Molecular and Cellular Biology, Oct. 1995, p. 5205–5213
0270-7306/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 15, No. 10

Ras p21^{Val} Inhibits Myogenesis without Altering the DNA Binding or Transcriptional Activities of the Myogenic Basic Helix-Loop-Helix Factors

YANFENG KONG, SALLY E. JOHNSON, ELIZABETH J. TAPAROWSKY, AND STEPHEN F. KONIECZNY*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907-1392

Received 17 May 1995/Returned for modification 20 June 1995/Accepted 30 June 1995

MRF4, MyoD, myogenin, and Myf-5 are muscle-specific basic helix-loop-helix transcription factors that share the ability to activate the expression of skeletal muscle genes such as those encoding α-actin, myosin heavy chain, and the acetylcholine receptor subunits. The muscle regulatory factors (MRFs) also exhibit the unique capacity to initiate the myogenic program when ectopically expressed in a variety of nonmuscle cell types, most notably C3H10T1/2 fibroblasts (10T1/2 cells). The commitment of myoblasts to terminal differentiation, although positively regulated by the MRFs, also is controlled negatively by a variety of agents, including several growth factors and oncoproteins such as fibroblast growth factor (FGF-2), transforming growth factor β1 (TGF-β1), and Ras p21^{Val}. The molecular mechanisms by which these varied agents alter myogenic terminal differentiation events remain unclear. In an effort to establish whether Ras p21^{Val} represses MRF activity by directly targeting the MRF proteins, we examined the DNA binding and transcription activation potentials of MRF4 and MyoD when expressed in 10T1/2 cells or in 10T1/2 cells expressing Ras p21^{Val}. Our results demonstrate that Ras p21^{Val} inhibits terminal differentiation events by targeting the basic domain of the MRFs, and yet the mechanism underlying this inhibition does not involve altering the DNA binding or the inherent transcriptional activity of these regulatory factors. In contrast, FGF-2 and TGF-β1 block terminal differentiation by repressing the transcriptional activity of the MRFs. We conclude that the Ras p21^{Val} block in differentiation operates via an intracellular signaling pathway that is distinct from the FGF-2 and TGF-β1 pathways.

The ability of the myogenic regulatory factors (MRFs) to initiate a muscle phenotype in a variety of nonmuscle cells makes skeletal myogenesis an excellent model system in which to study the regulation of tissue-specific gene expression. Skeletal muscle precursor cells, or myoblasts, proliferate when maintained in growth medium containing high levels of serum or specific serum growth factors. Upon induction for differentiation, i.e., removal of growth factors, myoblasts exit the cell cycle and fuse into multinucleate myotubes. These terminally differentiated cells express muscle-specific genes, many of which encode the sarcomeric proteins that are responsible for contraction.

The MRFs, which include MyoD (12), myogenin (16, 62), Myf-5 (6), and MRF4 (5, 39, 50), share a highly conserved basic helix-loop-helix (bHLH) domain that mediates DNA binding and protein-protein interactions (reviewed in references 15 and 45). The basic region of each MRF contains a conserved myogenic regulatory motif (MRM) that is required for the myogenic activity of the MRFs. The amino acid residues comprising the MRM are not found in any other known bHLH proteins (7, 13, 14). The myogenic activity of the MRFs also requires heterodimerization with ubiquitously expressed E proteins (such as E12, E47, or HEB) to form functional transcription complexes that bind to target E-box sequences (-CANNTG-) located within muscle-specific promoters or enhancers. The binding of the heterodimer complex to these specific E boxes is required to generate the tissue-specific gene expression patterns characteristic of skeletal muscle.

* Corresponding author. Phone: (317) 494-7976. Fax: (317) 496-2536.

The decision to differentiate into myotubes is influenced negatively by several factors. Treatment of myoblasts with fetal bovine serum (FBS), basic fibroblast growth factor 2 (FGF-2), or transforming growth factor β1 (TGF-β1) is known to inhibit differentiation of both primary myoblasts and established myoblast cell lines (reviewed in reference 43). The events responsible for the inhibition of differentiation by these agents are complex and occur via different molecular mechanisms. FBS suppresses myogenesis by increasing intracellular levels of Id (3, 8), a helix-loop-helix protein that heterodimerizes with E proteins and prevents the formation of myogenic MRF-E protein complexes (23). TGF-β1, on the other hand, suppresses the activity of the MRF protein myogenin by a mechanism that is independent of Id and that does not disrupt the DNA binding properties of the protein (8). Inhibition of DNA binding has been implicated as the mechanism through which FGF-2 represses myogenin activity, since FGF-2-induced phosphorylation of a conserved threonine residue in the basic domain inhibits myogenin from binding to DNA (32). Interestingly, this repression mechanism is not universal for all MRFs, since FGF-2 fails to induce phosphorylation of this conserved threonine in MRF4 (22) and MyoD mutants lacking the conserved threonine residue remain repressed by FGF-2 (28). These conflicting results suggest that FGF-2 functions to inhibit myogenesis by targeting myogenin directly and by repressing MRF4 and MyoD activities indirectly.

Myogenesis also is regulated negatively by many oncogenes, such as c-*myc*, c-*jun*, c-*fos*, H-*ras* and *E1a* (reviewed in reference 1). For many of these oncogenes, their expression in myoblasts leads to cell transformation and the repression of the MRF genes at the transcriptional level (10, 17, 27, 30). Oncoproteins also can attenuate the formation of active

MRF-E protein heterodimers by increasing levels of Id expression (1). Lastly, certain oncoproteins inhibit myogenesis by directly silencing the transcriptional activity of the MRFs. In the case of Fos and Jun, for example, a direct interaction between these oncoproteins and MyoD or myogenin blocks muscle-specific gene activation (4, 31).

Of the various oncogene-encoded proteins shown to inhibit myogenesis, Ras p21^{Val} may be the most intriguing. Ras p21^{Val} encodes a membrane-bound, constitutively active G protein which initiates signal transduction events through the mitogen-activated protein kinase (MAPK) pathway (41). Ras p21^{Val} represses the differentiation of myoblast cell lines independently of their continued proliferation (46). One documented mechanism by which Ras p21^{Val} inhibits myogenic differentiation involves repressing transcription of the endogenous *MyoD* gene. Although this inhibition can be overridden by forced overexpression of MyoD (27, 30), only a minimum number of cells overexpressing MyoD fully proceed through the myogenic program. In a manner similar to the Ras p21^{Val}-induced inhibition of myogenic differentiation, treatment of myoblasts with FGF-2 and TGF-β1 also down-regulates expression of the endogenous *MyoD* gene (58). Although this finding would support a role for Ras G-protein activation in the FGF-2 and TGF-β1 inhibition of myogenesis, it remains unknown whether the signaling pathways triggered by these distinct factors operate via the same downstream effector molecules.

In this study, we examined whether Ras p21^{Val} and growth factors inhibit muscle differentiation events through signaling pathways that modify well-characterized functions of the MRF proteins. We show that overexpression of MyoD or MRF4 in C3H10T1/2 fibroblasts (10T1/2 cells) is not sufficient to override the inhibitory effect of Ras p21^{Val} when tested in transient DNA transfections. Ras p21^{Val} inhibition of myogenesis is targeted at the conserved MRM region of the MRFs, but surprisingly, inhibition does not alter the dimerization, DNA binding, or inherent transcriptional activity of MRF4 or MyoD. This observation contrasts with the mechanisms utilized by FGF-2 and TGF-β1 to block myogenesis, which involve the direct inhibition of MRF transcription activities. The results reported here strongly suggest that Ras p21^{Val} inhibits myogenesis through a signal transduction pathway that is distinct from the FGF-2 and TGF-β1 pathways.

MATERIALS AND METHODS

Cell culture and DNA transfections. 10T1/2 cells were maintained in growth medium containing basal medium Eagle (GIBCO) supplemented with 10% FBS plus penicillin (100 U/ml) and streptomycin (100 μg/ml). C3H10T1/2 *ras neo* 11A and C3H10T1/2 *ras* 9B (10T1/2-Ras) cells (57, 61), which constitutively express the activated human H-*ras* oncogene encoding Ras p21^{Val-12}, were maintained in growth medium containing basal medium Eagle, with and without, respectively, 400 μg of G418 (GIBCO) per ml. Rev12N2 (10T1/2-Ras-K-rev) cells (generous gift of C. Ashendel) were derived from the C3H10T1/2 *ras* 9B cell line and constitutively express the human K-*rev*-1 cDNA. These cells were maintained as described above.

