MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3901–3908 Vol. 16, No. 7
0270-7306/96/$04.00+0

Tumor Cell Complementation Groups Based on Myogenic Potential: Evidence for Inactivation of Loci Required for Basic Helix-Loop-Helix Protein Activity

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Received 5 January 1996/Returned for modification 9 February 1996/Accepted 8 April 1996

Basic helix-loop-helix (bHLH) proteins mediate terminal differentiation in many lineages. By using the bHLH protein MyoD, which can dominantly activate the myogenic differentiation program in numerous cell types, we demonstrated that recessive defects in bHLH protein function are present in human tumor lines. In contrast to prior work with primary cell cultures, MyoD did not activate the myogenic program in six of the eight tumor lines we tested. Cell fusions between the MyoD-defective lines and fibroblasts restored MyoD activity, indicating that the deficiency of a gene or factor prevents bHLH protein function in the tumor lines. Fusions between certain pairings of the MyoD-defective lines also restored MyoD activity, allowing the tumor lines to be assigned to complementation groups on the basis of their ability to execute the myogenic program and indicating that multiple mechanisms exist for abrogation of bHLH protein activity. These groups provide a basis for identifying genes critical for bHLH-mediated differentiation and tumor progression by using genetic complementation.

Cancer cells have abnormalities in terminal differentiation and cell cycle control. The severity of these two abnormalities is correlated with tumor stage and aggressiveness (9). High-grade tumors have a higher mitotic index, exhibit fewer features of terminal differentiation, and have a poorer prognosis than do their low-grade counterparts (for a review, see reference 49). Tumor progression must therefore select for alterations in genes involved in cell cycle control and terminal differentiation.

A combination of biochemical and genetic approaches has led to the identification of numerous cell cycle regulatory genes (for reviews, see references 20 and 54), and many have been implicated as critical regulators of tumor cell progression. Recently, for example, mutations in p16, a regulator of cyclin-*cdk* complex activity, have been identified in tumors of many different lineages (33, 44, 69). However, there are many mechanisms that abrogate p16 cell cycle function, such as amplification of CDK4 or cyclin D1 or loss of Rb (26, 39). A recent study demonstrated that each of these mechanisms was associated with glioblastomas in a nonoverlapping fashion that accounted for over 80% of the tumors studied (53).

Less progress has been made in understanding abnormalities in tumor cell differentiation. Loss-of-function mutations analogous to those characterized in the general cell cycle machinery have not been widely identified, although a limited number of cell fusion experiments suggest that some tumor-derived cell lines contain loss-of-function alterations preventing terminal differentiation (4). An example of this is provided by cell lines derived from rhabdomyosarcomas, which express members of the MyoD family of basic helix-loop-helix (bHLH) proteins (13). The MyoD family orchestrates skeletal myogenesis (for reviews, see references 45 and 65), and a subset of rhabdomyosarcomas fails to differentiate into skeletal muscle because of the loss of a factor required for MyoD family activity (62). Since bHLH proteins mediate differentiation in many different lineages (6, 14, 23), tumors of nonmuscle lineage may also avoid differentiation by inactivating bHLH function, analogous to mechanisms for evasion of cell cycle control that occur across a wide spectrum of tumors.

We have examined this possibility in tumors of the neural lineage, since bHLH proteins such as the *achaete scute*, *atonal*, and *neuroD* family members are involved in neuronal differentiation (18, 22, 31, 36). We tested the activity of MyoD, as a surrogate bHLH protein, in a variety of neural tumors, reasoning that factors necessary for bHLH activity may be used in both neuronal differentiation and muscle differentiation. This notion was supported by observations that some tumors display aspects of both neuronal differentiation and myogenic differentiation (28, 52, 58). MyoD, rather than a neuronal bHLH protein, was used for this analysis since the target genes for MyoD have been identified. In contrast to many nontransformed cell types, where MyoD expression is sufficient to activate skeletal myogenesis (12, 50), forced expression of MyoD failed to convert a majority of tested neural tumor lines to muscle. In all cases, myogenesis was restored in heterokaryons between the tumor cells and fibroblasts, indicating that the inactivity of MyoD in the tumor cells reflected the deficiency of a factor necessary for myogenesis and allowing us to establish complementation groups among different tumors on the basis of the ability to execute a myogenic differentiation program. These tumor complementation groups provide evidence that multiple mechanisms exist for abrogation of bHLH protein activity and may have utility in identifying genes critical for bHLH-mediated differentiation.

MATERIALS AND METHODS

Cell culture. The RD and DAOY cell lines were obtained from the American Type Culture Collection. NLF, NSH, NGP, and NMB (3) were obtained from G. M. Brodeur (Washington University). UW18, UW228, SNB19, and HTB16 (29, 55, 56, 59) were obtained from M. Berger (University of Washington). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% calf serum (Hyclone), penicillin, and streptomycin. For differentiation, we used DMEM supplemented with 10 μg of insulin per ml, 10

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μg of transferrin per ml, and 1% heat-inactivated horse serum (Gibco). Transfections were performed with Ca-PO_4 precipitation. Following transfection, cells were washed once with DMEM and allowed to recover for 24 h in growth medium prior to placement in differentiation medium or trypsinization.

