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Cellular Aggregation Enhances MyoD-Directed Skeletal Myogenesis in Embryonal Carcinoma Cells

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When introduced into P19 embryonal carcinoma cells, recombinant genes encoding MyoD converted only a small percentage (<3%) of the transfected cells into skeletal muscle. We isolated stably transfected cells that expressed the MyoD transcript. These P19[MyoD] cells continued to express markers characteristic of undifferentiated stem cells but also expressed myf-5 and the myotonic dystrophy kinase, transcripts normally present in myoblasts but absent from P19 cells. Aggregation of P19[MyoD] cells induced the expression of myogenin, desmin, and the retinoblastoma protein and resulted in the rapid and abundant development of skeletal muscle. Both the embryonic and the slow isoforms of myosin heavy chain were present in this muscle, indicating that it resembled skeletal muscle formed from primary myoblasts. Since aggregation of P19 cells normally results in inefficient differentiation and the development of only low levels of cardiac muscle but no skeletal muscle, we conclude that MyoD imposes the skeletal muscle program on P19 cells and that the differentiation of these cells requires inductive events provided by cell aggregation.

Our understanding of myogenesis and the regulated expression of muscle genes is based largely on the myogenic family of basic helix-loop-helix transcription factors. MyoD was the first factor cloned by virtue of its ability to transform 10T1/2 fibroblast cells into skeletal muscle (11). MyoD and the other myogenic regulatory factors (MRFs), myf-5, myogenin, and myf-6/MRF-4/herculin (6, 7, 12, 30, 38, 61), bind DNA at E-box sites (consensus sequence CANNTG), which are found in many muscle-specific promoters, and activate the transcription of the genes encoding these MRFs (4, 24). MRFs share a basic DNA-binding motif and a helix-loop-helix dimerization domain. The active forms of the MRFs are heterodimers consisting of an MRF and a ubiquitous basic helix-loop-helix protein, such as E12, ITF1, or ITF2 (22, 23, 32). MRFs can activate the transcription of their own genes (5, 52), creating a positive feedback system that may be responsible for the maintenance of the differentiated state.

Each of the four MRFs has a distinct pattern of expression during the development of the mouse embryo, as shown by in situ hybridization (36), suggesting that each plays a distinct role. Gene knockout experiments have provided insight into the roles of three of the myogenic factors. Mice homozygous for a deletion of the *MyoD1* locus have normal muscle (39) but myf-5 mRNA levels are elevated, suggesting that myf-5 expression is normally repressed by MyoD. Mice lacking myf-5 also develop normal muscle, but embryos die at birth because of a failure in development of the rib cage (8). However, mice lacking both MyoD and myf-5 develop no muscle or myoblasts (42). Mice lacking myogenin develop myoblasts but have a severe reduction of all skeletal muscle (21, 33). These results suggest that either MyoD or myf-5 is necessary for myoblast formation, while myogenin is essential for the transition from myoblast to myotube (59).

Since MRFs depend on heterodimerization, their activities may be modulated by the repertoire of cellular factors available for interaction. This prediction is supported by studies of the susceptibility of different cell types to myogenic conversion. Although fibroblasts are in general susceptible to the actions of MyoD, expression of MyoD also activates myogenesis in cell types derived from all three germ layers, including melanomas, neuroblastomas, adipocytes, and perhaps some types of liver cells (26, 60). In many of these cell types, however, only certain components of the myogenic program are activated transiently. There are also some cells of nonmesodermal origin, such as HeLa, HepG2 (liver), and CV-1 (kidney) cells, which appear refractory to myogenic conversion by MyoD (46, 60). The failure of MyoD to activate myogenesis in these cells suggests either that the factors with which it cooperates are not ubiquitous or that there are inhibitory factors present.

P19 is a line of pluripotent embryonal carcinoma cells with a stable diploid karyotype (29, 41). The differentiation of these cells mimics early embryonic development (27). Aggregation of P19 cells induces expression of the mesoderm marker Brachyury T (57), but few of these cells differentiate. On the other hand, aggregates treated with dimethyl sulfoxide (DMSO) differentiate into cardiac and skeletal muscle along with other mesodermal and endodermal cell types (14). The resulting cardiocytes are embryonic in nature (40) and first appear at day 6 following DMSO treatment, while skeletal muscle does not appear until after day 9. When aggregated and treated with retinoic acid (RA), P19 cells differentiate into neuroectodermal derivatives (3, 29, 41).

Previous work has shown that expression of transcripts encoding MyoD did not induce myogenesis in P19 cells (37). In this study, we have examined how the ectopic expression of MyoD affects the developmental potential of P19 cells. We found that while the majority of P19 cells expressing MyoD retain stem cell characteristics, they also express some transcripts found in myoblasts, such as myf-5 and the myotonic dystrophy kinase (DMK) (44), but not others, such as desmin (54). Upon aggregation of the cells, these MyoD-expressing

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cells differentiate into skeletal muscle, suggesting that MyoD function is modulated by factors induced or repressed by cell-cell interaction during aggregation.

MATERIALS AND METHODS

Plasmid constructs. The construct PGK-MyoD(+UTR) contains the *Pgk-1* promoter driving an *Eco*RI fragment containing the entire MyoD cDNA (11), as described previously (37). The MyoD cDNA used to construct PGK-MyoD(−UTR) was an *Rsa*I-*Sau*3AI fragment in which the first 80 bp of 5′ untranslated region and the entire 3′ untranslated region were removed. The MyoD cDNA used to construct PGK-MyoD (+80 UTR) was an *Rsa*I-*Hin*dIII fragment in which the first 80 bp of 5′ untranslated region and all but the first 80 bp of 3′ untranslated region were removed. The construct CA-Puro contains a 450-bp *Pvu*II-*Ban*I (−440 to +6) fragment of the human cardiac α-actin (CA) promoter (37) driving the gene encoding puromycin resistance (55). The construct PGK-lacZ contains the *Pgk-1* promoter driving the LacZ cDNA, as described elsewhere (37).

