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MyoD induces growth arrest independent of differentiation in normal and transformed cells

(skeletal muscle/myoblasts/oncogenes/helix–loop–helix)

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ABSTRACT ***MyoD* is a gene involved in the control of muscle differentiation. We show that *MyoD* causes growth arrest when expressed in cell lines derived from tumors or transformed by different oncogenes. MyoD-induced growth inhibition was demonstrated by reduction in the efficiency of colony formation and at the single-cell level. We further show that MyoD growth inhibition can occur in cells that are not induced to activate muscle differentiation markers. The inhibitory activity of MyoD was mapped to the same 68-amino acid segment necessary and sufficient for induction of muscle differentiation, the basic–helix–loop–helix motif. Mutants with alterations in the basic region of MyoD that fail to bind or do not activate a muscle-specific enhancer inhibited growth; mutants with deletions in the helix–loop–helix region failed to inhibit growth. Thus, inhibition of cell growth by MyoD seems to occur by means of a parallel pathway to the one that leads to myogenesis. We conclude that *MyoD* is a prototypic gene capable of functionally activating intracellular growth inhibitory pathways.**

Our understanding of mechanisms promoting normal cell proliferation has increased in recent years. Much less is known about intracellular pathways that negatively regulate cell proliferation. Cell differentiation is, at least in some systems, a natural antagonist of cell proliferation. It has also been proposed as the mechanism by which normal cells, when fused to neoplastic cells, suppress tumorigenicity of the latter (1). However, what causes exit from the cell cycle during "terminal" differentiation of some cell types remains to be elucidated.

A classical example of terminal differentiation is that of skeletal muscle cells. Genes capable of inducing muscle differentiation have recently been identified. The regulatory gene *MyoD* (2) has been shown to induce a number of different cell types to differentiate into mature skeletal muscle cells (2, 3). MyoD is a phosphorylated (4) transcription factor able to bind specifically to a sequence found in the promoters of several muscle-specific genes, including muscle creatine kinase (5). MyoD has been shown *in vitro* to form homodimers and heterodimers with other transcription factors (6, 7) and with the putative negative regulatory protein Id (8). Two small regions of the molecule are sufficient to mediate most of the known properties of MyoD (4, 7). These are a basic region and the adjacent helix–loop–helix (HLH) motif (9); the latter has been found in a number of proteins involved in transcriptional regulation and cell differentiation (8). In addition, *MyoD* has been reported to inhibit the colony-forming efficiency (CFE) of a target fibroblast cell line in which the same gene induced muscle differentiation (2).

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MyoD may have either a direct or an indirect influence on the irreversible withdrawal from the cell cycle—namely, cell cycle withdrawal might be a consequence of the myogenic program activated by *MyoD*; alternatively, *MyoD* might induce withdrawal by activating an independent pathway. Because of our interest in studying mechanisms negatively regulating the cell cycle and aberrations in such pathways that may characterize cancer cells, we sought to investigate the antiproliferative activity of *MyoD*.

MATERIALS AND METHODS

Cells and Tissue Culture Media. All cell lines were from the American Type Culture Collection. H-*ras*-, *src*-, and *fos*-transformed cell lines were established in our laboratory. Growth medium (GM) was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Starvation medium (SM) was DMEM supplemented with insulin at 10 μg/ml, transferrin at 5 μg/ml, and 10 nM Na_2SeO_3.

Molecular Constructs and Transfections. The plasmid pSV2neo (10) was used to provide G418 resistance. The *MyoD* expression vector pEMC11s was described (2) as pEMSVscribe-MyoD. A modified form of this plasmid, pMM4, contains only the *MyoD* open reading frame. MyoD mutants have been described (4, 7). The epidermal growth factor receptor (EGFR) expression vector LTR-2/EGFR has been described (11). The corresponding expression vector LTR-2 (12) was used for CFE comparisons. Transfections were performed by the calcium phosphate precipitation technique (13).

Immunodetection. The following antisera and monoclonal antibodies (mAbs) were employed: MyoD, rabbit polyclonal antiserum (4); 5-bromo-2′-deoxyuridine (BrdU), mAb/DNase mixture provided in the cell proliferation kit (Amersham); myosin heavy chain (MHC), mAb MF-20 (14); desmin, anti-desmin mAb (Amersham); EGFR, mAb Ab-2 (Oncogene Science, Manhasset, NY). mAbs were detected by an affinity-purified, fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories). Anti-MyoD immunoreactivity was detected by an affinity-purified, rhodamine-conjugated, goat anti-rabbit IgG (Cappel Laboratories). In some experiments involving two mAbs, the anti-BrdU mAb was detected by a peroxidase-conjugated anti-mouse IgG antiserum supplied in the cell proliferation kit (Amersham).