Retroviral infection. Retroviruses containing resistance genes (27) for histidinol, neomycin, or hygromycin were used to establish resistant lines for hybrid analysis. A retrovirus containing the MyoD cDNA (50) was used to generate cell lines stably expressing MyoD. Retroviral stocks were prepared by harvesting medium from confluent retroviral packaging cell lines (40). Infections were performed by exposing cells to 1/10 dilutions of viral supernatant plus Polybrene (1 ng/ml) for 12 to 16 h. Following infection, cells were placed in appropriate selective medium for greater than 2 weeks. Infections generally resulted in polyclones with greater than 100 contributing subclones.

RNA analysis. Cells were grown in differentiation medium for 48 h, washed once with ice-cold Tris, and harvested for RNA extraction as previously described (2). Fifteen micrograms of total RNA was used for Northern (RNA) blot analysis on 1.5% agarose gels containing 6.7% formaldehyde. Gels were capillary transferred to Hybond-N and UV cross-linked. Blots were hybridized with probes for myosin light chain (MLC) (hA5-13 [American Type Culture Collection]), muscle creatine kinase (MCK) (pJN2CK-M [American Type Culture Collection]), myogenin (Myf-4 [American Type Culture Collection]), mouse MyoD (EMC11S), and 28s. Hybridizations were performed at 65°C in 7% sodium dodecyl sulfate–0.25 M Na_2PO_4–10 mg of bovine serum albumin per ml–1 mM EDTA for 12 to 16 h. Blots were washed at 60°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1× sodium dodecyl sulfate twice for 10 to 15 min each time. Blots were stripped in 95°C water for 5 min.

Immunohistochemistry test and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. Cells were washed once in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde for 7 min, washed twice in PBS, and permeabilized in 0.25% Triton for 10 min. Cells were incubated with a primary antibody for 90 min, washed three times in PBS, and incubated for 90 min with a secondary antibody conjugated to fluorescein isothiocyanate or rhodamine (Jackson IRL). Cells were washed three times in PBS, and the final wash contained 1 ng of 4′,6-diamidino-2-phenylindole (DAPI) per ml. The primary antibodies used were MF20 (monoclonal anti-myosin heavy chain [MHC]) and a polyclonal antiserum to MyoD. Cells to be stained with X-Gal were fixed in 0.5% glutaraldehyde for 5 min, washed three times in PBS, and incubated at 37°C for 2 h in PBS containing 1 mg of X-Gal per ml, 5 mM $K_4Fe(CN_6)$, 5 mM $K_3Fe(CN_6)$, and 2 mM $MgCl_2$.

Cell fusions. Cell lines were coplated at confluence in 3.5- or 6-cm-diameter dishes. After 24 h, cells were washed with DMEM and fused by using polyethylene glycol 8000 (Gibco) for 1.25 min. Cells were immediately washed three times with DMEM and allowed to recover for 24 h in DMEM plus bovine calf serum. Fusions to be analyzed transiently as heterokaryons were placed in differentiation medium for 48 h and prepared for immunohistochemistry testing or X-Gal staining. Cells to be grown as hybrids were split between two 10-cm-diameter dishes and grown in appropriate double-antibiotic selection medium. After 2 to 3 weeks in selection medium, hybrid colonies were cloned and amplified for further analysis.

RESULTS

MyoD fails to convert a majority of neural tumor lines to muscle. To assess the effects of MyoD expression in different tumor cells, eight independent tumor lines from three different classes of neural tumors—neuroblastoma, glioblastoma, and medulloblastoma—were infected with a retrovirus containing the MyoD cDNA (50) or a retroviral vector lacking the MyoD cDNA as a control. Primary human fibroblasts were similarly analyzed as a baseline for MyoD-induced muscle gene expression. After infection, cells were selected in G418 for 2 weeks and RNA from each polyclone was prepared after culture in differentiation medium for 48 h. The RNAs were Northern blotted and probed with the muscle markers MLC, MCK, and myogenin.

In contrast to previous work with primary cell lines (12), MyoD did not strongly induce skeletal muscle gene expression in six of eight tumor lines (Fig. 1). Only DAOY, a medulloblastoma, and UW18, a glioblastoma, showed up-regulation of muscle-specific markers comparable to that observed in primary fibroblasts (Fig. 1, lanes 1 and 2, 11 and 12, and 17 and 18). In contrast, two other glioblastoma lines, HTB16 and SNB19, showed only weak induction of muscle markers despite high levels of MyoD expression (lanes 13 to 16). The medulloblastoma UW228 (lanes 9 and 10) and three neuroblastoma lines, NLF, NSH, and NGP (lanes 3 to 8), failed to show