Transient DNA transfections were performed as previously described (63). Briefly, 10T1/2 cells were plated at 9×10^5/100-mm dish, and 10T1/2-Ras or 10T1/2-Ras-K-rev cells were plated at 6×10^5/100-mm dish. On the following day, DNA calcium phosphate precipitates were added to the dishes. DNA precipitates consisted of 2 or 5 μg of pEM-MRF4 or pEM-MyoD and 5 μg of α-actin CAT (chloramphenicol acetyltransferase), TnICAT, α-AchR-CAT, or (E-box)$_4$CAT reporter gene (see below). After 4 h, cells were subjected to osmotic shock for 2 min (for 10T1/2) or 1.5 min (for 10T1/2-Ras and 10T1/2-Ras-K-rev) in serum-free medium containing 20% glycerol and then fed growth medium containing 15% (for 10T1/2) or 10% (for 10T1/2-Ras and 10T1/2-Ras-K-rev) FBS. Differentiation medium containing 2% horse serum (HS) in low-glucose Dulbecco's modified Eagle medium (GIBCO) was added to the cultures on the following day, and the cultures were incubated for an additional 48 h. Where indicated, cells were treated with differentiation medium supplemented with 5 ng of FGF-2 or 5 ng of TGF-β1 (R & D Products) per ml as described previously (58). For GAL4 assays, DNA precipitates consisted of 5 μg of the reporter plasmid (GAL4)$_5$-E1bTATA-CAT (33) and 5 μg of one of the following plasmids: GAL4 (1-147) (33), GAL4-MRF4(1-60) (36), and GAL4-MyoD(1-66) (60). After glycerol treatment, the cultures were maintained in growth medium containing 10% FBS for 2 days. Cell extracts were harvested, and CAT assays were performed as described by Gorman et al. (19). The amount of extract used for each assay was normalized to the protein content of each sample. A minimum of three independent transfections were performed for each experimental group.

For immunohistocytochemistry, cultures were rinsed twice in cold phosphate-buffered saline and fixed in a 20:2:1 solution of 70% ethanol-formalin-acetic acid for 1 min at 4°C. Fixed cells were incubated with the antimyosin mouse monoclonal antibody MF-20 (2) and a biotinylated mouse secondary antibody. Immunoreactivity was visualized by using the Vectastain ABC reagent (Vector Laboratories, Inc.).

Expression plasmids. Reporter genes used for CAT assays included α-actin CAT, TnICAT, α-AchR-CAT, and (E-box)$_4$CAT. α-Actin CAT is the human α-actin reporter gene LK359CAT containing 359 bp of the α-actin promoter ligated to the CAT gene (40). TnICAT is the troponin I reporter gene TnICAT-1 containing the first intron enhancer of the quail troponin I gene (63). α-AchR-CAT is the 850-bp chicken α-subunit promoter fused to CAT (48). (E-box)$_4$CAT (also known as 4RTKCAT) contains four tandem copies of the muscle creatine kinase enhancer right E-box sequence cloned 5′ to the thymidine kinase CAT reporter gene (60). In some instances, a constitutively expressed β-actin CAT reporter gene was used as a positive control (18).

pEM-MRF4 and pEMc11s (pEM-MyoD) contain the rat MRF4 (50) and mouse MyoD (12) cDNAs, respectively, cloned into the eukaryotic expression vector pEMSVscribeα2 (12). Truncated and mutated MyoD and E12 gene constructs were a generous gift of A. Lassar, R. Davis, and H. Weintraub and have been previously described (14, 60). The expression plasmid pSVE2-5 contains the E2-5 cDNA and was used in conjunction with the (μE5+μE2)TATACAT reporter gene as described previously (51).

Nuclear extracts and electrophoretic mobility shift assays (EMSAs). Each 100-mm dish containing 10T1/2 or 10T1/2-Ras cells was transiently transfected with 30 μg of plasmid pEM-MRF4. The following day, cultures were treated with 2% HS or 2% HS supplemented with FGF-2 or TGF-β1 as described above. Nuclear extracts were prepared by washing cells with cold phosphate-buffered saline and then adding 2 ml of lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 100 μg of aprotinin per ml, 2 mM Na_3VO_4, 50 mM NaF, 5 mM $Na_4P_2O_7$) to each plate (29). Lysates were collected, and nuclei were pelleted by centrifugation for 5 min at 2,000 rpm at 4°C. Nuclei were resuspended at 2.5×10^7/ml in nuclear resuspension buffer (identical to lysis buffer except that 500 mM NaCl was included) and gently rocked for 1 h at 4°C. The resuspension was cleared by centrifugation at 10,000 rpm for 10 min, and the supernatant was aliquoted and stored frozen at −80°C.

Oligonucleotide probes containing the troponin I gene E-box site (34) were generated by labeling one DNA strand with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear). Unincorporated [γ-^{32}P]ATP was removed by G-50 spin columns. Labeled oligonucleotides then were mixed with a 10-fold excess of unlabeled complementary oligonucleotides. After denaturation by boiling for 5 min, the oligonucleotides were placed at room temperature and allowed to anneal.

Nuclear extracts were normalized to MRF4 levels by Western blot (immunoblot) analysis (see below) prior to EMSAs. Typically, 25 μg of nuclear extract was used for each 30 μl of binding reaction mixture in a buffer containing 20 mM HEPES (pH 7.6), 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. The mixture was incubated at 37°C for 15 min, and then 2 μg of poly(dI-dC) and 10 fmol of ^{32}P-labeled troponin I gene E-box probe were added prior to an additional incubation for 20 min at room temperature. In some instances, 1 μl of preimmune serum or 1 μl of MRF4, myogenin, or E12 antiserum was added to the reaction mixtures (53). DNA-protein complexes were analyzed by polyacrylamide gel (5%) electrophoresis in TBE (50 mM Tris [pH 8.0], 50 mM boric acid, 1 mM EDTA), and the dried gels were subjected to autoradiography. A minimum of three independent nuclear extract isolations and EMSAs were performed for each experimental group.

In vivo labeling and immunoprecipitations. In vivo labeling and immunoprecipitations were carried out as described by Hardy et al. (22). In brief, 10T1/2 and 10T1/2-Ras cells were transiently transfected with 10 μg of pEM-MRF4 per 100-mm dish, rinsed twice in Dulbecco's modified Eagle medium lacking methionine, and incubated for 1 h. The cells then were treated with the same medium containing 0.2 mCi of [^{35}S]methionine. After 4 h, cells were rinsed in ice-cold phosphate-buffered saline and harvested. Cells were pelleted at 4°C and resuspended in 1 ml of radioimmunoprecipitation buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.1% sodium deoxycholate, 0.1% Triton X-100) containing aprotinin, pepstatin, and leupeptin (each at 2 μg/ml) and incubated on ice for 30 min after vortexing. The suspension was cleared by a brief centrifugation, and the supernatant was removed. Ten microliters of MRF4 preimmune serum and 50 μl of protein A beads were added to the supernatant, the mixture was rocked for 1 h, and nonspecific complexes were removed by centrifugation. Ten microliters of MRF4 antibody plus 50 μl of

TABLE 1. Conversion of 10T1/2 and 10T1/2-Ras cells to a muscle phenotype[a]

Plasmid	Myosin staining	
	10T1/2	10T1/2-Ras
EMSV	0 (0)	0 (0)
MRF4	100 (474)	0 (0)
MyoD	91 (431)	5 (25)

[a] Plasmid pEMSVscribeα2 (EMSV), pEM-MRF4 (MRF4), or pEM-MyoD (MyoD) were used to transiently transfect 10T1/2 and 10T1/2-Ras cells as described in Materials and Methods. After 48 h in differentiation medium, cultures were fixed and stained with a monoclonal antibody directed against the skeletal myosin heavy chains. The number of nuclei contained in myosin-positive cells was determined by visual examination of 10 fields per dish per experiment. The values reported represent the percentage of myosin-positive cells observed relative to the number detected in the MRF4-transfected 10T1/2 cell group, which was set to 100%. Numbers in parentheses represent the average number of nuclei contained in myosin-positive cells per 10 fields. Parallel plates of 10T1/2 and 10T1/2-Ras cells transfected with pRSV-LacZ and analyzed for β-galactosidase expression revealed that the two cell types exhibit similar levels of transfection efficiency.