Abbreviations: CFE, colony-forming efficiency; GM, growth medium; SM, starvation medium; EGFR, epidermal growth factor receptor; BrdU, 5-bromo-2′-deoxyuridine; mAb, monoclonal antibody; MHC, myosin heavy chain; HLH, helix–loop–helix.

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Transient Expression Assay and Viability Tests. Cells were transfected in GM by using 10 μg of the appropriate expression vector and 30 μg of calf thymus DNA as carrier. For [^{3}H]thymidine incorporation, cells were incubated in GM added with [*methyl*-^{3}H]thymidine (3 TBq/mmol; NEN) at a concentration of 5 μCi/ml (1.85 × 10^5 Bq/ml). After appropriate incubation, cells were fixed, stained with anti-MyoD antiserum, washed with water, and overlaid with autoradiographic emulsion (NTB2, Kodak). Autoradiographs were developed 1–3 days later. For BrdU incorporation, cells were incubated in GM containing 10 μM BrdU. Phagocytosis was measured by incubating cells in suspension with 5 ml of fluorescent latex beads (1.16-mm diameter; Polysciences) for 3 hr at 37°C in a total volume of 0.5 ml of GM. To control for nonspecific bead-to-cell adherence, identical incubations were carried out at 4°C. After the incubation, the cells were seeded in chamber slides (Nunc), allowed 4 hr to reattach, stained for BrdU and MyoD, and scored. A cell was scored positive when ≥3 beads were present in its cytoplasm. Percentages result from at least 100 scored cells, unless otherwise indicated.

CFE Assay. Each dish containing 1.5 × 10^5 cells was transfected with 10 μg of pMM4, 0.1 μg of pSV2neo, and 29.9 μg of carrier DNA (two dishes per cell line or construct tested). G418 selection began 1 day after transfection, and dishes were scored 10–17 days later. The number of colonies in the *MyoD*-transfected dishes was compared to that in control dishes, transfected with pEMSVscribe. Identical experiments were performed with EGFR. The percentage of inhibition was calculated according to the formula: % inhibition = 100 − [(n_m/n_c) × 100], where n_m = number of colonies in MyoD dishes and n_c = number of colonies in control dishes.

RESULTS

Forced *MyoD* Expression Induces Growth Arrest. In order to express *MyoD*, we performed cotransfections of a *MyoD* expression vector (pEMC11s; see *Materials and Methods*) and the selectable marker pSV2neo, which confers resistance to the antibiotic G418. When a high MyoD/pSV2neo ratio was employed, we observed in a number of different cell lines effects similar to those described by Davis *et al.* (2) in the murine fibroblast cell line C3H/10T½ (10T½). Upon G418 selection, *MyoD*-transfected dishes of normal and tumorigenic cell lines demonstrated a marked reduction in CFE, when compared to control dishes transfected with the expression vector alone (see below). These results suggested that the growth inhibitory effect of *MyoD* could be exerted on growth control-impaired tumor cells as well as on nontransformed cells.

To investigate the mechanisms involved, we devised a transient expression assay to examine early events occurring after *MyoD* transfection. Mouse fibroblast cell lines 10T½ and NIH 3T3 were transfected with the *MyoD* expression vector pMM4. The transfected cells were incubated 48 hr later in the presence of either [^{3}H]thymidine or BrdU for 24–72 hr. Cells were then stained for MyoD by indirect immunofluorescence and subjected to either autoradiography for [^{3}H]thymidine or to immunodetection for BrdU. Under these conditions, 1–5% of the transfected cells scored as MyoD$^+$. Of these, >90% were thymidine$^-$ or BrdU$^-$. In contrast, >95% of the surrounding MyoD$^-$ cells demonstrated DNA synthesis (Fig. 1 *A–D*). Labeling with [^{3}H]thymidine or BrdU yielded identical results, and BrdU was selected for subsequent experiments. At the concentration used (10 μM), BrdU does not affect differentiation of cells expressing *MyoD* under the control of a constitutive promoter (ref. 15; M.C., unpublished results).