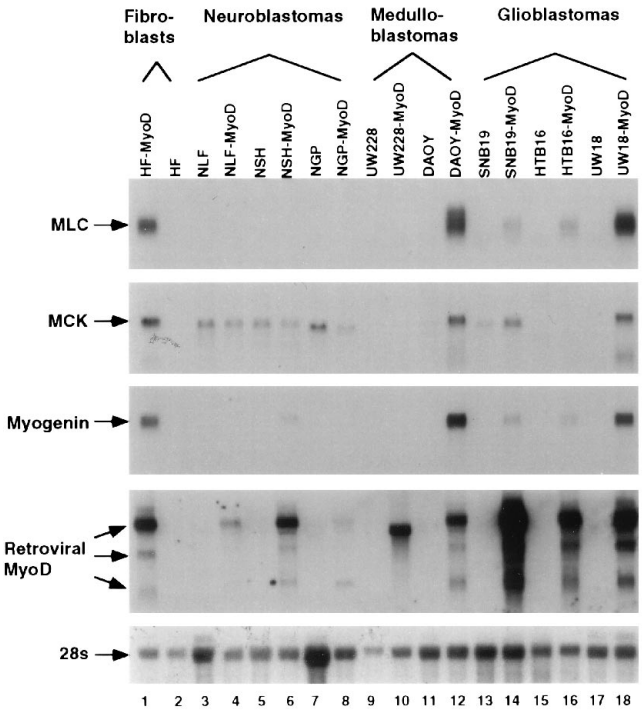

FIG. 1. Muscle marker expression in tumor lines infected with MyoD or control retrovirus. Lanes were loaded with ~15 μg of total RNA from each cell line. The blot was sequentially probed with MLC, MCK, and myogenin. A probe for mouse MyoD was used to show expression from the retrovirus. The three MyoD messages are alternate transcripts from the MyoD retrovirus, all of which can be translated and made into functional protein. A probe for 28s was used to control for loading.

MyoD-dependent transcription of skeletal muscle genes. While NLF and NGP have lower amounts of MyoD mRNA, additional data (see Fig. 3 and 5 and Table 1) show that high levels of MyoD in these cells does not induce myogenesis. Immunofluorescent staining (see, for example, Fig. 2d) demonstrated that MyoD protein was present in the nuclei of these tumor cells, indicating that the mRNA levels reflected expression and nuclear accumulation of MyoD protein in all of the tumor cell lines.

The MyoD-expressing tumor lines were also analyzed for conversion to muscle on the basis of myotube formation and MHC staining. After growth in differentiation medium for 48 to 72 h, the DAOY-MyoD line (Fig. 2b), the UW18-MyoD line (not shown), and primary fibroblasts with MyoD (Fig. 2a), exhibited fusion and elongation, characteristic features of muscle differentiation. In addition, both lines showed intense and extensive staining for MHC with indirect immunofluorescence. This correlates with the high levels of muscle marker RNA detected in these cell lines. In contrast, the HTB16-MyoD line had an unusual disassociation of the muscle phenotype and terminal marker expression. Under phase contrast, HTB16-MyoD exhibited extensive cell fusion (Fig. 2f); however, only a small percentage of fused cells were MHC positive (Fig. 2c) and Northern analysis (Fig. 1) showed only low levels of other muscle structural genes. Cell fusion was a consequence of MyoD expression, since HTB16 cells without MyoD did not exhibit this phenotype (Fig. 2e). Disassociation of cell fusion and muscle structural gene activation in this glioblastoma line may reflect separable MyoD-mediated pathways modulating myotube formation and gene activation. The third glioblas-

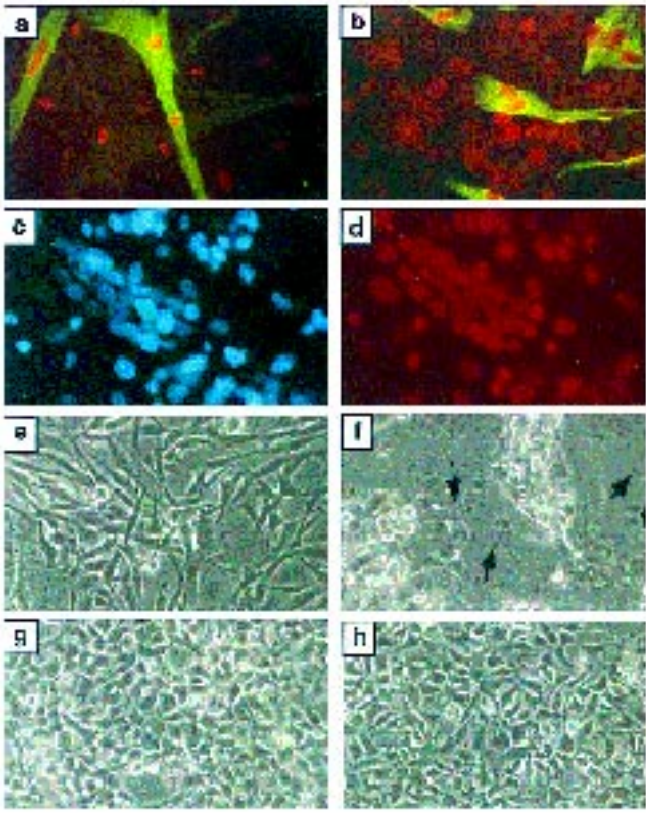

FIG. 2. Varying effects of MyoD expression on cell phenotype. (a and b) MHC-positive myotubes in human fibroblasts and DAOY cells infected with a retrovirus containing the MyoD cDNA. Cells were double stained for MHC (green) and MyoD (red) and visualized with indirect immunofluorescence. (c and d) HTB16-MyoD. (c) Double exposure for MHC (green) and DAPI staining (blue). (d) MyoD (red) staining. The grouping of cells in the center is an example of cell fusion without MHC expression. (e to h) Phase-contrast microscopy of HTB16 (e), HTB16-MyoD (f), NLF (g), and NLF-MyoD (h). Extensive fusion is evident in HTB16-MyoD cells (f), with arrows indicating several nuclei within a syncytium with more than 50 nuclei; in contrast, no fusion is evident in NLF-MyoD cells, which have a phenotype similar to that of the parent NLF line.