Cell culture and transfections. P19 embryonal carcinoma cells were cultured as described previously (41). Cells were transfected by the calcium phosphate method (9). For transient transfections, 10^6 cells in 5 ml of medium in a 60-mm-diameter dish were exposed for 6 to 8 h to a DNA precipitate containing 5 μg of PGK-MyoD and 1 μg of PGK-lacZ. Some of the cells were fixed in methanol at various times after transfection for analysis by immunofluorescence. The rest of the cells were harvested and analyzed on day 2 for β-galactosidase activity as described elsewhere (49) to normalize for transfection efficiency.

To isolate cells with transfected DNA stably integrated into the cellular genomes, transfections were performed with 3.5 μg of PGK-MyoD(+UTR), 7 μg of CA-Puro, and 5 μg of B17 (28). After 24 h, 2×10^6 cells were plated in a 150-mm-diameter dish and selected for puromycin resistance (2 μg/ml). After 7 days, colonies were either pooled or isolated for further studies. Cells expressing MyoD are termed P19[MyoD] cells.

Differentiation was induced by plating 5×10^5 P19 or P19[MyoD] cells into 60-mm-diameter bacterial dishes containing either 1 μM RA, 0.8% DMSO, or growth medium alone. Cells were cultured as aggregates for 5 days and then plated in tissue culture dishes and harvested for RNA, protein, or immunofluorescence on day 6. P19 cells do not form skeletal muscle under these conditions (41).

P19 cells were induced to differentiate into skeletal muscle by aggregation for 6 days in the presence of 1.5% DMSO and 3 nM RA and harvested on day 10, as described previously (49).

Immunofluorescence. P19 and P19[MyoD] cells were plated on gelatin-coated coverslips, fixed in methanol at −20°C for 5 min, rehydrated in phosphate-buffered saline (PBS) for 15 min at room temperature, and then incubated for 1 h at room temperature with the appropriate antibody. For muscle myosin staining, 50 μl of a mouse antimyosin monoclonal antibody supernatant, MF20 (2), was used; for neurofilament staining, the rabbit anti-neurofilament 68 antibody (NF68) was diluted 1:500 (Walter Mushinski, McGill University). For staining the embryonic isoform of myosin heavy chain (MHCemb) with BF-45 antibody and the slow isoform of myosin heavy chain (MHCslow) with D-5 antibody (47, 48), 1:5,000 and 1:200 dilutions of the mouse monoclonal antibodies were used, respectively. After three 5-min washes in PBS, cells were incubated for 30 min in 50 μl of PBS with 1 μl of goat anti-mouse immunoglobulin G fluorescein isothiocyanate (FITC)-linked antibody or 1 μl of goat anti-rabbit immunoglobulin G rhodamine-linked antibody (Tago, Inc., Burlingame, Calif.). For staining the stem cell-specific surface glycoprotein SSEA-1, the mouse monoclonal antibody AEC3A1-C3 was used (19). The cells were stained live and then fixed with methanol. Immunofluorescence was visualized on a Zeiss Axiophot microscope.

Cell sorting. Monolayer cultures of P19 and P19[MyoD] cells were rendered single-cell suspensions, and 2×10^6 cells were resuspended in 100 μl of Puck's saline (5 mM KCl, 140 mM NaCl, 5 mM D-glucose, 4 mM $NaHCO_3$, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.4). The cells were fluorescently labeled by the addition of 20 μl of FITC (500 μg/ml) for 15 min. The cells were layered over 10 ml of fetal calf serum and pelleted to remove the excess FITC. The labeled cells were resuspended in medium and mixed in a 1:1 ratio with either unlabeled P19 or P19[MyoD] cells in a bacterial petri dish. Aggregates were examined by fluorescence microscopy every day for 4 days.

Northern blot analysis. Total RNA was isolated from differentiated P19 and P19[MyoD] cell cultures by the lithium chloride-urea extraction method (1). Northern (RNA) blot analysis was performed as described previously (40). Total RNA (10 μg) was separated on a 1% agarose gel containing formaldehyde. Transfer to Hybond-N (Amersham Canada, Ltd., Oakville, Canada) was done by capillary blotting, and RNA was cross-linked by UV irradiation. The membrane was hybridized to DNA probes labeled with $>10^9$ cpm/μg with [α-^{32}P]dCTP by using a multiprime labeling kit (Amersham Canada, Ltd.). The probes were purified on a spin column of Sephadex G-50 (Pharmacia Biotech Inc., Baie d'Urfé, Quebec, Canada) and hybridized for 16 h at 42°C. Washing was performed for 30 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS) and for 15 min at 65°C in 0.2× SSC–0.2% SDS. Hybridization was visualized by autoradiography. The probes used were a 600-bp *Pst*I fragment from the human CA last exon (40), a 1.8-kb *Eco*RI fragment from the mouse MyoD cDNA (11), a 695-bp *Eco*RI-*Pst*I fragment from the rat myogenin cDNA (7), a 530-bp *Pst*I fragment from the human DMK cDNA (45), a 2-kb *Eco*RI fragment from the mouse myf-5 cDNA (Thomas Braun), a 1.3-kb *Eco*RI fragment of the mouse Oct-3 cDNA (34), and a 2.2-kb *Eco*RI fragment of the mouse EndoA cDNA (56). The cardiac muscle-specific probe was a 745-bp *Eco*RI fragment from the rat cardiac troponin I (CTnI) cDNA (31). The skeletal muscle-specific probe was a 600-bp *Eco*RI fragment from the rat myosin light-chain 1/3 (MLC 1/3) cDNA (15). The neuron-specific probe was a 180-bp *Kpn*I-*Bam*HI fragment of the mouse β_3-tubulin cDNA (A. Frankfurter).