As a specificity control, we transfected the unrelated EGFR cDNA and tested its effects on DNA synthesis at different times after transfection. Expression of high levels of transfected EGFR has no detectable effect on NIH 3T3 proliferation, in the absence of added epidermal growth factor (11). Fig. 1*E* shows a typical experiment in which, at 24–48 hr following transfection, 24% of EGFR-expressing

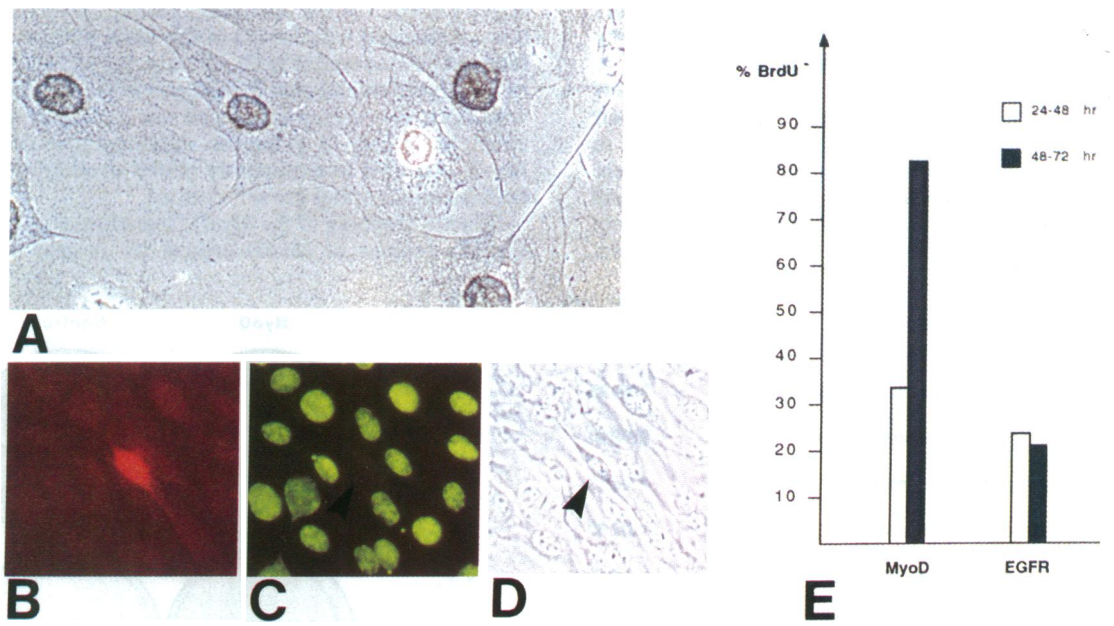

FIG. 1. Effects of *MyoD* expression on DNA synthesis. NIH 3T3 and 10T½ cells were transfected with either pMM4 or LTR-2/EGFR, and [^{3}H]thymidine or BrdU was added 48 hr later. Cells were incubated for 3 days ([^{3}H]thymidine) or 24 hr (BrdU). Greater than 95% of MyoD$^-$ or EGFR$^-$ cells replicated DNA. (*A*) [^{3}H]Thymidine incorporation. A MyoD-expressing 10T½ cell (red nucleus) did not incorporate thymidine, whereas black autoradiographic grains are seen on the surrounding nuclei. (*B–D*) BrdU incorporation. The same microscopic field is shown in *B–D*. A MyoD-expressing 10T½ cell (red nucleus in *B*) did not incorporate BrdU (absence of green signal, arrowhead in *C*). (*D*) Phase-contrast view. The arrowhead indicates the MyoD$^+$ cell. (*E*) Percentage of BrdU$^-$/MyoD$^+$ or BrdU$^-$/EGFR$^+$ cells. NIH 3T3 cells were incubated with BrdU 24–48 hr (open bars) or 48–72 hr (closed bars) after transfection. At least 100 MyoD$^+$ or EGFR$^+$ cells were counted at each time point.

cells failed to incorporate BrdU, compared to 33% of MyoD$^+$ cells. However, by 48–72 hr, the fraction of BrdU$^-$/MyoD$^+$ cells had increased to 83%, while BrdU$^-$/EGFR$^+$ cells decreased to 22%. We observed no significant reduction compared to controls transfected with the expression vector alone (data not shown). Thus, the fraction of BrdU$^-$/EGFR$^+$ cells observed in our transient assay represents a nonspecific background, and the magnitude of DNA synthesis inhibition above this level observed with *MyoD* likely reflects specific growth arrest induced by this gene. This interpretation is further substantiated by data presented on MyoD mutants (see below).