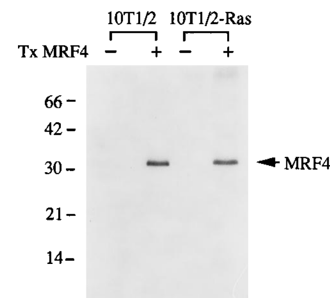

FIG. 1. Immunoprecipitation of MRF4 proteins expressed in 10T1/2 and 10T1/2-Ras cells. 10T1/2 and 10T1/2-Ras cells were transiently transfected (Tx) with pEM-MRF4 (+) and labeled with [^{35}S]methionine for 4 h. After labeling, the cells were lysed, the 33-kDa MRF4 protein was immunoprecipitated with an MRF4-specific antibody, and the products were resolved by SDS-polyacrylamide gel electrophoresis. Equivalent amounts of MRF4 protein are detected in the transfected 10T1/2 and 10T1/2-Ras cells. No MRF4 protein is detected in the untransfected (−) cell groups. Sizes are indicated in kilodaltons.

protein A beads were added to the supernatant, and after rocking for 2 h, specific complexes were isolated by centrifugation. The precipitates were washed in radioimmunoprecipitation assay buffer, boiled in SDS loading buffer, and analyzed by SDS-polyacrylamide gel (12%) electrophoresis and autoradiography.

Western analysis. Nuclear extracts were prepared from 10T1/2 and 10T1/2-Ras cells transfected with pEM-MRF4 as described above. Total nuclear proteins (25 μg) were electrophoretically separated on SDS–10% polyacrylamide gels. The gels were equilibrated in a transfer buffer composed of 50 mM Tris, 38 mM glycine, and 20% methanol, and the separated proteins were electrophoretically transferred to nitrocellulose for 40 min at 150 V, using a semidry blotter (Pharmacia). After completion of the transfer, the filter was stained with 0.5% Ponceau S in 1% acetic acid, and the positions of the molecular weight standards were noted. Nonspecific binding sites were blocked by incubation of the filter with 5% nonfat dry milk in Tris-buffered saline (TBS; 10 mM Tris [pH 8.0], 150 mM NaCl). Subsequently, the blot was incubated with a rabbit polyclonal antibody generated against the carboxy-terminal 20 amino acids of MRF4 (Santa Cruz Biotechnology, Inc.) diluted 1:300 in 5% nonfat dry milk in TBS-T (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20). After the filter was washed with TBS-T, the blot was incubated with a horseradish peroxidase-conjugated second antibody diluted in 5% nonfat dry milk in TBS-T. Detection of the immune complexes was achieved with an ECL (enhanced chemiluminescence) kit (Amersham) and by exposure to X-ray film. MRF4 proteins were quantified with a Molecular Dynamics PhosphorImager.

RESULTS

Expression of MRF4 or MyoD is not sufficient to override Ras p21^{Val}-dependent inhibition of myogenesis. Ras p21^{Val} is a potent transforming agent of mammalian cells and a negative regulator of muscle differentiation (reviewed in reference 1). One mechanism by which Ras p21^{Val} inhibits the differentiation of myoblasts involves repression of endogenous *MyoD* gene transcription (27, 30), although additional evidence suggests that in cells engineered to overexpress MyoD, Ras p21^{Val} also functions to inhibit the myogenic activity of the protein. In an effort to examine how Ras p21^{Val} blocks the activity of the MRFs, 10T1/2 cells and a 10T1/2 cell line stably expressing Ras p21^{Val} (57) were transiently transfected with pEM-MRF4 or pEM-MyoD. 10T1/2 cells expressing MRF4 or MyoD adopt a myogenic phenotype and fuse into small myotubes that express muscle-specific proteins such as the myosin heavy chains (Table 1). On the other hand, in 10T1/2-Ras cells, MRF4 and MyoD fail to induce myogenic events leading to myosin heavy-chain gene expression (Table 1), suggesting that transient expression of the MRFs cannot overcome the negative effects of Ras p21^{Val} in these cells. Similar results were obtained when 10T1/2 cells were transiently cotransfected with MRF4 and Ras p21^{Val} (data not shown).

As a first step toward establishing how the MRFs are blocked from generating a myogenic phenotype in 10T1/2-Ras cells, MRF4 protein levels were examined. Following incubation in differentiation medium, lysates were prepared from 10T1/2 cells and from 10T1/2-Ras cells transfected with pEM-MRF4, and the MRF4 protein in the lysates was immunoprecipitated with a MRF4-specific polyclonal antibody (53). As shown in Fig. 1, equivalent amounts of MRF4 protein are expressed in both 10T1/2 and 10T1/2-Ras cells, confirming the presence of MRF4 in both transfected populations. The MRF4 protein, as well as the MyoD protein, also efficiently translocates to the nucleus in both cell types (data not shown). Thus, the MRF proteins are expressed stably in the 10T1/2-Ras cells and yet remain unable to induce a muscle phenotype.

MRF4 and MyoD retain their heterodimerization and DNA binding activities in 10T1/2-Ras cells. The failure of MRF4 and MyoD to initiate a myogenic program in the 10T1/2-Ras cell line may be due to the production of dysfunctional proteins. In an effort to examine the structural properties of the MRFs in 10T1/2-Ras cells, nuclear extracts were prepared from normal and transfected cells and normalized for MRF4 protein content by Western analysis (Fig. 2A). Equivalent amounts of MRF4-containing extracts then were incubated with a ^{32}P-labeled E-box oligonucleotide probe and subjected to EMSA. Nuclear extracts isolated from 10T1/2 cells transiently transfected with MRF4 produce a novel shifted protein-DNA complex which is not present in control extracts (Fig. 2B). This complex can be competed for by the addition of excess wild-type E-box DNA but not mutant E-box DNA (data not shown). The MRF4-DNA complex does not form when an anti-MRF4 or anti-E12 antibody is added to the incubation mixture, whereas addition of an antimyogenin antibody has no effect on the binding pattern of the protein-DNA complex, demonstrating that the shifted complex observed in these extracts contains an MRF4-E protein heterodimer (Fig. 2B). The two non-MRF4-containing complexes detected in these assays are E-box specific, since they are effectively competed for by excess wild-type E-box DNA but not by mutant E-box DNA (data not shown). These complexes do not contain E12, however, since incubation with the anti-E12 antibody does not alter the mobility or reduce formation of either complex (Fig. 2B).

Similar assays using nuclear extracts from 10T1/2-Ras cells demonstrated that the MRF4 protein retains its DNA binding and protein heterodimerization properties. As shown in Fig. 2C, the three protein-DNA complexes detected in 10T1/2-MRF4 extracts also are observed in 10T1/2-Ras-MRF4 ex-

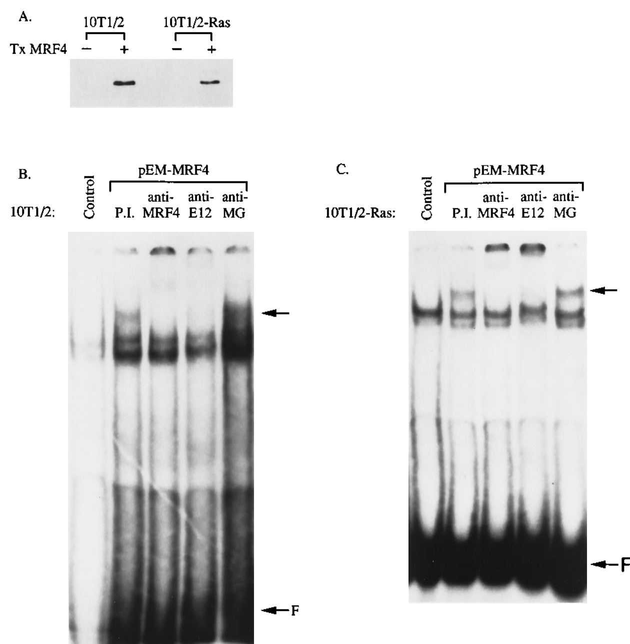

FIG. 2. The DNA binding activity of MRF4 is not inhibited in 10T1/2-Ras cells. (A) Nuclear extracts derived from 10T1/2 and 10T1/2-Ras cells transiently transfected (Tx) with MRF4 were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with an MRF4-specific antibody. (B and C) Nuclear extracts from the cells in panel A were incubated with a ^{32}P-labeled E-box probe. MRF4 preimmune serum (P.I.), MRF4 antiserum (anti-MRF4), E12 antiserum (anti-E12), or myogenin antiserum (anti-MG) was added, and the binding mixtures were separated by nondenaturing polyacrylamide gel electrophoresis. Untransfected control 10T1/2 and 10T1/2-Ras nuclear extracts also were tested. Arrows denote the specific protein-DNA band containing the MRF4-E12 protein complex. F marks the migration of the free probe.

tracts. Once again, formation of the MRF4-DNA complex is inhibited when an anti-MRF4 or anti-E12 antibody is included in the reaction mixtures, confirming that in 10T1/2-Ras cells, MRF4-E12 heterodimers form and efficiently bind to E-box DNA. Identical results are obtained when extracts prepared from 10T1/2 and 10T1/2-Ras cells that were transiently transfected with the pEM-MyoD construct are examined by EMSA (data not shown). These data suggest that Ras p21^{Val} does not inhibit the MRF proteins from dimerizing with E-protein partners or inhibit the MRF-E protein complexes from binding to their normal DNA target sequences in vitro.