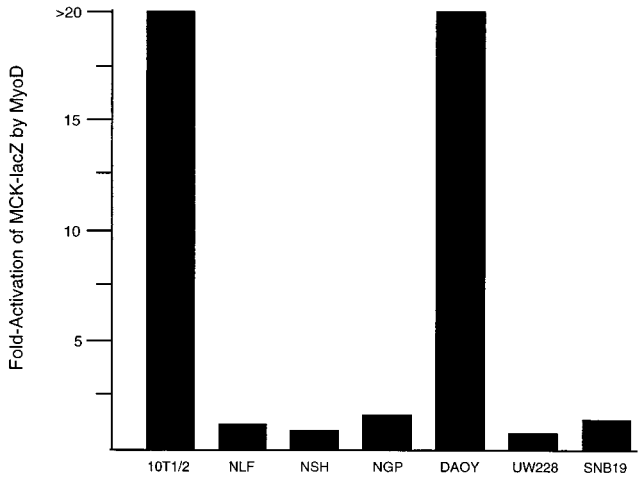

FIG. 3. MyoD fails to induce an MCK-*lacZ* reporter in tumor lines. Bar graph of the ratio of the number of *lacZ*-positive cells per plate cotransfected with pCS2-MyoD and pMCK-lacZ compared with plates of cells cotransfected with a control pCS2 vector and pMCK-lacZ. Results are averages of three separate experiments.

toma line, SNB19, exhibited more modest phenotypic changes with MyoD expression. Less than 1% of cells showed morphologic conversion to muscle on the basis of both fusion and MHC staining (data not shown). This is similar to the frequency of spontaneous differentiation observed in rhabodmyosarcomas (48). The phenotype of NLF (Fig. 2g,h), NSH, NGP, and UW228 was unaffected by MyoD expression, with no apparent myotube formation or MHC-positive cells.

Since MyoD can activate muscle reporter constructs in many cell lines (68), we tested whether the inability of MyoD to activate the endogenous myogenic program in the tumor lines was mirrored by inactivity on a transfected MCK template. A MyoD expression vector, pCS2-MyoD, or a control vector, pCS2, was cotransfected with pMCK-lacZ, a reporter containing 3.3 kb of the MCK regulatory region driving *lacZ* (67). The relative *trans* activation of MCK-*lacZ* by MyoD was calculated as the ratio of *lacZ*-positive cells on dishes cotransfected with pCS2-MyoD to *lacZ*-positive cells on dishes of cells cotransfected with the pCS2 control vector (Fig. 3). MyoD increased the number of cells expressing the MCK-*lacZ* construct more than 20-fold in both 10T1/2 mouse fibroblasts and myogenic DAOY cells, although the absolute level of activation was higher in the fibroblast cell line. In contrast, UW228, SNB19, and the three neuroblastoma lines did not show MyoD-mediated induction of MCK-*lacZ* expression. This correlates with data obtained for activation of the endogenous MCK locus and indicates that myogenic failure in the tumor lines is not caused by a *cis*-acting modification (e.g., methylation) of the target gene that prevents activation.

Myogenic defects in tumor lines are complemented in heterokaryons. Two remaining explanations for the relative inactivity of MyoD in the tumor cell lines are that (i) the tumor cells express factors that repress MyoD protein function in *trans*, as described for activated oncogenes such as *ras*, *jun*, and *myc* (5, 41, 63), and (ii) the tumor cells are deficient in factors required for myogenesis, perhaps reflecting genetic or epigenetic silencing of loci necessary for cell differentiation. One way to distinguish between these possibilities is to establish heterokaryons between the nonconverting tumor lines and a MyoD-responsive cell line such as 10T1/2 cells. In the first case, if a tumor line expresses factors which repress MyoD activity in *trans*, heterokaryons with 10T1/2 cells should be nonmyogenic, since these factors should also repress MyoD activity in 10T1/2 nuclei. In the second case, however, if a tumor line has silenced a locus necessary for myogenesis, heterokaryons with 10T1/2 cells should be myogenic, since 10T1/2 cells should supply the genes and gene products necessary for MyoD activity in the heterokaryon. We therefore prepared heterokaryons between the nonconverting tumor lines stably expressing MyoD from the retrovirus and 10T1/2 cells by using polyethylene glycol-mediated cell fusion. Less than 5% of the cells in these fusions were heterokaryons, as identified by differential DAPI staining of mouse and human nuclei. However, more than 50% of the MHC-positive cells were heterokaryons (Table 1). Thus, MHC staining was induced with heterokaryon formation (for example, see Fig. 4a and b). The somatic cell complementation of the myogenesis-defective phenotype argues that the tumor lines are deficient in factors required for MyoD-mediated differentiation. This conclusion was strengthened by the finding that MHC-positive heterokaryons often had more human than mouse nuclei (data not shown), making it unlikely that 10T1/2 cells were simply titrating out myogenic repressors.

To confirm that MyoD is a more potent activator of muscle genes in the tumor cell nucleus following heterokaryon forma-

TABLE 1. Complementation of myogenesis in tumor lines via heterokaryon formation with fibroblasts

Tumor line	No. of MHC-positive heterokaryons[a]	Total no. MHC-positive cells	No. of MCK-*lacZ*-positive cells in:	
			Fusions with 10T1/2 cells[b]	Self-fusions
UW228	22	22	ND[c]	ND
HTB16	28	35	>200	17
SNB19	17	30	>200	12
NLF	5	5	123	0
NSH	5	6	21	0
NGP	40	40	91	0

[a] Tumor lines expressing retroviral MyoD were fused with 10T1/2 mouse fibroblasts and stained for MHC after 48 h in differentiation medium. Heterokaryons were identified on the basis of intense centromeric DAPI staining in mouse nuclei.