***MyoD*-Induced Growth Arrest Is Independent of Differentiation.** It has been proposed (2) that the reduced CFE observed in *MyoD*-transfected cells might be explained by the induction of terminal differentiation early after transfection. Thus, high levels of MyoD achieved during transient expression might trigger muscle differentiation despite the continuous presence of serum, which normally prevents the expression of muscle differentiation markers (16). To test this hypothesis, 10T½ or NIH 3T3 cells were transfected with *MyoD* as described and then cultured for 2 days in GM containing 10% fetal bovine serum or in serum-free SM. The cells were then immunostained to detect expression of MyoD and the differentiation marker MHC, a muscle-specific protein. About 20% of the *MyoD*-transfected 10T½ cells expressed MHC when starved for 48 hr as previously described (4). However, nonconfluent cells grown in the presence of serum very rarely showed expression of differentiation markers (<1% of all MyoD$^+$ 10T½ cells). Identical results were obtained with another differentiation marker, desmin (data not shown). Moreover, the vast majority of growth-arrested, MyoD-expressing cells did not show MHC or desmin expression in GM, although they did so when incubated in SM (data not shown). Thus, we conclude that MyoD-induced growth arrest is not mediated by differentiation.

Having demonstrated the ability of *MyoD* to inhibit DNA synthesis, we asked whether this was due to true growth arrest or to cell death due to a nonspecific toxic effect, produced by excessive or inappropriate expression of *MyoD*. Because the viability of the growth-arrested cells could not be established by their capacity to replicate, we sought to determine their ability to perform such functions as adherence, differentiation, and phagocytosis. To determine their ability to adhere, cells were incubated for 48 hr with BrdU in GM (beginning 48 hr after *MyoD* transfection). The cells were then trypsinized, reseeded, allowed 4–8 hr to reattach, and double-stained for MyoD and BrdU. Control cells were not trypsinized. If cell death resulted from *MyoD* expression, MyoD$^+$, BrdU$^-$ cells should be preferentially lost upon trypsinization and replating. This would result in a relative enrichment for MyoD$^+$, BrdU$^+$ cells in the trypsinized sample. The pooled results of five separate experiments showed no significant differences in the percentages of BrdU$^-$, MyoD$^+$ cells, which were 71.6 ± 19.5% and 73.6 ± 8.9% for the trypsinized and control cells, respectively. A similar experiment with EGFR-transfected cells yielded analogous results: the percentages of BrdU$^-$, EGFR$^+$ cells were 27% and 29% in the trypsinized and control samples, respectively.

To test whether arrested cells could be induced to differentiate, we incubated *MyoD*-transfected cells for 26–48 hr in GM with BrdU and then in SM with BrdU for 24–48 hr. The cells were then double stained for MHC and BrdU. An estimated 5% of the MyoD$^+$ cells differentiated within the first 24 hr of starvation. The great majority of these (92/101) were BrdU$^-$. Similarly, after 48 hr of starvation, the number of BrdU$^-$/MHC$^+$ cells was 92/114. These results indicated that differentiation could be activated after a period of MyoD-induced growth arrest. Phagocytosis is a complex function requiring energy production as well as membrane and microfilament integrity (17). *MyoD*-transfected cells were incubated for up to 80 hr with BrdU. Cells were then trypsinized, incubated with latex beads for 3 hr, replated, and stained for MyoD and BrdU. Among MyoD$^+$ cells, 94% scored positive for phagocytosis; 90% of these were BrdU$^-$. In the same sample, 94% of the MyoD$^-$ cells scored positive for phagocytosis.

***MyoD* Induces Growth Arrest in Transformed as Well as in "Normal" Cell Lines.** To determine the range of tumor and transformed cell lines in which *MyoD* could cause growth arrest, we evaluated a series of both tumor-derived and *in vitro*-transformed cell lines. These included three mouse tumor cell lines, two of which were of epithelial origin, and several NIH 3T3 cell lines transformed by various oncogenes in tissue culture. NIH 3T3 lines transformed with the H-*ras*, *src*, or *fos* oncogenes were growth-inhibited to a similar extent as the parental NIH 3T3 (Fig. 2). These results indicated that *MyoD* can overcome the growth-promoting activity of each of the oncogenes tested. All three tumor cell lines challenged with *MyoD* in the same CFE assay showed reduced CFE as well (Fig. 2). Of note, the average colony size in the *MyoD*-transfected dishes was noticeably smaller than with both the vector alone and the inactive mutants (see examples in Fig. 2). From the above data we conclude that *MyoD* was able to dominantly inhibit proliferation of tumor cells lacking normal growth control.