Transcriptional activity of MRF4 in 10T1/2-Ras cells. Despite retaining the ability to heterodimerize with E proteins and to bind in a sequence-specific manner to DNA, MRFs remain nonfunctional in 10T1/2-Ras cells, implying that their role as transcriptional activators may be impaired by Ras p21^{Val}. To investigate this possibility, 10T1/2 and 10T1/2-Ras cells were cotransfected transiently with pEM-MRF4 and with the α-actin CAT or α-AchR-CAT reporter gene as described in Materials and Methods. For 10T1/2 cells, both muscle-specific reporter genes are transcriptionally activated when pEM-MRF4 is included (Fig. 3). In contrast, the α-actin CAT and α-AchR-CAT genes are only minimally activated in 10T1/2-Ras cells cotransfected with MRF4. The inability of muscle-specific reporter genes to be expressed in 10T1/2-Ras cells cannot be attributed to a general repression of transcription by Ras p21^{Val}, since β-actin CAT is activated to equivalent levels in both 10T1/2 and 10T1/2-Ras cells (Fig. 3). The mechanism of inhibition of MRF4 activity by Ras p21^{Val} also does not

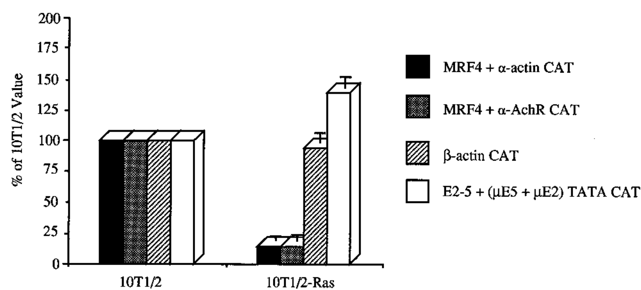

FIG. 3. Ras p21^{Val} inhibits MRF4 from *trans* activating expression of the α-actin CAT and α-AchR-CAT genes. 10T1/2 and 10T1/2-Ras cells were co-transfected with pEM-MRF4 or the control plasmid pEMSV and the α-actin CAT or α-AchR-CAT reporter gene. The fold increase in CAT activity obtained with pEM-MRF4 over pEMSV was established and set to 100% in all 10T1/2 groups. 10T1/2-Ras cells do not support the MRF4-induced expression of the muscle-specific genes, while both cell types express high levels of β-actin CAT and (μE5+μE2)TATACAT (see text for details). Error bars reflect the standard error of the mean.

involve specific inhibition of E-protein function, since the bHLH transcription factor E2-5, which binds as a homodimer to μE5 and μE2 E-box DNA, activates a (μE5+μE2)TATA-CAT reporter gene to very high levels in both control and Ras p21^{Val}-expressing 10T1/2 cells (Fig. 3).

The 10T1/2-Ras cells used in these experiments represent an established cell line that was selected for its Ras-transformed phenotype. To address the possibility that the inability of the MRFs to activate muscle-specific gene expression in 10T1/2-Ras cells is caused by secondary events associated with the long-term expression of Ras p21^{Val} in these cells, we examined the activity of MRF4 in a second, independently isolated 10T1/2-Ras cell line (61) and in 10T1/2-Ras cells expressing the K-*rev*-1 cDNA. K-*rev*-1, also known as *rap*1A and *smg* p21A (26), encodes a low-molecular-weight G protein with a high degree of amino acid similarity to Ras p21, especially across the effector domain. In many cell types, including 10T1/2 (17a), overexpression of K-*rev*-1 reverses the transformed phenotype initiated by Ras p21^{Val}. As shown in Fig. 4, MRF4 is incapable of efficiently activating expression of the myosin heavy-chain and α-acetylcholine receptor genes in this 10T1/2-Ras cell line. However, 10T1/2-Ras-K-rev cells respond to overexpression of MRF4 by activating the myogenic program and restoring expression of the α-acetylcholine receptor and myosin heavy-

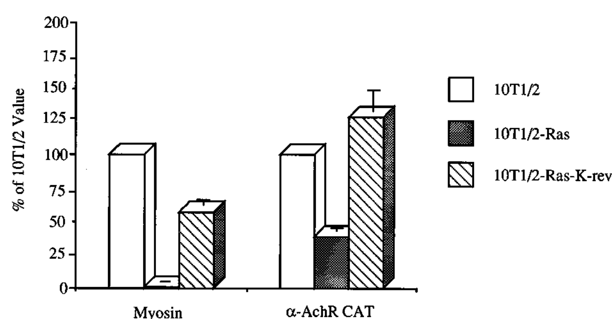

FIG. 4. K-*rev*-1 reverses Ras p21^{Val}-mediated inhibition of myogenesis. 10T1/2, 10T1/2-Ras, and 10T1/2-Ras-K-rev cells were cotransfected with pEM-MRF4 and the α-AchR-CAT reporter gene. Cells were harvested for CAT activity or stained with an antimyosin antibody to determine the number of differentiated cells. All values are reported relative to the level of differentiation obtained with the 10T1/2 cell groups, which was set to 100%. Error bars reflect the standard error of the mean.

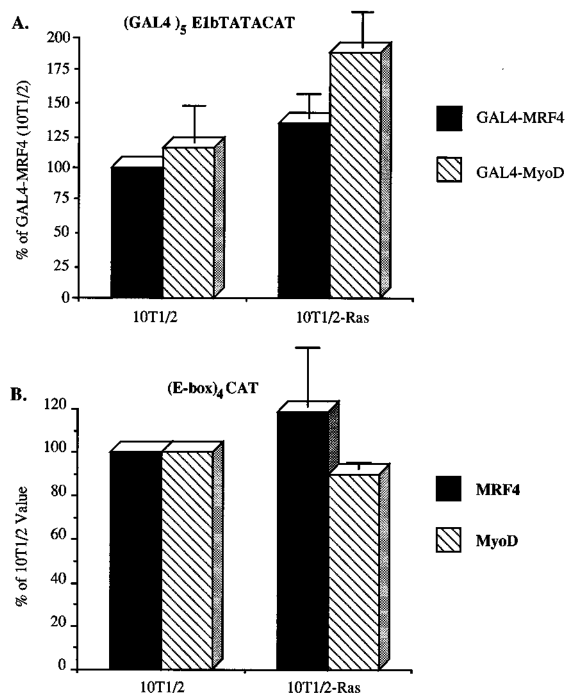

FIG. 5. The transcription activation domains of MRF4 and MyoD are not inhibited in 10T1/2-Ras cells. (A) GAL4-MRF4(1-60) and GAL4-MyoD(1-66) containing the transcription activation domains of MRF4 and MyoD, respectively, were cotransfected into 10T1/2 and 10T1/2-Ras cells with the $(GAL4)_5$-E1bTATA-CAT reporter gene. The transcription activation domains of MRF4 and MyoD function equivalently in both cell types. (B) 10T1/2 and 10T1/2-Ras cells were cotransfected with pEM-MRF4 or pEM-MyoD and $(E\text{-box})_4$CAT. Both MRF4 and MyoD activate transcription of the E-box reporter gene in 10T1/2 and in 10T1/2-Ras cells. Error bars reflect the standard error of the mean.

chain genes. This result suggests that the inability of MRF4 to activate muscle-specific gene expression in the 10T1/2-Ras cells is linked to a signaling pathway activated by Ras p21^{Val}, since the molecular events triggered by K-*rev*-1 revert the Ras-transformed phenotype of these cells and now allow MRF4 to induce muscle-specific gene expression.