Western blot (immunoblot) analysis. Protein immunoblots were performed with an anti-retinoblastoma protein (anti-RB protein) antibody on P19 and P19[MyoD] cells at various times of differentiation as described previously (50). The desmin monoclonal antibody D3 was diluted 1/30 for immunoblots (10).

RESULTS

MyoD induces myogenesis inefficiently in P19 stem cells. Transient transfection of P19 cells with expression vectors encoding MyoD induced skeletal muscle in a small percentage of the transfected cells (Fig. 1). About 30% of the 25,000 cells plated from these transfected P19 cultures expressed β-galactosidase from a cotransfected plasmid carrying PGK-lacZ, but fewer than 3% of these β-galactosidase-expressing cells ex-

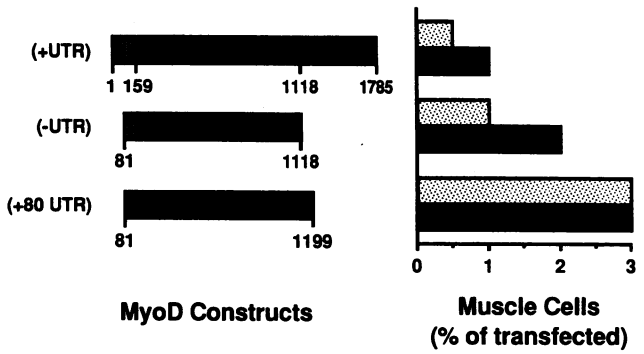


FIG. 1. MyoD inefficiently induces the development of bipolar myocytes following transient transfection into P19 cells. Three PGK-MyoD constructs contained various lengths of 5′ and 3′ untranslated regions as indicated by the nucleotide position numbers shown on the left (shaded boxes). The coding region of MyoD (black boxes) was constant in the constructs, beginning with nucleotide 159 and ending with nucleotide 1118. The plasmid carrying the PGK-MyoD construct was mixed with one carrying PGK-LacZ and cotransfected into P19 cells. Cells were stained with antibody MF20, and the myosin-positive cells were counted 2 (dotted bars) and 3 (striped bars) days after transfection. The percentage of muscle cells was calculated as the number of muscle myosin-positive cells divided by the number of cells expressing β-galactosidase from the cotransfected *Pgk-lacZ* gene. For example, following transfection with PGK-MyoD(+UTR), aliquots of 25,000 cells contained 225 myosin-positive cells and 7,500 β-galactosidase-positive cells.

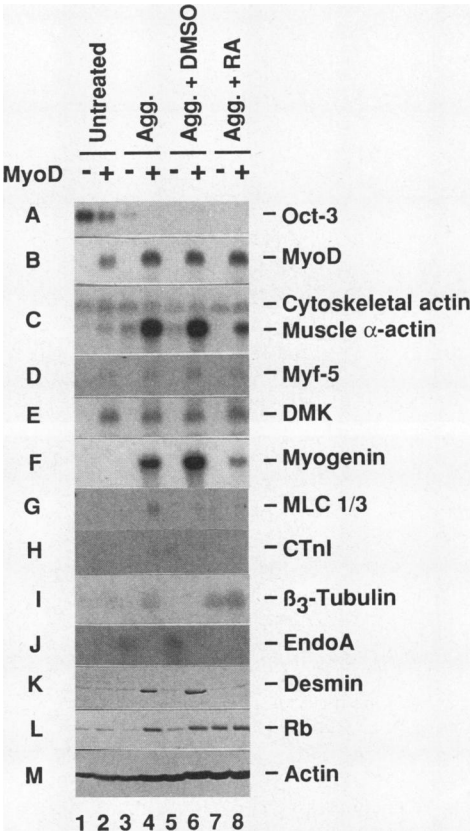


FIG. 2. Differentiating P19[MyoD] cells express skeletal-muscle-specific markers. Cultures of P19 (lanes 1, 3, 5, and 7) or P19[MyoD] (lanes 2, 4, 6, and 8) were either untreated on plastic surfaces or aggregated (Agg.) and treated with no drug, DMSO, or RA as indicated above the lanes. RNA was harvested on day 6, and duplicate Northern blots containing 10 μg of total RNA were probed for the transcripts indicated on the right (A to J). The CA probe reacts with both cytoskeletal and muscle-specific α-actin mRNAs. The former serves as a loading control, as it is present in all cells. Protein was harvested from parallel cultures, and immunoblots were probed with antibodies to desmin (K), the RB protein (L), and actin (M).

pressed muscle-specific myosin as detected by the MF20 antibody. MF20-positive bipolar cells were first observed 2 days after transfection of *MyoD*. The number of induced myocytes increased slightly with the removal of 5′ and 3′ untranslated regions of the MyoD cDNA. Thus, MyoD induced myogenesis inefficiently in P19 cells.