Genetic Mapping of the *MyoD* Growth Arrest Function. To identify regions of MyoD critical to its growth inhibitory

Cell line	Derivation	No. of colonies (MyoD/Control)	CFE (% reduction)
C3H-10T1/2	embryo fibroblasts	39/430	91
NIH-3T3	embryo fibroblasts	28/280	90
NIH-3T3/H-ras	transformed fibroblasts	26/257	90
NIH-3T3/src	transformed fibroblasts	14/220	94
NIH-3T3/fos	transformed fibroblasts	14/210	93
L cells	connective tissue sarcoma	146/374	61
KLN 205	squamous cell carcinoma	3/ 47	94
LL/2	lung carcinoma	148/609	76

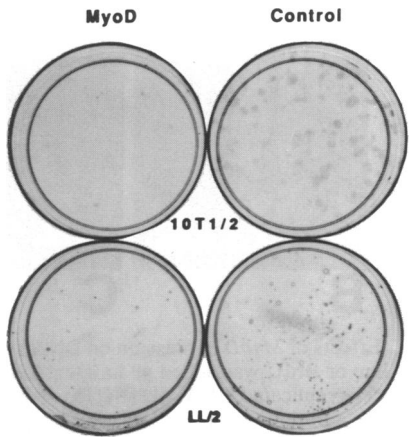

FIG. 2. CFE of normal and transformed cells transfected with *MyoD*. Examples are shown on the right. The circular appearance of the largest LL/2 colonies is due to cell detachment during fixation.

activity, two sets of MyoD mutants were evaluated with both transient expression and CFE assays. The first set comprised a series of MyoD deletion mutants (ref. 4; see Fig. 3). A mutant with a deletion of amino acids 3–56 (DM:3–56) lacks the amino-terminal acidic region; DM:63–99 lacks a domain containing clustered cysteines and histidines, DM:102–135 lacks the basic region and the helix 1 domain of the HLH motif (as defined in ref. 7), and DM:143–162 lacks part of the HLH motif. In addition, we studied a truncation mutant (TM:167) lacking the carboxyl-terminal half of the molecule and a mutant encoding only the basic and HLH regions (DM:4–101;TM:167). All of the mutants except DM:102–135 and DM:143–162 caused at least some degree of growth inhibition, as compared to the expression vector alone, transfected under identical conditions. This was reflected by reduced CFE (Fig. 3) as well as average colony size (data not shown). In the transient assay, not all of the mutants could be detected by immunofluorescence, as previously reported (4). However, each immunorecognizable mutant showed a degree of growth inhibitory activity that closely paralleled the CFE results (Fig. 3). Because DM:143–162 reproducibly showed no detectable effect on CFE, we believe that the 24% $BrdU^-$ cells constitutes the background of the transient assay, in accordance with our findings with the EGFR. In addition, the close agreement between inhibition of BrdU incorporation and reduction of CFE strongly supports the conclusion that reduced CFE is a reflection of the growth arrest observed in the transient assay. Of note, the deletion mutants that had no detectable growth inhibitory activity in our assays have been shown previously to be unable to induce differentiation (4). Both of these mutants lack part of the HLH motif. In addition DM:4–101;TM:167, which encodes a short 68-amino acid fragment including the HLH domain, induced a 31% reduction in CFE. From these results we conclude that the small region encompassing the basic and HLH domains of MyoD is sufficient to induce growth arrest and that the HLH motif is necessary for this activity.