The promoters and enhancers responsible for regulating expression of the contractile protein genes, such as those encoding α-actin and the α-acetylcholine receptor subunits, contain regulatory elements in addition to E boxes that are required for muscle-specific transcription (40, 48). Ras p21^{Val} may inhibit the expression of the α-actin and α-acetylcholine receptor genes by targeting the MRF transcription activation domains or by repressing the activities of other enhancer-binding proteins. In an attempt to analyze whether the transcription activation domains of MRF4 and MyoD are inhibited directly by Ras p21^{Val}, we used the GAL4 activator/reporter gene system initially derived from *Saccharomyces cerevisiae*. A CAT reporter gene containing GAL4 protein binding sites was introduced into 10T1/2 and 10T1/2-Ras cells along with a construct encoding the GAL4 protein DNA binding domain fused to the transcription activation domain of MRF4 or MyoD (36, 60). As shown in Fig. 5A, the transcription activation domains of MRF4 and MyoD efficiently activate the GAL4-CAT reporter gene to equal levels in both cell types, demonstrating that Ras p21^{Val} does not inhibit the overall function of the MRF transcription activation domains.

Given that the MRFs expressed in 10T1/2-Ras cells retain a functional transcription activation domain and yet do not activate muscle-specific expression, we decided to examine whether Ras p21^{Val} can inhibit an MRF-E protein complex from activating transcription of a reporter gene controlled by four copies of an E-box site and no other known regulatory elements. 10T1/2 and 10T1/2-Ras cells were cotransfected with MRF4 or MyoD and with the $(E\text{-box})_4$CAT reporter gene, and CAT activity was monitored after 2 days in differentiation medium. High levels of CAT expression from this reporter gene were obtained in both cell types (Fig. 5B), even though muscle differentiation (i.e., myosin heavy-chain expression and myotube formation) was repressed in the 10T1/2-Ras cultures (Table 1). Identical results were obtained when 10T1/2 cells were transiently cotransfected with MRF4 and Ras p21^{Val} (data not shown). In all cases, the effects observed were dependent on the presence of wild-type MRF4 or MyoD protein, since control cells expressing a nonfunctional MRF protein do not produce significant levels of CAT expression from the $(E\text{-box})_4$ reporter genes (data not shown). Thus, in this in vivo context, the MRFs bind to muscle-specific E-box elements and efficiently activate expression of a reporter gene whose expression is controlled exclusively by MRF binding sites.

The Ras p21^{Val} signaling pathway targets the MRF basic domain in inhibiting myogenesis. Analysis of the transcriptional activity of the MRFs in the context of a GAL4 fusion protein or with respect to activating an artificial $(E\text{-box})_4$CAT reporter gene does not address all aspects of muscle-specific *trans* activation by MRFs. Many studies have suggested that it is the conserved MRM within the basic domain of the MRF proteins that is crucial for the induction of myogenesis in cells (7, 13, 14). To test whether the Ras p21^{Val} inhibition of MRF activity is targeted at the MRM region, 10T1/2 and 10T1/2-Ras cells were transfected with constructs expressing various mutant MyoD proteins. As previously discussed, the full-length MyoD protein induces myogenesis in 10T1/2 cells but is inhibited from functioning in that capacity when expressed in 10T1/2-Ras cells. As predicted, a truncated MyoD protein (ΔN, ΔC-MyoD), containing only the bHLH region, is severely impaired in its ability to convert 10T1/2 cells to a muscle phenotype or to activate muscle-specific reporter genes (Fig. 6). Addition of the VP16 transcriptional activation domain (ΔN, ΔC-MyoD-VP16) to this truncated MyoD protein fully restores myogenic activity in 10T1/2 cells but not in 10T1/2-Ras cells, suggesting that the Ras p21^{Val} inhibition of MyoD in fact is targeted at the bHLH domain. To further examine the specific molecular region that is subject to Ras p21^{Val} inhibition, MyoD and MyoD-VP16 constructs containing a substituted E12 basic domain [MyoD-E12(B) and MyoD-E12(B)-VP16] were tested for activity and found to be nonfunctional in both cell types. As expected, the E12 protein also does not generate a myogenic phenotype. However, when the basic domain of MyoD is substituted for the basic domain in E12 [E12-MyoD(BJ)], the E protein becomes myogenic in 10T1/2 cells and, like MyoD, remains nonfunctional in 10T1/2-Ras cells [compare E12 and E12-MyoD(BJ) in Fig. 6]. These experiments demonstrate that the Ras signaling pathway likely targets the MRM regions of the MRFs, although the inhibition does not involve overt changes in dimerization or in the DNA binding properties which are specified by these regions of the proteins (Fig. 2).

Ras p21^{Val} inhibits myogenesis through regulatory pathways that are distinct from the FGF-2 and TGF-β1 pathways. We have demonstrated that Ras p21^{Val} inhibits MRF-dependent myogenesis but does not alter the DNA binding or the inherent transcriptional activities of the muscle regulatory factors. Several other agents, most notably FGF-2 and TGF-β1, also inhibit MRF-dependent myogenesis through poorly characterized signaling pathways (8, 22, 32, 38, 50, 63). To examine

	Myosin		TnI CAT	
	10T1/2	Ras	10T1/2	Ras
MyoD	100	4	100	8
ΔN, ΔC-MyoD	7	0	47	3
ΔN, ΔC-MyoD-VP16	109	5	152	9
MyoD-E12(B)	0	0	6	3
MyoD-E12(B)-VP16	0	0	14	3
E12	0	0	nd	nd
E12-MyoD(BJ)	14	0	nd	nd

FIG. 6. Ras p21^{Val} inhibition of myogenesis is targeted at the basic domain of the MRFs. 10T1/2 and 10T1/2-Ras cells were cotransfected with the indicated MyoD or E12 expression construct and a TnICAT reporter gene. Following 48 h in 2% HS, cells were harvested for CAT activity or stained for myosin expression (see Materials and Methods for details). The number of nuclei present in myosin-positive cells was determined for 10 fields per dish per experiment. The values reported represent the number of myosin-positive cells observed relative to the number detected in the MyoD-transfected 10T1/2 cell groups, which was set to 100%. Similarly, all TnICAT expression values were normalized to the value obtained in the MyoD-transfected 10T1/2 cell group, which was set to 100%. E12-MyoD(BJ), which expresses an E12 with a substituted MyoD basic domain, is myogenic in 10T1/2 cells but not in 10T1/2-Ras cells. nd, not determined.

whether Ras p21^{Val} and growth factors inhibit terminal differentiation events by similar mechanisms, nuclear extracts prepared from 10T1/2 and 10T1/2-Ras cells transfected with MRF4 and maintained in either 2% HS (differentiation medium), 2% HS supplemented with FGF-2, or 2% HS supplemented with TGF-β1 were normalized for MRF4 content and used for EMSA with a ^{32}P-labeled E-box probe. As shown in Fig. 7, extracts from 10T1/2 cells expressing MRF4 and maintained in differentiation medium contain MRF4-E protein complexes that bind to E-box DNA. Interestingly, identical binding complexes were detected in the extracts prepared from cells maintained in FGF-2 and TGF-β1, demonstrating that inhibition of MRF4 activity by FGF-2 and TGF-β1 does not involve inhibiting protein heterodimerization or protein-DNA interactions (Fig. 7). Similarly, 10T1/2-Ras cells contain an active MRF4-E protein binding complex, regardless of whether the 10T1/2-Ras cells were maintained in differentiation medium (2% HS) or in medium supplemented with FGF-2 or TGF-β1 (Fig. 7). Comparable results also were obtained for nuclear extracts prepared from 10T1/2 and 10T1/2-Ras cells transiently expressing MyoD and maintained in 2% HS in the presence or absence of FGF-2 and TGF-β1 (data not shown). Thus, transfected 10T1/2-Ras cells and wild-type 10T1/2 cells maintained in FGF-2 or in TGF-β1 produce MRF proteins that exhibit the appropriate DNA binding and heterodimerization activities, even though the proteins are repressed from inducing a myogenic phenotype.