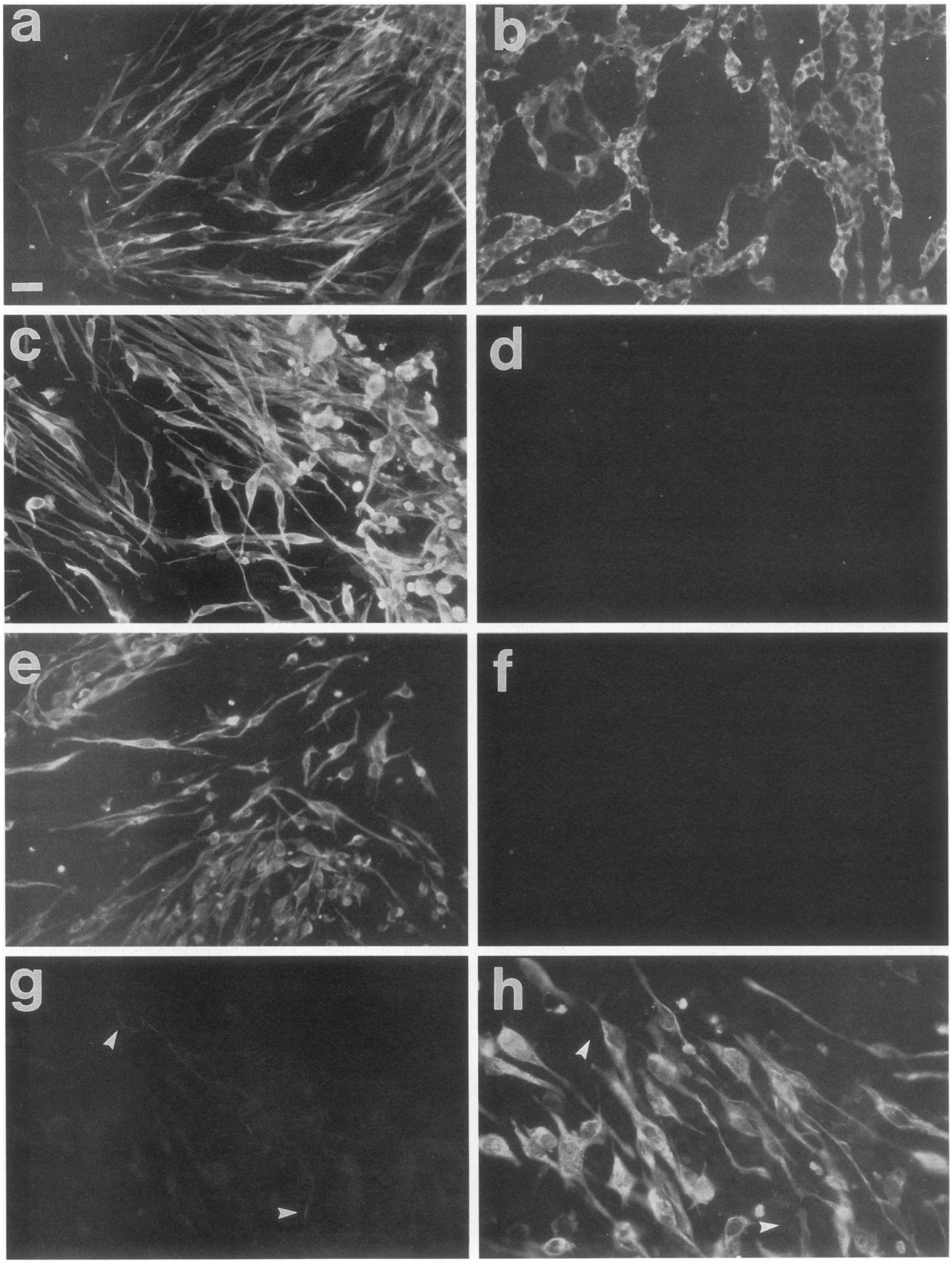
It is often difficult to isolate clones of P19 cells stably expressing a transfected gene because expression from transfected genes can be gradually lost as the cells are passaged. Thus, a method of positive selection was used to ensure that MyoD was expressed in all growing cells. P19 cells were transfected with two plasmids, one containing PGK-MyoD (+UTR) and the other carrying the CA promoter driving the gene for puromycin resistance (CA-Puro). The cells were selected in puromycin. The CA promoter is normally active only in muscle cells but is activated by MyoD in nonmuscle cells (37, 49). Thus, only P19[MyoD] cells should contain active CA promoters and become puromycin resistant. Puromycin-resistant colonies developed efficiently after 1 week of selection. More than 96% of these colonies consisted of cells with embryonal carcinoma morphology, and <4% of the colonies contained bipolar myocytes. When CA-Puro was transfected without PGK-MyoD, we recovered <3% of the puromycin-resistant colonies. Three P19[MyoD] clones as well as pooled colonies were examined in detail, and all gave similar results. The results presented in this paper are from one of the P19[MyoD] clones.

The P19[MyoD] cells retained stem cell characteristics of the parental P19 cells. Immunofluorescence with antibody AEC3A1-C3 (19) demonstrated that both P19 and P19[MyoD] cells expressed the stem cell-specific surface glycoprotein SSEA-1 (data not shown).