[b] Tumor cells were cotransfected with pCS2-MyoD and pMCK-lacZ and fused with 10T1/2 cells or the untransfected parent line (next column). Cells were stained with X-Gal after 48 h in differentiation medium.

[c] ND, not determined.

tion with 10T1/2 cells, nonconverting lines were transfected with pCS2-MyoD and pMCK-lacZ. The transfected cells were fused either to 10T1/2 cells or to themselves. In all of the cell lines tested, there was a marked increase (greater than 20-fold) in the number of *lacZ*-positive cells in 10T1/2 fusions compared with control self-fusions (Table 1). An example of this assay is shown in Fig. 4c and d. This example also highlights the characteristic morphology of the differentiated myotube frequently exhibited by *lacZ*-positive cells. The results of both sets of fusion experiments indicate that 10T1/2 cells are able to complement the inactivity of MyoD in the six nonmyogenic tumor lines. This is consistent with a model in which genes required for MyoD-mediated myogenesis are silenced, either genetically or epigenetically, in the nonconverting tumor lines. By this criterion, the tumor lines have "recessive" defects preventing MyoD activity.

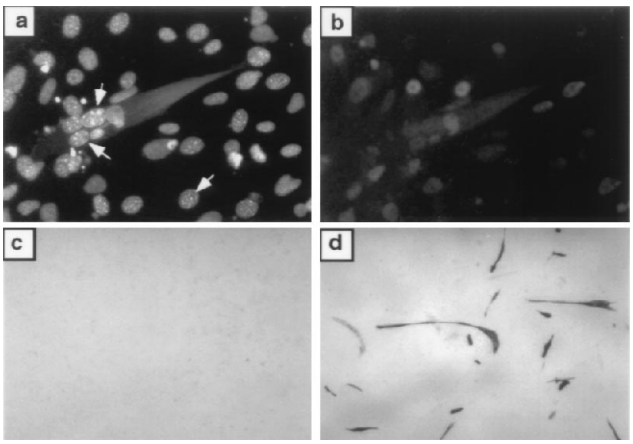

FIG. 4. Two assays for complementation of myogenesis with heterokaryon formation. (a and b) Fusions between UW228-MyoD and 10T1/2. (a) Staining for MHC (cytoplasmic) and with DAPI (nuclear). (b) Staining for MyoD. Mouse nuclei exhibit punctate staining with DAPI (arrows), while human nuclei have a homogeneous appearance with DAPI staining. The MHC-positive cell is therefore a heterokaryon. Note the lack of MHC staining in the other cells, which are not heterokaryons, despite detectable MyoD staining. (c and d) SNB19 cells cotransfected with pCS2-MyoD and pMCK-lacZ and fused with untransfected SNB19 (c) or 10T1/2 (d) cells. Cells were X-Gal stained after 48 h in differentiation medium.

Distinct recessive defects prevent myogenesis in tumor lines. The recessive, nonconverting phenotype of these tumor lines is similar to the previously characterized recessive phenotype of several rhabdomyosarcoma cell lines, which express MyoD and myogenin endogenously yet are not fully myogenic (30, 62). To determine whether the nonmyogenic phenotypes of the rhabdomyosarcomas and the neural tumors are due to a deficiency of the same factor or factors, we analyzed hybrids and heterokaryons among the tumors for complementation of myogenesis. One would predict that whole-cell hybrids formed between two lines lacking a common factor would be nonmyogenic, while two lines with functionally distinct defects would yield myogenic hybrids. Northern analysis of RD × NLF, RD × NSH, RD × UW228, and RD × SNB19 hybrids exemplified both possibilities (Fig. 5). RD × NLF hybrids (Fig. 5, lanes 6 to 8) showed high levels of MLC and MCK expression in comparison with the NLF-MyoD (lane 3) and RD (lane 5) lines, as did RD × UW228 (lanes 9 and 10) and RD × NSH (lanes 13 and 14) hybrids in comparison with control RD × RD hybrids (lanes 15 and 16). In contrast, RD × SNB19 hybrids (lanes 11 and 12) did not exhibit muscle marker upregulation, although myogenin expression, inherited from the parent RD line, was detectable.

The hybrids were also analyzed for muscle morphology. After 48 h under differentiation conditions, RD × NLF (Fig. 6a), RD × UW228 (Fig. 6b), and RD × NSH (not shown) hybrids adopted a dramatic fusion phenotype, in contrast to the RD × RD controls (Fig. 6d). Therefore, hybrids between RD and either NLF, UW228, or NSH were myogenic both morphologically and with regard to gene expression. This is strong evidence that these three neural tumor lines have myogenic differentiation defects which are distinct from that of the rhabdomyosarcoma. Interestingly, despite low levels of muscle marker expression (Fig. 5), RD × SNB19 hybrids exhibited some fusion and myotube formation (Fig. 6c). This is similar to the phenotype of HTB16 with MyoD expression (Fig. 1 and 2) and supports the notion that separable pathways may govern fusion to form myotubes versus activation of downstream muscle genes such as MCK. SNB19 and RD may share defects in loci important for muscle gene activation but not for myotube formation.