FGF-2, TGF-β1, and Ras p21^{Val} inhibit myogenesis through mechanisms that are independent of MRF DNA binding activity, since MRF-E protein complexes form and bind to DNA under each of these conditions. To examine how specific growth factors function in 10T1/2 and 10T1/2-Ras cells to inhibit the transcriptional activity of MRF4, both cell types were transfected with pEM-MRF4 and the (E-box)$_4$CAT reporter gene in the presence or absence of FGF-2 or TGF-β1. In 10T1/2 cells, FGF-2 decreases the E-box-mediated transcriptional activity of MRF4 by approximately 50%, while high levels of (E-box)$_4$CAT expression are maintained in 10T1/2-

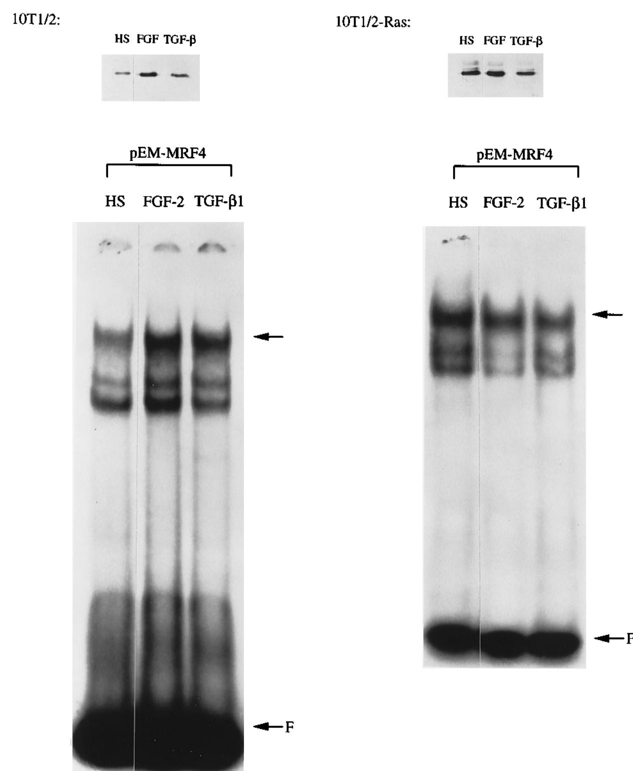

FIG. 7. Western analysis and EMSA using extracts from 10T1/2 and 10T1/2-Ras cells transfected with pEM-MRF4 and maintained in differentiation medium (HS) or in differentiation medium supplemented with FGF-2 or TGF-β1. Equivalent amounts of MRF4-containing nuclear extracts (as determined from the Western analysis) were incubated with ^{32}P-labeled E-box probes, and the protein-DNA complexes were separated by nondenaturing polyacrylamide gel electrophoresis. MRF4-containing complexes are indicated by arrows. F denotes the migration of the free probe.

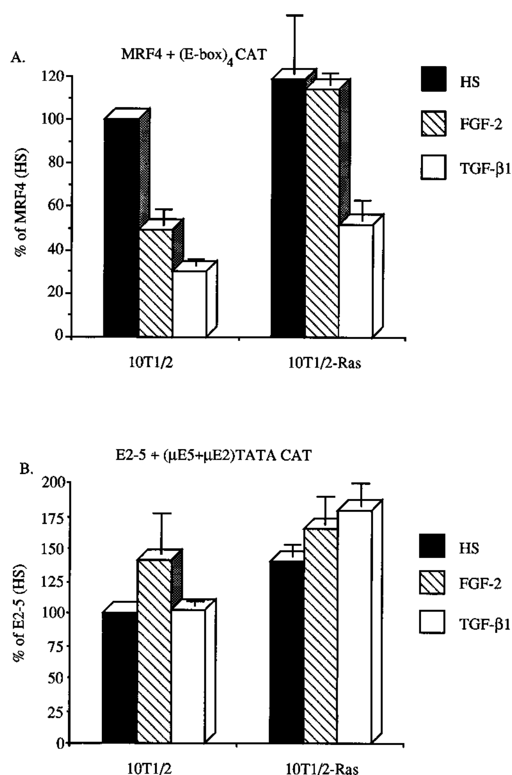

FIG. 8. Transcriptional activity of MRF4 and E2-5 in 10T1/2 and 10T1/2-Ras cells maintained in FGF-2 and in TGF-β1. 10T1/2 and 10T1/2-Ras cells were transfected with MRF4 and (E-box)$_4$CAT or with E2-5 and (μE5+μE2)TATA CAT as described for Fig. 3 and 5. FGF-2 inhibits (E-box)$_4$CAT expression in 10T1/2 cells but not in 10T1/2-Ras cells. TGF-β1 inhibits (E-box)$_4$CAT expression in both cell types. Ras p21^{Val}, FGF-2, and TGF-β1 do not inhibit E2-5 activity. Error bars reflect the standard error of the mean.

Ras cells maintained in the presence or absence of FGF-2 (Fig. 8A). In contrast, exposure of 10T1/2 and 10T1/2-Ras cells to TGF-β1 produces a similar decrease in the E-box-dependent CAT expression in both cell types (Fig. 8A). As a control, the effects of Ras p21^{Val}, FGF-2, and TGF-β1 on the activity of the ubiquitous bHLH protein E2-5 also were examined, and similar levels of (μE5+μE2)TATACAT gene expression were obtained in both cell types regardless of whether the cells were maintained in 2% HS, TGF-β1, or FGF-2 (Fig. 8B). Our results suggest that Ras p21^{Val} abrogates the signal transduction pathway utilized by FGF-2 in 10T1/2 cells and that FGF-2, TGF-β1, and Ras p21^{Val} do not operate through identical pathways to inhibit MRF-induced myogenesis.

DISCUSSION

The myogenic properties of myoblasts expressing the oncogenic form of Ras p21 (Ras p21^{Val}) are similar to the properties of myoblasts that are maintained in the presence of serum growth factors; i.e., the cells remain mononuclear and do not express muscle-specific genes such as the α-actin and myosin genes (27, 30). This observation implies that Ras p21^{Val} and growth factor signaling pathways may converge at a single point to inhibit terminal differentiation events. However, unlike growth factor pathways which block myogenesis by inhibiting the DNA binding (8) and/or transcriptional activities of the muscle regulatory factors (8, 22, 63), MRF proteins in Ras p21^{Val} cells correctly enter the nucleus, heterodimerize efficiently with partner E proteins, bind to E-box elements in DNA, and transcriptionally activate reporter genes controlled by multiple E-box sites.

The molecular target of Ras p21^{Val}-mediated inhibition of MRF activity appears to be the basic domain. Both ΔN, ΔC-MyoD-VP16, which expresses a fusion protein containing the bHLH domain of MyoD and the VP16 activation domain, and E12-MyoD(BJ), which expresses an E12 protein modified with the MyoD basic domain, are inactive in the 10T1/2-Ras cells. The basic domain of all four MRFs contains a conserved region, MRM (7, 13, 14), which maps to the alanine and threonine at amino acid positions 114 and 115, respectively, of MyoD. Interestingly, the MRM is absent from all nonmyogenic bHLH proteins (7), and if MRM motifs are introduced into other bHLH proteins by site-directed mutagenesis, the chimeric proteins now exhibit myogenic activity (14). This is convincing evidence that the MRM of the MRFs imparts muscle specificity on the bHLH factors. The basic region of the MRFs also serves as the contact point for the major groove of DNA (35), suggesting that the MRM functions in DNA recognition. Although Ras p21^{Val} appears to target the basic domain of the MRFs, it is surprising that the DNA binding and the heterodimerization of the MRFs is unaffected. How Ras p21^{Val} inhibits a muscle phenotype is unknown, but one possibility is that the Ras signaling pathway disrupts additional protein-protein interactions involving the MRF proteins. For example, the MRM has been shown to serve as a protein interface for other transcription factors, most notably the myocyte enhancer factor MEF2 (24). Although MEF2 efficiently activates a (MEF2)$_2$CAT reporter gene in 10T1/2-Ras cells (unpublished data), it remains formally possible that MRF-MEF2 interactions are altered in Ras cells so that MyoD and MEF2 remain competent to activate a simple multimerized E-box and multimerized MEF2 reporter but not a complex muscle promoter containing both E-box and MEF2 sites. Studies addressing this model are under way.

Understanding how serum growth factors control skeletal muscle events by regulating the MRF function also has been the subject of recent investigations (reviewed in references 43 and 44). In the present study, we have confirmed that myogenic differentiation is repressed by FGF-2 and TGF-β1 through mechanisms that target the transcriptional activity of the MRF proteins independently of their ability to heterodimerize with E proteins and to bind to DNA. The mechanism by which the transcriptional activity of the MRFs is inhibited in FGF-2- and TGF-β1-treated cells remains unknown but may involve dimerization with partners that lack activation domains or may be a consequence of a posttranslational modification. Indeed, Li et al. (32) have shown that FGF-2 induces the phosphorylation of a conserved threonine residue in myogenin which affects the ability of myogenin to bind to DNA. In the case of MRF4 and MyoD, however, FGF-2 treatment does not lead to detectable changes in the phosphorylation status of the proteins (reference 22 and unpublished observations) and does not prevent these MRFs from dimerizing with E proteins and binding to DNA (Fig. 7).