RNA was isolated from P19 and P19[MyoD] cultures grown in monolayers and subjected to Northern blot analysis (Fig. 2). Both P19 (lane 1) and P19[MyoD] (lane 2) cultures expressed the stem cell marker Oct-3 (34) (Fig. 2A). P19[MyoD] cells expressed MyoD, low levels of muscle actin, myf-5, and DMK (Fig. 2B to E, respectively), while P19 cells expressed none of these muscle-specific gene products. Neither P19 nor P19 [MyoD] cells expressed the skeletal muscle markers myogenin (Fig. 2F) and MLC 1/3 (Fig. 2G). Western blot analysis of proteins from these cultures indicated that neither P19 nor P19[MyoD] cells contained desmin, a protein expressed in myoblasts (54) (Fig. 2K). Thus, expression of MyoD in P19 cells activated the expression of myf-5, DMK, and low levels of CA but did not induce myogenesis or inhibit the expression of the stem cell markers Oct-3 and SSEA-1.

Aggregation of P19[MyoD] cells induces myogenesis. In order to determine whether the expression of MyoD altered the P19 cell differentiation programs, P19 and P19[MyoD] cells were aggregated for 5 days with or without DMSO or RA, plated into tissue culture dishes on day 5, and harvested on day 6. About 25 to 35% of the P19[MyoD] cells developed into bipolar skeletal muscle under all the conditions examined (Fig. 3a, c, and e). P19 cells did not develop skeletal muscle under these conditions (Fig. 3b, d, and f).

P19 cells aggregated in the absence of drug normally differentiate inefficiently into endoderm and mesoderm (14), al-


a
b
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h

FIG. 3. P19[MyoD] cells differentiate into bipolar cells resembling skeletal muscle. P19 (b, d, and f) and P19[MyoD] (a, c, e, g, and h) cells were aggregated for 5 days in DMSO (a and b), in the absence of drug (c and d), or in RA (e, f, g, and h) and stained on day 6 with MF20 (2), an anti-muscle myosin antibody (a to f). Bipolar muscle myosin-containing cells developed in all P19[MyoD] cultures (a, c, e, and h), while cardiac-like cells were found in DMSO-treated P19 cultures (b). Double immunofluorescence with antibodies to muscle myosin (h) and to neurofilament 68 (g) was performed on P19[MyoD] cultures 6 days after treatment with RA. Panels g and h represent the same field of view. The neurofilaments (arrowheads) and muscle myosin were present in distinct populations of cells. Bar, 40 (a to f) or 20 (g and h) μm.

though a variable amount of spontaneous differentiation may occur. In these experiments, P19 cells aggregated in the absence of drug (Fig. 2, lane 3) underwent relatively high levels of spontaneous differentiation. Oct-3 expression was reduced (Fig. 2A), and EndoA levels increased (56) (Fig. 2J), indicating the development of epithelial cells. These aggregated P19 cultures also contained muscle α-actin mRNA (Fig. 2C) but no myf-5, DMK, myogenin, MLC1/3, or desmin, indicating that some cardiac muscle had developed.

P19[MyoD] cells aggregated in the absence of drug (Fig. 2, lane 4) lost expression of Oct-3 (Fig. 2A) and differentiated into skeletal muscle, as indicated by expression of desmin (Fig. 2K) and the muscle-specific transcripts of α-actin, myogenin, and MLC 1/3 (Fig. 2C, F, and G). In addition, aggregated P19[MyoD] cells differentiated into cardiac muscle and neurons but not epithelial cells, as indicated by the presence of cardiac troponin I (Fig. 2H) and the neuronal marker β_3-tubulin (Fig. 2I) and by the absence of EndoA expression (Fig. 2J).

In P19 cells treated with DMSO, no skeletal muscle was observed in the cultures harvested on day 6, as indicated by the lack of expression of myf-5, DMK, myogenin, MLC 1/3, and desmin (Fig. 2D to G and K, lane 5). Differentiation into cardiac muscle was evidenced by the presence of low levels of muscle α-actin and CTnI mRNA (Fig. 2C and H) and by the presence of MHC within mononucleate stellate cells (Fig. 3b). Differentiation into endoderm was demonstrated by EndoA transcripts (Fig. 2J).

In DMSO-treated P19[MyoD] cultures, the induction of skeletal muscle was extensive on day 6 but no cardiac muscle developed (compare Fig. 2H, lanes 5 and 6, and Fig. 3a and b). No neuronal or endoderm markers were present (Fig. 2I and J, respectively).

Neuronal development occurred in RA-treated P19 and P19[MyoD] cells, as demonstrated by immunofluorescent staining with an anti-neurofilament antibody, NF68 (Fig. 3g), and β_3-tubulin expression (Fig. 2I, lanes 7 and 8), which are two neuron-specific markers. Double labeling of RA-treated P19[MyoD] cultures with both NF68 and MF20 indicated that the neurons did not coexpress muscle myosin (compare Fig. 3g and h). No endoderm or cardiac muscle developed in RA-treated P19 or P19[MyoD] cultures (Fig. 2J and H). The skeletal muscle produced from P19[MyoD] cultures expressed all skeletal markers except for desmin (Fig. 2K).

The RB protein may be essential for skeletal muscle differentiation (16) and for the cell growth-inhibitory activity of MyoD (13). P19 and P19[MyoD] cells express very low levels of RB protein (50) (Fig. 2L, lanes 1 and 2); however, RB protein levels increased 8- to 12-fold during P19[MyoD] differentiation into skeletal muscle (Fig. 2L, lanes 4, 6, and 8). Similar increases in RB protein in P19 cultures occurred only in the presence of RA (Fig. 2L, lane 7). Thus, cellular aggregation activated the expression of the RB gene in P19[MyoD] cells. This is probably not a direct effect of MyoD on the RB promoter, since MyoD did not transactivate the RB promoter during transient transfection experiments with P19 cells under conditions in which the CA promoter is transactivated (data not shown).