To further investigate the complexity of defects represented by the nonconverting lines, whole-cell hybrids were established between DAOY and NSH-MyoD and between NGP and NSH-MyoD. As one would predict (since DAOY cells convert to muscle), DAOY × NSH-MyoD hybrids were myogenic (Fig. 7, lanes 3 and 4), with marked myotube formation and muscle marker expression. In contrast, NGP × NSH-MyoD hybrids showed no evidence of myogenesis (Fig. 7, lanes 1 and 2). This suggests that neuroblastomas NGP and NSH share a recessive defect, although firm conclusions regarding shared defects are compromised by the possibility that complementing chromosomes may segregate during hybrid formation, a process which only demands retention of the parental chromosomes harboring the selectable resistance markers in the hybrid.

This caveat, coupled with difficulties in obtaining vigorously growing hybrid lines between a number of neural line combinations, prompted us to utilize activation of the pMCK-lacZ reporter in heterokaryons between tumor lines as an assay system for complementation of differentiation defects. Complementation of pMCK-lacZ activation by pCS2-MyoD in neuroblastoma lines NLF and NSH via heterokaryon formation with other tumor lines was tested. The NLF and NSH lines were cotransfected with pCS2-MyoD and pMCK-lacZ and

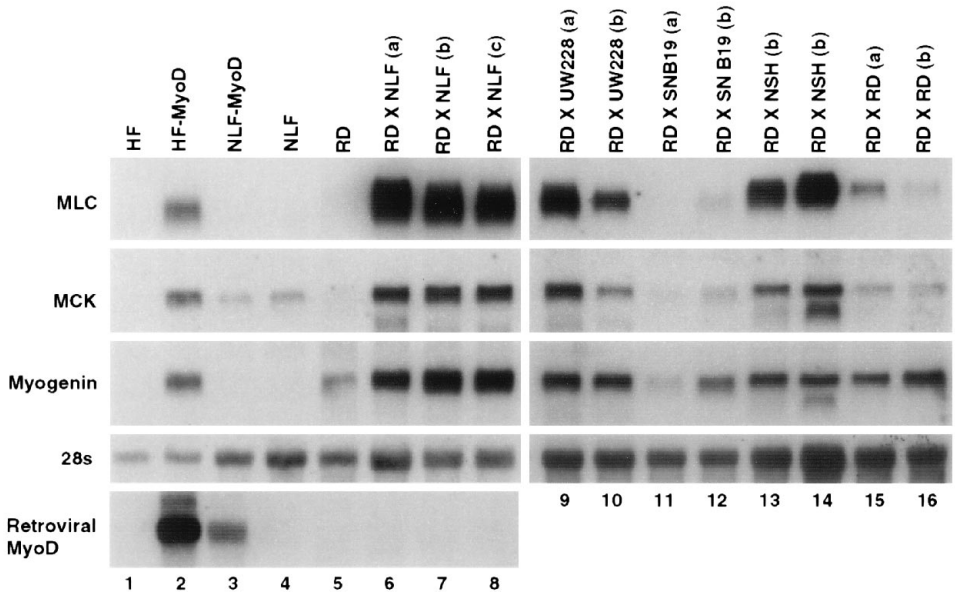

FIG. 5. Muscle marker expression in hybrids of NLF, NSH, UW228, and SNB19 with RD. Each lane in the Northern blot was loaded with ~15 μg of RNA. Hybrids [e.g., RD × NLF(a)] are independent clones obtained from fusions following greater than 2 weeks of selection.

fused pairwise to themselves, NGP, RD, and SNB19. All three neuroblastoma lines failed to complement each other for pMCK-lacZ activity (Table 2), while RD and SNB19 cells displayed a low but reproducible level of complementing activity in fusions with both NLF and NSH (Table 2). This is concordant with the whole-cell hybrid data, and taken together, these results indicate that different tumor cell lines have functionally distinct defects in factors necessary for myogenesis. One class of defect is represented by the rhabdomyosarcoma RD and likely contains the glioblastoma SNB19, as well as several other independent rhabdomyosarcoma lines (62). A second category is represented by the three neuroblastoma cell lines. These results demonstrate that cell hybrid and heterokaryon formation can be used to establish somatic cell complementation groups (47, 70) among tumor cell lines with respect to myogenic differentiation.

MyoD binds its cognate site as a heterodimer with an E protein, and the inactivity of MyoD in the complementation groups could be due to E protein deficiency or overexpression of one of the Id proteins, bHLH proteins that compete with MyoD for E protein dimerization. To determine if a relative E protein deficiency was the cause of MyoD inactivity in the tumor groups, we cotransfected RD and NLF cells with pMCK-lacZ and expression vectors for MyoD and the E12 protein or with pMCK-lacZ and a chimeric protein that fuses MyoD and E47 into a single protein resulting in a "forced dimer" that is relatively insensitive to inhibition by the Id proteins (43). Neither the forced dimer nor E12 increased MyoD activity (Table 3), suggesting that limiting amounts of an E protein were not responsible for the relative inactivity of MyoD in either the RD or NLF complementation group.