The mechanism by which Ras p21^{Val}, FGF-2, and TGF-β1 inhibit myogenesis is distinct from the mechanism by which FBS blocks myogenesis. In serum, DNA binding by the MRFs is inhibited (8), presumably because serum-treated cells express elevated levels of Id (3), a helix-loop-helix protein that lacks a basic domain. The protein Id dimerizes efficiently with E proteins and results in an overall decrease in the amount of E-protein partners available for binding to the MRFs. For cells expressing Ras p21^{Val}, or for cells treated with FGF-2 or TGF-β1, Id mRNA is detected at a constant, low level (unpublished

results), which correlates well with the sustained ability of the MRFs to bind to DNA under these conditions. Thus, a mechanism other than Id-mediated inhibition of myogenesis must be evoked to explain the inhibition of MRF activity by Ras $p21^{Val}$, FGF-2, and TGF-β1.

An interesting and somewhat unexpected finding of this study is that 10T1/2-Ras cells do not respond to FGF-2 signaling in the same manner as 10T1/2 cells with respect to inhibiting MRF activities. In 10T1/2-Ras cells treated with FGF-2, the MRFs continue to bind to specific DNA elements and transcriptionally activate E-box reporter genes, even though FGF-2 inhibits MRF transcriptional activity in normal 10T1/2 cells. This finding suggests that Ras $p21^{Val}$ antagonizes the action of FGF-2 by interfering with one specific component of the FGF-2 signal pathway, possibly the FGF receptor complement on cells. Alternatively, Ras $p21^{Val}$ and FGF-2 may function through similar intracellular pathways but exert different effects on specific components of the respective pathway. One candidate pathway is the MAPK cascade, which is known to function as a signal transduction pathway in cells exposed to FGF-2 or to Ras $p21^{Val}$ (41). Interestingly, for the MM14 muscle cell line, FGF-2 signaling stimulates MAPK kinase activity but not MAPK (9). The block in the MAPK pathway appears to be the result of a specific MAPK phosphatase activity which is present in MM14 cell extracts. Thus, it is possible that Ras cells can overcome the FGF-2-induced block in the MAPK cascade and generate a profile of MRF activities that is characteristic of Ras $p21^{Val}$-expressing cells.

Previous studies from several laboratories have demonstrated that constitutive expression of a stably transfected MyoD cDNA partially restores the ability of myoblasts expressing Ras $p21^{Val}$ to differentiate (27, 30). The 10T1/2-Ras cell lines used in this study also are capable of partially differentiating into muscle cells when stably transfected with pEM-MRF4 or pEM-MyoD (unpublished data). In transient assays, however, MyoD and MRF4 proteins fail to initiate myogenesis. A block in differentiation also is observed following cotransfection of 10T1/2 cells with MyoD and Ras $p21^{Val}$ expression constructs (unpublished results), indicating that long-term expression of Ras $p21^{Val}$ is not a prerequisite for this effect. The failure of MyoD and MRF4 to induce a myogenic phenotype in Ras cells may reflect instability in the RNA and/or protein levels of muscle-specific genes or of the MRFs themselves. However, examination of MRF protein levels in these studies revealed that transiently transfected 10T1/2 cells express a much higher level of MRF protein than is generated in a stable transfection (unpublished results), indicating that loss of myogenic activity in Ras cells is not due to a reduced level of MRF expression. It remains formally possible that MRF proteins need to be expressed for longer periods of time in 10T1/2-Ras cells in order to override the Ras-induced inhibition. Extended expression periods may be necessary for the MRFs to activate downstream genes, such as those encoding specific kinases and/or phosphatases, that function to antagonize the Ras signaling pathway. Recent studies (42, 55) have indicated that expression of the MRFs may increase intracellular phosphatase activities that are critical for normal muscle differentiation, since inhibition of phosphatase activity inhibits myogenesis (25, 47). Whether MRF4 or MyoD plays a direct role in the regulation of phosphatase activity during myogenesis has yet to be examined.

A hallmark of skeletal muscle differentiation is the requirement for myoblasts to withdraw from the cell cycle and arrest in the G_1 phase. Several pieces of evidence suggest that the MRFs play a dual role both in the inhibition of cell cycle progression and in the establishment of the myogenic phenotype (11, 20, 54, 56). Halevy et al. (21) have proposed that one mechanism by which MyoD may promote cell cycle exit is by inducing the expression of $p21^{CIP1}$. $p21^{CIP1}$ serves as a cellular inhibitor of the cyclin-dependent kinases (cdks), enzymes implicated in the phosphorylation and inactivation of the G_1 repressor Rb (37, 59). From this finding, one can postulate that Ras $p21^{Val}$ cells may be refractile to myogenic differentiation as a result of the lack of a functional $p21^{CIP1}$. However, when $p21^{CIP1}$, alone or in conjunction with MyoD, is overexpressed in 10T1/2-Ras cells, the cultures fail to exhibit a myogenic phenotype, suggesting that Ras $p21^{Val}$ inhibits differentiation through pathways independent of $p21^{CIP1}$ expression (unpublished results). Alternatively, myogenic inhibition by Ras $p21^{Val}$ may involve a change in the phosphorylation status of the MRFs within the context of the cell cycle. In support of this idea, overexpression of cyclin D1, a component of the G_1 cdk complex, has been shown to induce hyperphosphorylation of MyoD and inhibit myogenesis (49, 52). Therefore, it is possible that some aspect of the Ras signaling pathway is involved in the activation of the cyclin D1-cdk complex which promotes the phosphorylation of the MRFs. While studies from our laboratory indicate that this phosphorylation event is not likely to involve the threonine residue located within the MRF MRM domain, it may target other amino acids important for the proper interaction of MRFs with the transcriptional machinery. The goal of additional experiments will be to define each of the downstream control points associated with the Ras $p21^{Val}$ signaling pathway and to integrate those points into the pathways that specify cell cycle progression and that are triggered by the treatment of cells with FGF-2 or TGF-β1. Clearly, it is the synergy between the positive and negative effects of these cellular pathways that coordinates the molecular regulation of skeletal muscle differentiation.

ACKNOWLEDGMENTS

We are indebted to R. Benezra, R. Davis, T. Kadesch, L. Kedes, A. Lassar, H. Weintraub, and J. Merlie for supplying plasmids used in these studies and to C. Ashendel for generously allowing the use of the 10T1/2-Ras-K-rev-1 cell line prior to publication. In addition, we thank C. Philbrook for preparation of the manuscript.

This work was supported by grants to S.F.K. from the National Institutes of Health and from the American Heart Association and to E.J.T. from the National Science Foundation. S.E.J. was supported by an NIH training grant. E.J.T. is a Scholar of the Leukemia Society of America, and S.F.K. is an Established Investigator of the American Heart Association.

REFERENCES

1. **Alema, S., and F. Tato.** 1994. Oncogenes and muscle differentiation: multiple mechanisms of interference. Cancer Biol. **5:**147–156.
2. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell. Biol. **95:**763–770.
3. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61:**49–59.
4. **Bengal, E. L., L. Ransone, R. Scharfmann, V. J. Dwarki, S. Tapscott, H. Weintraub, and I. M. Verma.** 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. Cell **68:**507–519.
5. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9:**821–831.
6. **Braun, T., E. Buschausen-Denker, E. Tannich, and H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8:**701–717.
7. **Brennen, T. J., T. Chakraborty, and E. N. Olson.** 1991. Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. Proc. Natl. Acad. Sci. USA **88:**5675–5679.
8. **Brennan, T. J., D. G. Edmondson, L. Li, and E. N. Olson.** 1991. Transforming growth factor β represses the action of myogenin through a mechanism independent of DNA binding. Proc. Natl. Acad. Sci. USA **88:**3822–3826.

9. **Campbell, J. S., M. P. Wenderoth, S. D. Hauschka, and E. G. Krebs.** 1995. Differential activation of mitogen-activated protein kinase in response to basic fibroblast growth factor in skeletal muscle cells. Proc. Natl. Acad. Sci. USA **92:**870–874.
10. **Caruso, M., F. Martelli, A. Giordano, and A. Felsani.** 1993. Regulation of MyoD gene transcription and protein function by the transforming domains of adenovirus E1A oncoprotein. Oncogene **8:**267–278.
11. **Crescenzi, M., T. P. Fleming, A. B. Lassar, H. Weintraub and S. A. Aaronson.** 1990. MyoD induces growth arrest independent of differentiation in normal and transformed cells. Proc. Natl. Acad. Sci. USA **87:**8442–8446.
12. **Davis, R., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51:**987–1000.
13. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60:**733–746.
14. **Davis, R. L., and H. Weintraub.** 1992. Acquisition of myogenic specificity by placement of three amino acid residues from MyoD into E12. Science **256:** 1027–1030.
15. **Edmondson, D., and E. 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.
16. **Edmondson, D. G., and E. N. Olson.** 1993. Helix-loop-helix proteins as regulators of muscle-specific transcription. J. Biol. Chem. **268:**755–758.
17. **Enkemann, S. A., S. F. Konieczny, and E. J. Taparowsky.** 1990. Adenovirus 5 E1A represses muscle-specific enhancers and inhibits expression of the myogenic regulatory factor genes, MyoD and myogenin. Cell Growth Differ. **1:**375–382.