Myogenesis occurs efficiently in P19[MyoD] cells aggregated with P19 cells. When cultured on plastic surfaces, P19[MyoD] cells grow continuously and closely resemble the P19 cell parent; however, when aggregated, these P19[MyoD] cells rapidly differentiate into skeletal muscle. To investigate this requirement for aggregation, we coaggregated P19[MyoD] cells with P19 cells and looked at the development of skeletal muscle at 6 days. As the proportion of P19[MyoD] cells in the aggregates increased, both MyoD and muscle α-actin transcript levels increased in a linear manner, compared with the tubulin standard (Fig. 4A). Immunofluorescence was performed on cells from these cultures, and myosin-positive cells were counted (Fig. 4B). As the percentage of P19[MyoD] cells in the aggregates increased, the percentage of myosin-positive

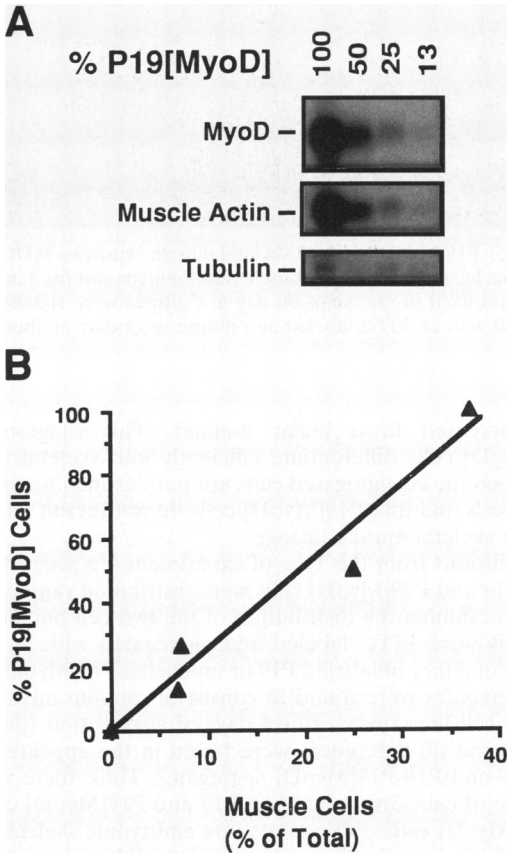

FIG. 4. P19[MyoD] cells differentiate efficiently when aggregated with P19 cells. P19 and P19[MyoD] cells were coaggregated for 5 days in proportions ranging from 13 to 100% P19[MyoD] cells. RNA was isolated on day 6 and analyzed for the presence of MyoD and CA transcripts. Tubulin transcripts were probed as a loading control (A). On day 6 after aggregation, cells were methanol fixed and the proportion of myosin-positive cells was determined following immunofluorescent staining with the MF20 antibody. The proportion of muscle cells was plotted against the proportion of input P19[MyoD] cells (B).

