and J. M. Trent. 1997. Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma. *Oncogene* **15**:453-457.
26. Dias, P., M. Dilling, and P. Houghton. 1994. The molecular basis of skeletal muscle differentiation. *Semin. Diagn. Pathol.* **11**:3-14.
27. Edmondson, D. G., and E. N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* **3**:628-640.
28. Fornerod, M., M. Ohno, M. Yoshida, and I. W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export. *Cell* **90**:1051-1060.
29. Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**:308-311.
30. Gariglio, M., M. D. Andrea, M. Lembo, M. Ravotto, C. Zappador, G. Valente, and S. Landolfo. 1998. The murine homolog of the HIN 200 family, Ifi204, is constitutively expressed in myeloid cells and selectively induced in the monocyte/macrophage lineage. *J. Leukoc. Biol.* **64**:608-614.
31. Gariglio, M., S. Panico, G. Cavallo, C. Divakar, P. Lengyel, and S. Landolfo. 1992. Impaired transcription of the poly rI:C- and interferon-activatable 202 gene in mice and cell lines from the C57BL/6 strain. *Virology* **187**:115-123.
32. Gerace, L. 1995. Nuclear export signals and the fast track to the cytoplasm. *Cell* **82**:341-344.
33. Gerner, W., C. Schuller, and H. Ruis. 1999. Being at the right place at the right time: the role of nuclear transport in dynamic transcriptional regulation in yeast. *Biol. Chem.* **380**:147-150.
34. Gribaudo, G., S. Ravaglia, L. Guandalini, L. Riera, M. Gariglio, and S. Landolfo. 1997. Molecular cloning and expression of an interferon-inducible protein encoded by gene 203 from the gene 200 cluster. *Eur. J. Biochem.* **249**:258-264.
35. Grounds, M. D., K. I. Garrett, M. C. Lai, W. E. Wright, and M. W. Beilharz. 1992. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res.* **267**:99-104.
36. Harhaj, E. W., and S. C. Sun. 1999. Regulation of RelA subcellular local-

- ization by a putative nuclear export signal and p50. *Mol. Cell. Biol.* **19**:7088–7095.
37. Hertel, L., M. DeAndrea, B. Azzimonti, A. Rolle, M. Gariglio, and S. Landolfo. 1999. The interferon-inducible 204 gene, a member of the Ifi200 family, is not involved in the antiviral state induction by IFN- α , but is required by the mouse cytomegalovirus for its replication. *Virology* **262**:1–8.
 38. Hopper, A. K. 1999. Nucleocytoplasmic transport: inside out regulation. *Curr. Biol.* **9**:R803–R806.
 39. Ito, N., G. Gribaudo, E. Toniato, A. Thakur, Y. Yagi, J. Barbosa, M. Karmarck, F. H. Ruddle, and P. Lengyel. 1989. Interferons as gene activators: identification of an interferon responsive “GA” box in a murine gene. *UCLA Symp. Mol. Cell. Biol. New Ser.* **103**:169–178.
 40. Jen, Y., H. Weintraub, and R. Benezra. 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A proteins. *Genes Dev.* **6**:1466–1479.
 41. Johnstone, R. W., and J. A. Trapani. 1999. Transcription and growth regulatory functions of the HIN-200 family of proteins. *Mol. Cell. Biol.* **19**:5833–5838.
 42. Kitzmann, M., M. Vandromme, V. Schaeffer, G. Carnac, J. C. Labbe, N. Lamb, and A. Fernandez. 1999. cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol. Cell. Biol.* **19**:3167–3176.
 43. Komeili, A., and E. K. O’Shea. 1999. Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. *Science* **284**:977–980.
 44. Koul, D., R. Lapushin, H. J. Xu, G. B. Mills, J. U. Gutterman, and D. Choubey. 1998. p202 prevents apoptosis in murine AKR-2B fibroblasts. *Biochem. Biophys. Res. Commun.* **247**:379–382.
 45. Kudo, N., B. Wolff, T. Sekimoto, E. P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, and M. Yoshida. 1998. Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* **242**:540–547.