We also studied a second set of mutants described by Davis *et al.* (7). This group includes a single-amino-acid mutant in which the alanine at position 114 in the basic region was changed to a proline (B2ProB3) and three chimeras in which the MyoD basic region was substituted with homologous regions from the HLH-containing proteins E12 (9), *Drosophila* T4 AS-C (18), and myc. The last three mutants are designated E12Basic, T4Basic, and MycBasic, respectively. Fig. 4 shows that each of these mutants caused a reproducible inhibition of CFE. This was again reflected in both the reduced number and average size of colonies observed (see mean number of cells per colony in Fig. 4). All of the mutants in the second set have been previously demonstrated to be

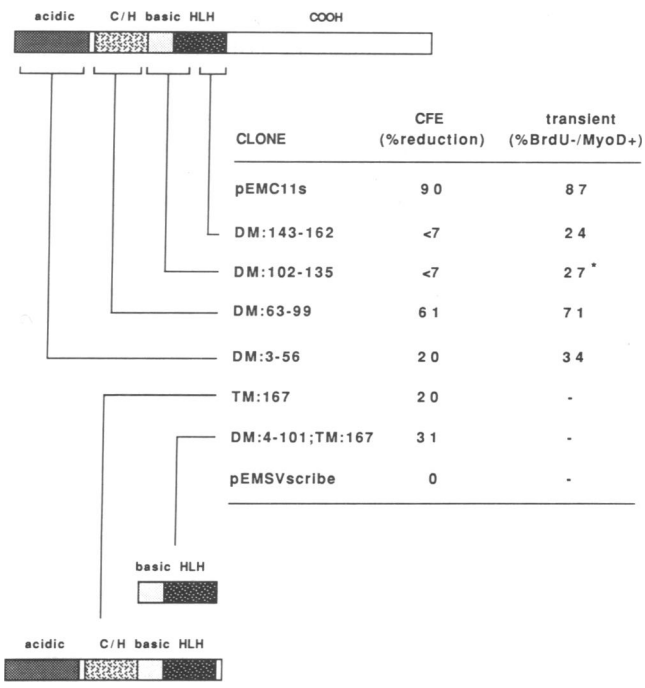

FIG. 3. MyoD deletion mutants. The top schematic shows the structural features of the MyoD molecule described in the text. Brackets indicate the approximate extension of the deletions. Bottom schematics represent deleted and/or truncated versions of MyoD. Mutants are identified as in ref. 4. DM, deletion mutant; TM, truncation mutant; C/H, cysteine/histidine-rich region; numbers indicate deleted amino acids, inclusive. Mutant and control constructs were transfected into 10T½ cells. CFE results are shown as the percent reduction in number of colonies in *MyoD*- or mutant-transfected dishes as compared to vector (pEMSVscribe)-transfected dishes. Each construct tested was transfected in two identical dishes and results were pooled. Intra- and inter-experiment variation in CFE experiments was less than 7%. Transient expression results are shown as the percentage of $BrdU^-/MyoD^+$ cells. All mutants were tested together, and all experiments were repeated twice. —, Mutant undetectable by immunofluorescence. *, A total of only 51 positive cells could be scored in two experiments, due to difficult detection of DM:102–135.

CLONE	CFE (% reduction)	Cells/colony (mean ± S.D.)
pEMC11s	96	105 ± 22
B2ProB3	43	468 ± 188
E12Basic	39	815 ± 272
T4Basic	13	1482 ± 313
MycBasic	33	847 ± 437
DM:143-162	<7	ND
pEMSVscribe	0	3298 ± 942

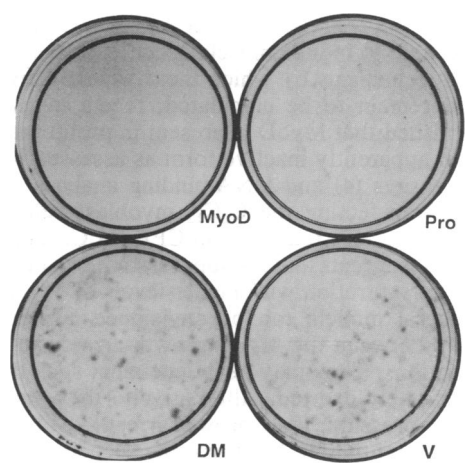

FIG. 4. MyoD basic region mutants. (*Upper*) CFE assay with MyoD basic region mutants. DM:143–162 was added as a negative control. ND, not determined. Experiments were performed as described in the legend to Fig. 3. (*Lower*) Examples of CFE assays. MyoD, pEMC11s; Pro, B2ProB3; DM, deletion mutant DM:143–162; V, pEMSVscribe. In the pEMC11s and B2ProB3 dishes, CFE reduction appears more pronounced than is shown in the table because most of the colonies counted in these two dishes were extremely small in size.

incapable of inducing differentiation (7). This constitutes further confirmation that differentiation cannot be responsible for the observed growth arrest. More importantly, B2ProB3, which does not bind the high-affinity binding site in the muscle creatine kinase enhancer (7), exhibited a relatively high level of growth inhibition. This allows us to conclude that this specific DNA-binding activity is not required by MyoD to induce growth arrest.