17a. **Fernandez, E., and C. Ashendel.** Unpublished results.

18. **Fregien, N., and N. Davidson.** 1986. Activating elements in the promoter region of the chicken β-actin gene. Gene **48:**1–11.
19. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
20. **Gu, W., J. W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard.** 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell **72:**309–324.
21. **Halevy, O., B. G. Novitch, D. B. Spicer, S. X. Skapek, J. Rhee, G. J. Hannon, D. Beach, and A. B. Lassar.** 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science **267:**1018–1021.
22. **Hardy, S., Y. Kong, and S. F. Konieczny.** 1993. Fibroblast growth factor inhibits MRF4 activity independently of the phosphorylation status of a conserved threonine residue within the DNA-binding domain. Mol. Cell. Biol. **13:**5943–5956.
23. **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.
24. **Kaushal, S., J. W. Schneider, B. Nadal-Ginard, and V. Mahdavi.** 1994. Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. Science **266:**1236–1240.
25. **Kim, S.-J., K. Y. Kim, S. J. Tapscott, T. S. Winokur, K. Park, H. Fujiki, H. Weintraub, and A. B. Roberts.** 1992. Inhibition of protein phosphatases blocks myogenesis by first altering MyoD binding activity. J. Biol. Chem. **267:**15140–15145.
26. **Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda.** 1989. A ras-related gene with transforming suppressor activity. Cell **56:**77–84.
27. **Konieczny, S. F., B. L. Drobes, S. L. Menke, and E. J. Taparowsky.** 1989. Inhibition of myogenic differentiation by the H-ras oncogene is associated with the down regulation of the MyoD1 gene. Oncogene **4:**473–481.
28. **Lassar, A., and A. Münsterberg.** 1994. Wiring diagrams: regulatory circuits and the control of skeletal myogenesis. Curr. Biol. **6:**432–442.
29. **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.
30. **Lassar, A. B., M. T. Thayer, R. W. Overell, and H. Weintraub.** 1989. Transformation by activated ras or fos prevents myogenesis by inhibiting expression of MyoD. Cell **58:**659–667.
31. **Li, L., J. C. Chambard, M. Karin, and E. Olson.** 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. Genes Dev. **6:**676–689.
32. **Li, L., J. Zhou, G. James, R. Heller-Harrison, M. P. Czech, and E. N. Olson.** 1992. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell **71:**1181–1194.
33. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) **338:**39–44.
34. **Lin, H., K. E. Yutzey, and S. F. Konieczny.** 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol. Cell. Biol. **11:** 267–280.
35. **Ma, P. C. M., M. A. Rould, H. Weintraub, and C. O. Pabo.** 1994. Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcription. Cell **77:**451–459.
36. **Mak, K., R. To, Y. Kong, and S. F. Konieczny.** 1992. The MRF4 activation domain is required to induce muscle-specific gene expression. Mol. Cell. Biol. **12:**4334–4346.
37. **Marshall, C. J.** 1991. Tumor suppressor genes. Cell **64:**313–326.
38. **Martin, J. F., L. Li, and E. N. Olson.** 1992. Repression of myogenin function by TGF-β1 is targeted at the basic helix-loop-helix motif and is independent of E2A products. J. Biol. Chem. **267:**10956–10960.
39. **Miner, J. H., and B. J. Wold.** 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA **87:**1089–1093.
40. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-actin gene. Mol. Cell. Biol. **7:**2803–2813.
41. **Moodie, S. A., B. M. Willumsen, M. J. Weber, and A. Wolfman.** 1993. Complexes of Ras-GTP with raf-1 and mitogen-activated protein kinase. Science **260:**1658–1661.
42. **Ohishi, S., E. Masahiko, T. Kobayashi, T. Terasawa, T. Murakami, M. Onoda, M. Ohnishi, T. Itoh, S. Tsuiki, and S. Tamura.** 1992. Enhanced expression of type 2C protein phosphatase gene during myogenic differentiation of C3H10T1/2 cells. Biochem. Int. **28:**345–351.
43. **Olson, E. N.** 1992. Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. **154:**261–272.
44. **Olson, E. N., T. J. Brennan, T. Chakraborty, T.-C. Cheng, P. Cserjesi, D. Edmondson, G. James, and L. Li.** 1991. Molecular control of myogenesis antagonism between growth and differentiation. Mol. Cell. Biochem. **104:**7–13.
45. **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.
46. **Olson, E. N., G. Spizz, and M. A. Tainsky.** 1987. The oncogenic forms of N-ras and H-ras prevent skeletal myoblast differentiation. Mol. Cell. Biol. **7:**2104–2111.
47. **Park, K., M. Chung, and S. Kim.** 1992. Inhibition of myogenesis by okadiac acid, an inhibitor of protein phosphatase, 1 and 2A, correlates with the induction of AP1. J. Biol. Chem. **267:**10810–10815.
48. **Prody, C. A., and J. P. Merlie.** 1991. A developmental and tissue-specific enhancer in the mouse skeletal muscle acetylcholine receptor α-subunit gene regulated by myogenic factors. J. Biol. Chem. **266:**22588–22596.
49. **Rao, S. S., C. Chu, and D. S. Kohtz.** 1994. Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. Mol. Cell. Biol. **14:**5259–5267.
50. **Rhodes, S., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of muscle regulatory factor gene family. Genes Dev. **3:**1089–1093.
51. **Ruezinsky, D., H. Beckmann, and T. Kadesch.** 1991. Modulation of the IgH enhancer's cell type specificity through a genetic switch. Genes Dev. **5:**29–37.
52. **Skapek, S. X., J. Rhee, D. B. Spicer, and A. B. Lassar.** 1995. Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science **267:**1022–1024.
53. **Smith, T. H., N. E. Block, S. J. Rhodes, S. F. Konieczny, and J. B. Miller.** 1993. A unique pattern of expression of the four muscle regulatory factor proteins distinguishes somitic from embryonic, fetal and newborn mouse myogenic cells. Development **117:**1125–1133.
54. **Sorrentino, V., R. Pepperkok, R. L. Davis, W. Ansorge, and L. Philipson.** 1990. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. Nature (London) **345:**813–815.
55. **Srinivasan, M., and N. Begum.** 1994. Regulation of protein phosphatase 1 and 2A activities by insulin during myogenesis in rat skeletal muscle cells in culture. J. Biol. Chem. **269:**12514–12520.
56. **Tapscott, S. J., M. J. Thayer, and H. Weintraub.** 1993. Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. Science **259:**1450–1453.
57. **Teegarden, D., E. J. Taparowsky, and C. Kent.** 1990. Altered phosphatidylcholine metabolism in C3H10T1/2 cells transfected with the Harvey-*ras* oncogene. J. Biol. Chem. **265:**6042–6047.
58. **Vaidya, T. B., S. J. Rhodes, E. J. Taparowsky, and S. F. Konieczny.** 1989. Fibroblast growth factor and transforming growth factor β represses transcription of the myogenic regulatory gene MyoD1. Mol. Cell. Biol. **9:**3576–3579.
59. **Weinberg, R. A.** 1991. Tumor suppressor genes. Science **254:**1138–1146.
60. **Weintraub, H., V. J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S. J. Tapscott.** 1991. Muscle-specific transcriptional activation by MyoD. Genes Dev. **5:**1377–1386.
61. **Weyman, C. M., E. J. Taparowsky, M. Wolfson, and C. L. Ashendel.** 1988. Partial down-regulation of protein kinase C in C3H10T1/2 mouse fibroblasts transfected with the human Ha-*ras* oncogene. Cancer Res. **48:**6535–6541.
62. **Wright, W., D. Sassoon, and V. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD1. Cell **56:**607–617.
63. **Yutzey, K. E., S. J. Rhodes, and S. F. Konieczny.** 1990. Differential *trans*-activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. Mol. Cell. Biol. **10:**3934–3944.