 46. Landolfo, S., G. Gribaudo, and D. Lembo. 1998. The Ifi 200 genes: an emerging family of IFN-inducible genes. *Biochimie* **80**:721–728.
 47. Lassar, A. B., R. L. Davis, W. E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell* **66**:305–315.
 48. Lassar, A. B., S. X. Skapek, and B. Novitsch. 1994. Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr. Opin. Cell Biol.* **6**:788–794.
 49. Lembo, M., C. Sacchi, C. Zappador, G. Bellomo, M. Gaboli, P. P. Pandolfi, M. Gariglio, and S. Landolfo. 1998. Inhibition of cell proliferation by the interferon-inducible 204 gene, a member of the Ifi 200 cluster. *Oncogene* **16**:1543–1551.
 50. Lengyel, P. 1982. Biochemistry of interferons and their actions. *Annu. Rev. Biochem.* **51**:251–282.
 51. Lengyel, P., D. Choubey, S.-J. Li, and B. Datta. 1995. The interferon-activatable gene 200 cluster: from structure toward function. *Semin. Virol.* **6**:203–213.
 52. Liu, C. J., H. Wang, and P. Lengyel. 1999. The interferon-inducible nucleolar p204 protein binds the ribosomal RNA-specific UBF1 transcription factor and inhibits ribosomal RNA transcription. *EMBO J.* **18**:2845–2854.
 53. Lyden, D., A. Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O’Reilly, B. L. Bader, R. O. Hynes, Y. Zhuang, K. Manova, and R. Benezra. 1999. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature* **401**:670–677.
 54. Megey, L. A., and M. A. Rudnicki. 1995. Determination versus differentiation and the MyoD family of transcription factors. *Biochem. Cell Biol.* **73**:723–732.
 55. Melnikova, I. N., M. Bounpheng, G. C. Schatteman, D. Gilliam, and B. A. Christy. 1999. Differential biological activities of mammalian Id proteins in muscle cells. *Exp. Cell Res.* **247**:94–104.
 56. Min, W., S. Ghosh, and P. Lengyel. 1996. The interferon-inducible p202 protein as a modulator of transcription: inhibition of NF- κ B, c-Fos, and c-Jun activities. *Mol. Cell. Biol.* **16**:359–368.
 57. Miner, J. H., and B. Wold. 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. *Proc. Natl. Acad. Sci. USA* **87**:1089–1093.
 58. Molkenin, J. D., and E. N. Olson. 1996. Defining the regulatory networks for muscle development. *Curr. Opin. Genet. Dev.* **6**:445–453.
 59. Nakielnny, S., and G. Dreyfuss. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell* **99**:677–690.
 60. Olson, E. N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.* **154**:261–272.
 61. Olson, E. N., and W. H. Klein. 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* **8**:1–8.
 62. Ossareh-Nazari, B., F. Bachelier, and C. Dargemont. 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278**:141–144.
 63. Quandt, K., K. Frech, H. Karas, E. Wingender, and T. Werner. 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res.* **23**:4878–4884.
 64. Rawls, A., and E. N. Olson. 1997. MyoD meets its maker. *Cell* **89**:5–8.
 65. Reynaud, E. G., K. Pempel, M. Guiller, M. P. Leibovitch, and S. A. Leibovitch. 1999. p57(Kip2) stabilizes the MyoD protein by inhibiting cyclin E-Cdk2 kinase activity in growing myoblasts. *Mol. Cell. Biol.* **19**:7621–7629.
 66. Reznikoff, C. A., D. W. Brankow, and C. Heidelberger. 1973. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* **33**:3231–3238.
 67. Rhodes, S. J., and S. F. Konieczny. 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* **3**:2050–2061.
 68. Rudnicki, M. A., and R. Jaenisch. 1995. The MyoD family of transcription factors and skeletal myogenesis. *Bioessays* **17**:203–209.
 69. Salzberg, S., S. Vilchik, S. Cohen, A. Heller, and Y. Kronfeld-Kinar. 2000. Expression of a PKR dominant-negative mutant in myogenic cells interferes with the myogenic process. *Exp. Cell Res.* **254**:45–54.