Several other genes have been identified that regulate the activity of MyoD. MEF2C, the muscle isoform of the MADS family of transcription factors, interacts with MyoD to activate transcription (42). Also, MyoD activity is diminished in cells that lack retinoblastoma protein Rb (21). To determine if

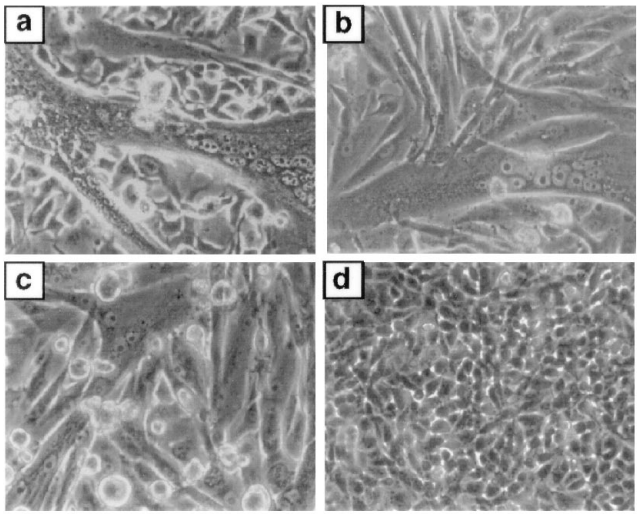

FIG. 6. Myotube formation in tumor hybrids. Phase-contrast micrographs of tumor hybrids following 48 h in differentiation medium. a, RD × NLF; b, RD × UW228; c, RD × SNB19; d, RD × RD.

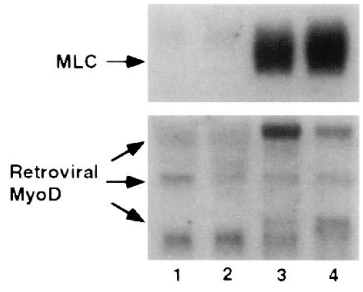

FIG. 7. MLC expression in DAOY × NSH-MyoD and NGP × NSH-MyoD hybrids. A Northern blot with RNA from the independent hybrid clones indicated was hybridized to probes for MLC and MyoD. NGP × NSH-MyoD hybrids do not express MLC despite MyoD expression.

TABLE 2. Effect of cell fusion on MCK-*lacZ* activity in NLF and NSH cells[a]

Cotransfected cell line	No. of blue cells after fusion with:					
	10T1/2*	RD*	SNB19*	NLF	NSH	NGP
NLF	398	17	21	1	0	0
NSH	130	17	20	1	0	0

[a] NLF and NSH cells were cotransfected with pCS2-MyoD and pMCK-lacZ. Transfected cells were fused with 10T1/2, RD, SNB19, NLF, NSH, or NGP. Cells were stained with X-Gal after 48 h in differentiation medium. The values shown are the total numbers of blue cells from five independent experiments. Values in columns with asterisks are statistically significantly different ($P < 0.05$) from those in columns without asterisks as determined by nonparametric analysis of the raw data.

deficiency of either MEF2C or Rb was responsible for the relative inactivity of MyoD in the tumor cell lines, we cotransfected RD and NLF cells, as representative members of distinct complementation groups, with expression vectors for MyoD and either Rb or MEF2C. MyoD activity was assessed by the activation of a cotransfected pMCK-lacZ reporter construct (Table 3) and by phenotypic conversion to muscle based on fusion and MHC staining (data not shown). Neither MEF2C nor Rb increased the activity of MyoD measured by these assays, indicating that other factors are responsible for the inactivity of MyoD in these cells. Interestingly, both MEF2C and Rb modestly activated the pMCK-lacZ reporter in NLF cells but failed to synergize with MyoD, suggesting that the myogenic defect in NLF cells may be due to an inability of MyoD to cooperate with these cofactors.

DISCUSSION

Forced expression of MyoD from a retroviral vector was not sufficient to convert a majority of neural tumor lines to muscle. Since previous work has demonstrated that MyoD can convert many primary cells and cell lines to muscle (12, 50), the inactivity of MyoD in the tumor cell lines is unusual and indicates that during tumor progression there may be a selection for alterations that prevent bHLH activity. Alternatively, the tumors may have arisen from a lineage position where the activity of MyoD, and perhaps other bHLH proteins, is precluded. To further characterize the basis for MyoD inactivity in the tumor cell lines, we analyzed heterokaryons established between the nonconverting tumor lines and 10T1/2 mouse fibroblasts. Heterokaryon formation restored MyoD activity in all six nonconverting lines, arguing that the tumor cells are deficient for factors necessary for MyoD-mediated myogenesis and that 10T1/2 cells can complement this deficiency. All six lines can therefore be considered to have a "recessive" defect which prevents myogenic differentiation, similar to the deficiency of a factor necessary for MyoD activity that we demonstrated for a group of rhabdomyosarcomas (62). Deletion, epigenetic silencing, and loss of heterozygosity coupled with an inactivating mutation are possible causes of the recessive defects observed in these tumor lines. Loss-of-function events are a major mechanism for disrupting cell cycle regulation in tumor cells (19, 46, 60); the high proportion of tumor lines with recessive inhibition of differentiation suggests that this may be a major mechanism for disruption of differentiation during tumorigenesis as well.

Since the myogenic defects in the tumor lines are recessive, we were able to group the tumor lines on the basis of phenotypic complementation in somatic cell hybrids and transient heterokaryons. A combination of stable hybrids and transient heterokaryons was used since chromosome segregation can occur during hybrid formation. Hybrids formed by neuroblastoma cell lines NSH and NLF with the RD rhabdomyosarcoma line were myogenic, whereas all three neuroblastomas (NSH, NLF, and NGP) failed to complement each other for MyoD-mediated *trans* activation, establishing that these three neural tumors represent a distinct complementation group from the rhabdomyosarcoma.