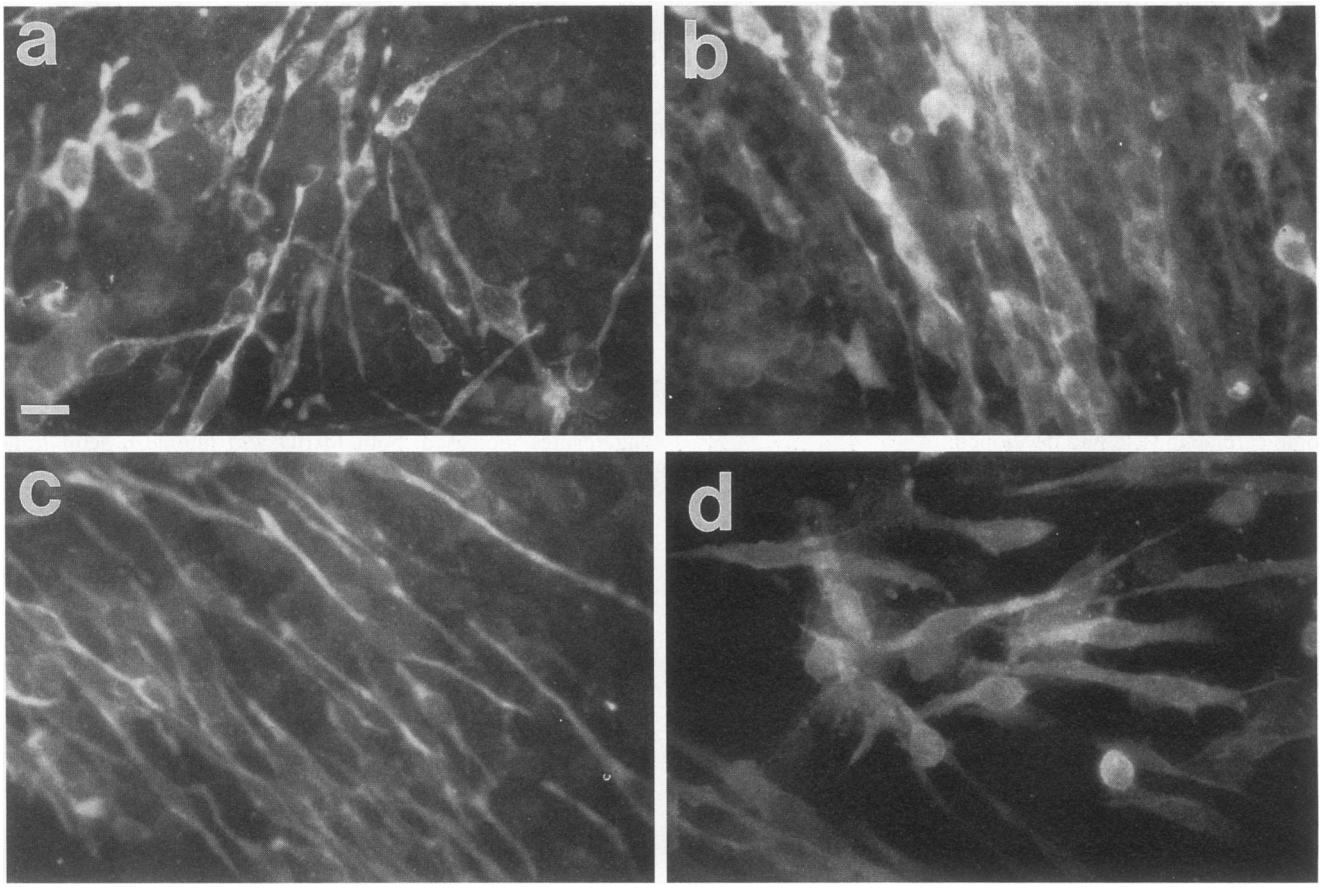

FIG. 5. P19[MyoD]-derived skeletal muscle expresses MHCemb and MHCslow. P19 cells (a and b) were aggregated in DMSO and RA under conditions leading to skeletal muscle development and fixed in methanol on day 10. P19[MyoD] cells (c and d) were aggregated without drug for 5 days and fixed in methanol on day 6. Cultures were stained with antibodies against either MHCemb (a and c) or MHCslow (b and d) and visualized with an FITC-labeled anti-mouse secondary antibody. Bar, 25 μm.

cells increased in a linear manner. This suggests that P19[MyoD] cells differentiate efficiently into skeletal muscle even when the coaggregated cells are not destined for skeletal myogenesis and that P19[MyoD] cells do not recruit P19 cells into the skeletal muscle lineage.

Conclusions from this type of experiment are accurate only if the P19 and P19[MyoD] cells were distributed randomly. In order to examine the distribution of the two cell populations, P19 cells were FITC labeled and aggregated with an equal number of either unlabeled P19 or unlabeled P19[MyoD] cells. The aggregates were found to consist of random mixtures of the two cell lines, even after 4 days of aggregation (data not shown), and no differences were found in the appearance of P19-P19 and P19-P19[MyoD] aggregates. Thus, there was no evidence of cell sorting between P19 and P19[MyoD] cells.

P19[MyoD] cells differentiate into embryonic skeletal muscle. Embryonic skeletal muscle contains MHCslow and MHC emb (58). The skeletal muscle from aggregated day 6 P19 [MyoD] cultures reacted with antibodies directed against both MHCemb and MHCslow (Fig. 5c and d). P19 cells aggregated in the presence of both DMSO and RA and cultured for 10 days developed small amounts of skeletal muscle (<5%) that also reacted with antibodies to both MHCemb and MHCslow (Fig. 5a and b). Thus, the skeletal muscle in these cultures resembled that formed from primary embryonic myoblasts.

Muscle formed in cultures of P19[MyoD] cells initially consisted of bipolar mononucleate myocytes. Fusion was not extensive until around day 10 (Fig. 6a), when about 5 to 40 nuclei were found per myotube or myosheet. Striations of myosin could be observed in some regions (Fig. 6b), indicating proper assembly of thick muscle filaments.

DISCUSSION

The efficacy with which MyoD induces P19 cells to undergo skeletal myogenesis appears to depend on the status of the cell, as summarized in Table 1. Cells growing in monolayers are relatively resistant to MyoD. In transient expression experiments, only 1 to 3% of transfected cells were transformed into skeletal muscle. Cell lines that expressed transfected PGK-MyoD could be readily recovered, and these P19[MyoD] cells resembled the parental P19 cells in expressing the stem cell markers SSEA-1 and Oct-3. However, P19[MyoD] cells also express myoblast-specific transcripts, such as DMK and myf-5, not expressed by the parental P19 cells. Upon cellular aggregation, P19[MyoD] cells rapidly initiated skeletal myogenesis, with 25 to 35% of the cells in these cultures expressing MHC along with a variety of myocyte-specific transcripts, such as myogenin, α-actin, and MLC 1/3. Aggregated P19 cells do not develop into skeletal muscle. Thus, there seem to be two different types of muscle genes directly or indirectly responsive to MyoD expression: those such as *myf-5* and the *DMK* gene