 70. Sartorelli, V., P. L. Puri, Y. Hamamori, V. Ogryzko, G. Chung, Y. Nakatani, J. Y. Wang, and L. Kedes. 1999. Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol. Cell* **4**:725–734.
 71. Schafer, B. W., B. T. Blakely, G. J. Darlington, and H. M. Blau. 1990. Effect of cell history on response to helix-loop-helix family of myogenic regulators. *Nature* **344**:454–458.
 72. Sen, G. C., and R. M. Ransohoff. 1998. Transcriptional regulation in the interferon system. Chapman & Hall, New York, N.Y.
 73. Soeno, Y., Y. Shimada, and T. Obinata. 1999. BDM (2,3-butanedione monoxime), an inhibitor of myosin-actin interaction, suppresses myofibrillogenesis in skeletal muscle cells in culture. *Cell Tissue Res.* **295**:307–331.
 74. Song, A., Q. Wang, M. G. Goebel, and M. A. Harrington. 1998. Phosphorylation of nuclear MyoD is required for its rapid degradation. *Mol. Cell. Biol.* **18**:4994–4999.
 75. Stark, G. R., B. R. G. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem.* **67**:227–262.
 76. Tannenbaum, C. S., J. Major, Y. Ohmori, and T. A. Hamilton. 1993. A lipopolysaccharide-inducible macrophage gene (D3) is a new member of an interferon-inducible gene cluster and is selectively expressed in mononuclear phagocytes. *J. Leukoc. Biol.* **53**:563–568.
 77. Trapani, J. A., M. Dawson, V. A. Apostolidis, and K. A. Browne. 1994. Genomic organization of IFI16, an interferon-inducible gene whose expression is associated with human myeloid cell differentiation: correlation of predicted protein domains with exon organization. *Immunogenetics* **40**:415–424.
 78. Trouche, D., M. Grigoriev, J. I. Lenormand, P. Robin, S. A. Leibovitch, P. Sassone-Corsi, and A. Harel-Bellan. 1993. Repression of c-fos promoter by MyoD on muscle cell differentiation. *Nature* **363**:79–82.
 79. Wang, H., G. Chatterjee, J. J. Meyer, C. J. Liu, N. A. Manjunath, P. Brayward, and P. Lengyel. 1999. Characteristics of three homologous 202 genes (Ifi202a, Ifi202b, and Ifi202c) from the murine interferon-activatable gene 200 cluster. *Genomics* **60**:281–294.
 80. Wang, H., C. J. Liu, Y. Lu, G. Chatterjee, X.-Y. Ma, R. N. Eisenman, and P. Lengyel. 2000. The interferon- and differentiation-inducible p202a protein inhibits the transcriptional activity of c-Myc by blocking its association with Max. *J. Biol. Chem.*, in press.
 81. Weber, H., D. Valenzuela, G. Lujber, M. Gubler, and C. Weissmann. 1987. Single amino acid changes that render human IFN- α 2 biologically active on mouse cells. *EMBO J.* **6**:591–598.
 82. Weintraub, H., R. Davis, D. Lockshon, and A. Lassar. 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. *Proc. Natl. Acad. Sci. USA* **87**:5623–5627.
 83. Weintraub, H., R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, and S. Hollenberg. 1991. The myoD gene family: nodal point during specification of the muscle cell lineage. *Science* **251**:761–766.
 84. White, R. L., and B. A. Wittenberg. 1993. NADH fluorescence of isolated ventricular myocytes: effects of pacing, myoglobin, and oxygen supply. *Biophys. J.* **65**:196–204.
 85. Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* **56**:607–617.
 86. Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* **270**:725–727.
 87. Yokota, Y., A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. 1999. Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* **397**:702–706.
 88. Zhang, J. M., X. Zhao, Q. Wei, and B. M. Paterson. 1999. Direct inhibition of G(1) cdk kinase activity by MyoD promotes myoblast cell cycle withdrawal and terminal differentiation. *EMBO J.* **18**:6983–6993.