These somatic cell complementation groups for tumor cells based on differentiation in response to MyoD are similar to complementation groups which have been previously established for disorders such as xeroderma pigmentosum, ataxia telangiectasia, and Fanconi's anemia (32, 70). For example, cell fusions with lines derived from xeroderma pigmentosum patients were used to form groupings based on DNA repair in response to UV irradiation (35). Several of the xeroderma pigmentosum complementation groups have been subsequently demonstrated to have distinct underlying genetic abnormalities (24, 51, 64), and the groupings have proved useful in identifying loci responsible for xeroderma pigmentosum by using cloning strategies based on complementation (8, 34). The myogenic complementation groups for tumor cells may have similar utility. Molecular identification of the defects responsible for each group will also definitively determine if the observed complementation is intergenic or intragenic.

While the tumor cell lines have complex genetic and epigenetic alterations, forced MyoD expression in tumor cells can be a useful approach for dissecting differentiation pathways. For example, the glioblastoma HTB16 exhibited extensive fusion after 48 h in differentiation medium, yet almost all of the myotubes, some with more than 10 nuclei, failed to stain positively with MHC. A similar disassociation between fusion and muscle structural gene activation was evident in hybrids between SNB19 and RD cells, indicating that different genes in the pathway between MyoD expression and fusion were deficient in each tumor. Myotube formation in the absence of structural gene activation has not been demonstrated previously, although the converse, muscle gene expression without myotube formation, has been observed (1, 16, 17). The disassociation of MyoD-mediated fusion and MyoD-mediated activation of muscle structural genes suggests that distinct regulatory pathways may control subsets of MyoD-activated genes, thereby establishing a hierarchy governing the temporal and spatial patterns of gene expression during myogenesis.

TABLE 3. E proteins, MEF2C, and RB fail to increase MyoD activity in RD or NLF cells[a]

Cotransfected cell line	No. of blue cells after cotransfection with:							
	MyoD	MyoD-E47 dimer	MyoD + E12	MEF2C	MEF2C + MyoD	Rb	Rb + MyoD	CMV-*lacZ*
RD	1	0	0	0	0	0	0	$>10^3$
NLF	2	0	1	62	30	53	13	$>10^3$

[a] Values represent the total number of blue cells on a dish. Cells were cotransfected with the indicated cDNAs and pMCK-lacZ and stained with X-Gal after 48 h in differentiation medium.

A number of possibilities exist for the genes which are inactivated in each complementation group. In the most simple models, the recessive inability of MyoD to induce myogenesis in the tumor cell lines could reflect either (i) deficiency of a factor that positively interacts with MyoD or (ii) loss of an inhibitor of a factor or signalling system that inhibits MyoD-mediated myogenesis. An example of the first case might be the loss of a recognition factor, or coactivator, that has been postulated as necessary for MyoD activity and may partly account for the specificity of bHLH-mediated gene activation (15, 66). An example of the second case, the deficiency of an inhibitor of an inhibitor, might be genes that inhibit signal transduction pathways mediated by transforming growth factor beta or fibroblast growth factor that suppress MyoD activity (7, 38). Similarly, *myc*, *jun*, *fos*, and *ras* can inhibit MyoD activity and the loss of factors that suppress the activation of these proto-oncogenes could inhibit myogenesis (37, 41, 61, 63). We have not been able to complement MyoD activity with MEF2C, Rb, or E proteins; therefore, the tumor differentiation groupings may be useful in identifying novel members of pathways critical for bHLH-mediated differentiation and tumor formation.

The interaction of cell cycle regulatory proteins with proteins that regulate the expression of terminal differentiation genes is only beginning to be understood. MyoD activates p21 expression (25), suggesting a mechanism through which MyoD might mediate cell cycle withdrawal. While MyoD influences the cell cycle, the cell cycle regulators can also influence MyoD activity, since cyclin D1 overexpression can prevent MyoD-mediated gene transcription, associated with increased phosphorylation of the MyoD protein (57). Therefore, the ability of MyoD to activate transcription of muscle genes in different tumors may depend on the underlying mechanisms that have abrogated cell cycle controls. Conversely, efficient evasion of cell cycle control in some tumors may be accomplished by inactivation of genes, such as *myoD*, that induce cell cycle withdrawal during terminal differentiation.

The use of MyoD as a surrogate bHLH protein to assess the differentiation potential of tumor lines is a new approach for identifying genes that normally mediate differentiation which are inactivated in tumor cells. Proteins of the bHLH family mediate differentiation in many cell lineages, and perturbations in their function, perhaps reflected here by MyoD inactivity, may be frequent events during the generation of poorly differentiating tumors. Chromosome transfer and genomic or cDNA transfections are established techniques which can be used to identify the inactive loci responsible for each MyoD-based grouping (10, 11). Identification of the loci responsible for each MyoD-based complementation group will aid in understanding the regulation of normal differentiation and, potentially, how cells avoid terminal differentiation during tumor formation, particularly in lineages in which differentiation is mediated by bHLH proteins.

ACKNOWLEDGMENTS

We thank M. Berger, G. Brodeur, and Dusty Miller for cell lines and E. Olson for the MEF2C plasmid. We also thank Mark Groudine, Keith Fournier, and members of the Tapscott laboratory for helpful comments.

A.G. was supported by a Merck fellowship, and S.T. was supported by the American Cancer Society and a Shannon Award from the National Cancer Institute.

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