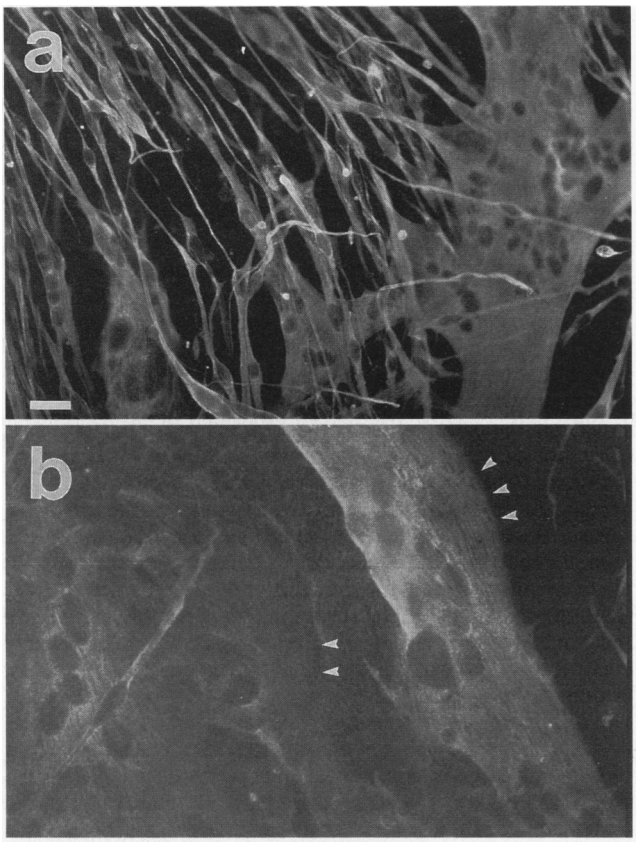

FIG. 6. P19[MyoD] cells fuse to form multinucleated myotubes and myosheets exhibiting striations. Cells were aggregated for 5 days and stained with MF20 on day 10. Striations in the myofiber bundles (arrowheads) are indicated. Bar, 40 (a) or 20 (b) μm.

that are activated by MyoD expression in P19[MyoD] cells, and those such as the myogenin, α-actin, desmin, *MLC 1/3*, and *RB* genes that require an additional activity provided by cellular aggregation.

How cell aggregation cooperates with MyoD to initiate myogenesis is not clear. P19[MyoD] cells differentiated into skeletal muscle efficiently when coaggregated with P19 cells, suggesting that the requirement for aggregation can be provided by cells not destined for myogenesis. Aggregation may

TABLE 1. Summary of stem cell- and skeletal muscle-specific genes expressed in P19 and P19[MyoD] cells before and after cellular aggregation

Cell type and gene	Expression in P19 cells		Expression in P19[MyoD] cells	
	Day 0	Day 6	Day 0	Day 6
Stem cells				
Oct-3	+	−	+	−
SSEA-1	+	−	+	−
Myoblasts				
DMK	−	−	+	+
myf-5	−	−	+	+
Desmin	−	−	−	+
Myotubes				
Myogenin	−	−	−	+
MLC 1/3	−	−	−	+

TABLE 2. MyoD expression alters cell types formed in differentiating P19 cell cultures

Cell type	Presence on:					
	Day 6 with DMSO		Day 9 with DMSO		Day 6 with RA	
	Without MyoD	With MyoD	Without MyoD	With MyoD	Without MyoD	With MyoD
Cardiac muscle	+	−	+	−	−	−
Skeletal muscle	−	+	+	+	−	+
Epithelial cells	+	−	+	−	−	−
Neurons	−	−	−	−	+	+

cause an increase in the translation of MyoD mRNA or a change in the posttranslational modification of the MyoD protein, such as phosphorylation (25). Alternatively, aggregation may initiate the induction of an auxiliary transcription factor or the loss of an inhibitor of MyoD. That aggregation of P19 cells can effect gene expression is evidenced by the induction of Brachyury T expression in these cells following their aggregation (57).

Although aggregation of P19[MyoD] cells efficiently induces them to develop into skeletal muscle, it is notable that 65 to 75% of these cells do not form muscle. The permissive condition conferred by aggregation may not be achieved by all cells in an aggregated culture, the expression of the transfected MyoD transcript in some cells may be reduced upon aggregation, or specific inhibitors may turn on in cells destined for other lineages.

There are many examples of regulators which modify MRF activity (18). MyoD does not activate muscle-specific genes in hepatocytes but does activate them after fusion of the hepatocytes to fibroblasts (46). A cellular factor stimulates the DNA-binding activity of purified MyoD and E47 (53), while rhabdomyosarcomas are deficient in a factor required for MyoD transactivation (51). Low levels of MyoD are expressed ubiquitously throughout the *Xenopus laevis* embryo following the midblastula transition (17, 20, 43), but no muscle genes are activated. Thus, it seems likely that the activities of MRFs are modulated by other cellular proteins and that the nonuniform behavior of P19[MyoD] cells is a consequence of the distribution of these modulatory factors in heterogeneous populations of differentiating cells.

In contrast to P19 cells, P19[MyoD] cells did not differentiate into cardiac muscle or epithelial cells in the presence of DMSO, but, like P19 cells, P19[MyoD] cells did differentiate into neurons in the presence of RA. These results, summarized in Table 2, imply that MyoD imposes the myogenic program on cells destined for mesodermal or epithelial lineages but not on neuroectoderm. Although MyoD has been shown to activate certain aspects of myogenesis in cells derived from all three germ layers, the most susceptible cell types have been shown to be those derived from the mesoderm (35).

Myosin was expressed only in bipolar myocytes or in myotubes, while the neurons formed from P19[MyoD] cultures did not express muscle myosin. Thus, muscle development in P19 [MyoD] cultures followed an all-or-none pathway, in contrast to other tissue culture systems, in which partial activation of myogenesis by MyoD may occur (60).

Isoforms of MHC can indicate the type of muscle produced. Embryonic or primary muscle expresses MHCemb and MHC-slow, while neonatal or secondary muscle expresses MHCemb and MHCneo (58). Both the endogenous P19-derived skeletal muscle at day 10 and the MyoD-induced skeletal muscle at day

6 express MHCemb and MHCslow and thus resemble embryonic muscle. These results indicate that MyoD did not change the type of muscle formed by P19 cells but accelerated the process and greatly expanded the number of cells destined to become skeletal muscle, from <5% in P19 cells to >25% in P19[MyoD] cells.

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