DISCUSSION

Our present studies demonstrate that, in addition to its well-documented function in differentiation, *MyoD* is able to cause growth arrest when expressed in a variety of cells. That growth arrest induced by *MyoD* was not due to a toxic effect of the protein was demonstrated by several criteria. These included the ability of cells growth arrested by MyoD to attach to a substrate, differentiate, and phagocytize. These functions require, minimally, cytoskeleton integrity, competence to transcribe and translate previously silent genes, energy production, and membrane integrity. Growth arrest was further shown to be independent of the expression of muscle-specific genes, in that it was observed with the wild-type gene under conditions in which differentiation does not occur and with a number of MyoD mutants incapable of triggering differentiation. Although the growth-arrest activity of MyoD was preserved under conditions in which its differentiation function could not be exerted, the two activities map to the same small region of MyoD, comprising its basic and HLH domains.

The growth-arresting activity observed might be due to an excessive and/or ectopic expression of MyoD and not to a normal function of the molecule. For example, MyoD could aberrantly interact with an HLH-containing protein or be a competitive substrate for a cellular enzyme, perhaps a protein kinase. Either molecule might be a limiting factor for progression in the cell cycle. By such a model, the excessive or inappropriate expression of *MyoD* might divert these proteins from their normal functions, thus inhibiting cell proliferation. Nevertheless, investigation of the mechanisms of MyoD growth inhibition should still lead to a better understanding of the machinery controlling cell proliferation.

Although we show that *MyoD* induces growth arrest in a variety of cell lines, myoblasts are able to proliferate despite its expression (4). Moreover, under proliferative conditions, MyoD is unable to induce muscle-specific gene expression. While the mechanisms by which these MyoD activities are suppressed remain to be elucidated, recent investigations have established that MyoD is present in proliferating myoblasts in an apparently inactive form as assessed by *in vivo* functional assays (4) and DNA-binding analysis (19). Evidence that transfecting the C_2C_{12} myoblast cell line with *MyoD* induces a 95% reduction in CFE (M.C., unpublished observations) suggests that the suppressor mechanism can be overcome by saturation with higher levels of MyoD.

Two general models can be envisioned concerning the relationship between the *MyoD* growth-arrest and differentiation functions. They may be independent such that either can be separately exerted. Alternatively, they may require sequential expression. If so, growth arrest must be a prerequisite for differentiation. We favor the second model on the basis of several observations. Expression of muscle-specific genes is not observed in cycling cells (20). Although in some systems nonfused, biochemically differentiated cells can return to a proliferating state, this is associated with loss of differentiation marker expression (21). Moreover, several of the MyoD mutants tested by us were able to induce growth arrest but not differentiation, whereas the converse was not observed. Thus, we propose that growth inhibition is one of the natural functions of *MyoD*, connected with its role in muscle differentiation. In its natural context, MyoD may induce myoblasts to exit the cell cycle, a step that must precede differentiation (22). Growth inhibition may also be required by terminally differentiated myotubes to prevent cytokinesis, which could disrupt their syncytial structure.

The molecular mechanisms mediating MyoD growth-inhibitory activity remain to be determined. However, the results obtained with the mutant B2ProB3 demonstrate that MyoD does not require the ability to bind the muscle creatine kinase enhancer in order to induce growth arrest. Although we cannot exclude the possibility that this mutant may retain the ability to bind other DNA sequences, our observations suggest that the growth-inhibitory activity of MyoD may be mediated by protein–protein interactions through its HLH motif.

A striking finding was that MyoD could efficiently induce growth arrest in neoplastic cells. The constitutive activation of oncogenes plays a major role in the acquisition of the transformed phenotype. We demonstrated that *MyoD* is able to override the proliferative stimuli of several different oncogenes. Moreover, *MyoD* was able to induce growth arrest even in tumor derived cell lines, in which multiple aberrations involving different growth control genes are likely to be present. Thus, MyoD exhibits the properties of a dominantly acting antagonist of cell proliferation. As such, it provides a tool to address negative growth control mechanisms at the molecular level.

Note Added in Proof. Cytofluorometric analysis shows that *MyoD*-transfected NIH 3T3 cells are arrested in G_1, analogous to terminally differentiated muscle cells.

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