MOLECULAR AND CELLULAR BIOLOGY, Sept 1996, p. 5048–5057 Vol. 16, No. 9
0270-7306/96/$04.00+0

Amplification of *MDM2* Inhibits MyoD-Mediated Myogenesis

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Received 20 February 1996/Returned for modification 3 April 1996/Accepted 22 May 1996

One obvious phenotype of tumor cells is the lack of terminal differentiation. We previously classified rhabdomyosarcoma cell lines as having either a recessive or a dominant nondifferentiating phenotype. To study the genetic basis of the dominant nondifferentiating phenotype, we utilized microcell fusion to transfer chromosomes from rhabdomyosarcoma cells into C2C12 myoblasts. Transfer of a derivative chromosome 14 inhibits differentiation. The derivative chromosome 14 contains a DNA amplification. *MDM2* is amplified and overexpressed in these nondifferentiating hybrids and in the parental rhabdomyosarcoma. Forced expression of *MDM2* inhibits MyoD-dependent transcription. Expression of antisense *MDM2* restores MyoD-dependent transcriptional activity. We conclude that amplification and overexpression of *MDM2* inhibit MyoD function, resulting in a dominant nondifferentiating phenotype.

Rhabdomyosarcomas are one of the most common solid tumors of childhood. Sarcomas have traditionally been classified as rhabdomyosarcomas on the basis of morphology and the expression of muscle structural genes, such as that for the myosin heavy chain (MHC) or desmin. Expression of MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas (4, 38). Rhabdomyosarcomas are grouped by histologic and cytogenetic criteria as either embryonal or alveolar rhabdomyosarcomas: a balanced translocation between chromosomes 2 and 13, t(2;13)(q35;q14), is associated with alveolar rhabdomyosarcomas (1). The *PAX3* gene has been shown to be fused to a member of the forkhead gene family in the t(2;13) translocation (1, 39). Loss of heterozygosity on the short arm of chromosome 11 encompassing 11p15.5 is associated not only with embryonal rhabdomyosarcomas (38) but also with a number of other solid tumors (26), suggesting the location of a tumor suppressor gene(s) for multiple tumor types in this region.

Recently, we have begun an analysis of five rhabdomyosarcoma cell lines (RD, Rh18, Rh28, Rh30, and RhJT) for expression and function of the MyoD family (42). We showed that even though MyoD is expressed in rhabdomyosarcoma cells, it is nonfunctional in inducing differentiation. Heterokaryon formation between 10T1/2 cells and RD, Rh28, Rh30, and RhJT cells results in differentiation of the heterokaryons into muscle and restoration of transcriptional activation by MyoD, indicating that these tumor lines display a recessive nondifferentiating phenotype. In contrast, heterokaryon formation with the rhabdomyosarcoma Rh18 and 10T1/2 cells did not result in myogenesis, suggesting that Rh18 cells display a dominant nondifferentiating phenotype.

In this paper, we show that transfer of a derivative chromosome 14 from Rh18 cells into the differentiation-competent myoblast cell line C2C12 inhibits muscle differentiation and the ability of MyoD to function as a transcription factor. The derivative chromosome 14 contains a region of amplified DNA originating from chromosome 12 and contains a number of genes often amplified in sarcomas, including *GLI*, *SAS*, *CDK4*, and *MDM2*. Testing of the amplified genes for the ability to inhibit muscle-specific gene expression indicates that forced expression of *MDM2* inhibits MyoD function and consequently inhibits muscle differentiation. In addition, expression of antisense *MDM2* in C2C12 microcell hybrids containing the derivative chromosome 14 restores MyoD-dependent transcriptional activation. Thus, amplification and overexpression of *MDM2* inhibit MyoD function, resulting in dominant inhibition of muscle differentiation.

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MATERIALS AND METHODS

Cells. C2C12 cells were from the American Type Culture Collection. Rh18 and Rh30 cells were provided by P. Houghton (13). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% calf serum (HyClone Laboratories). Rh18 cells were stably transfected with pRSV NEO by electroporation (at 300 V and 960 μF in phosphate-buffered saline) (Bio-Rad), and approximately 2,000 clones were pooled and expanded for use as donors in microcell fusions. Myogenic differentiation was induced by growing cells to confluence and then incubating them in DMEM with 2% horse serum (differentiation medium).

Microcell-mediated chromosome transfer. Rh18 cells or the C2(Rh18) primary microcell hybrids were micronucleated by treatment with 10.0 or 0.06 μg, respectively, of colcemid per ml in DMEM plus 15% calf serum for 48 h. The micronucleate cell populations were enucleated by centrifugation in the presence of 5μg of cytochalasin B (Sigma) per ml, and the isolated microcells were fused to C2C12 recipients as described previously (7a, 20a). Microcell hybrid clones were isolated by using cloning cylinders after 3 to 4 weeks of selection in medium containing 500 μg of Geneticin (Gibco) per ml.

Cell transfections. Cells were transiently transfected by the Lipofectamine (Gibco-BRL) method. Approximately 3×10^5 cells were plated 1 day prior to transfection into 60-mm-diameter tissue culture plates. On the day of transfection, the cells were washed twice with serum-free DMEM. Transfection mixtures consisted of a total DNA content of 6 μg, with 0.5 to 1.0 μg being represented by the reporter construct. The remaining amount of DNA consisted of other constructs mentioned in Results. The lipid-DNA mixtures were added to the washed cells and brought to a final volume of 2 ml with serum-free DMEM. The transfection mixture was allowed to remain on the cells for 6 h, after which the transfection solution was removed by aspiration. DMEM containing 15% calf serum was added to the cells, and the cells were harvested after approximately 48 h.

CAT assays. Chloramphenicol acetyltransferase (CAT) activity was measured by a phase extraction procedure (38a). In brief, 48 h after transfection, cell extracts were generated by freeze-thawing cell pellets in 100 μl of 0.25 M Tris (pH 7.5). Following treatment at 65°C for 15 min to inactivate endogenous acetylases, 30 μl of extract was assayed with 0.2 mCi of [^{3}H]chloramphenicol (Dupont-New England Nuclear) and 250 nM butyryl coenzyme A (Sigma) in a total volume of 100 μl. The reaction was allowed to proceed for 2 to 12 h at 37°C

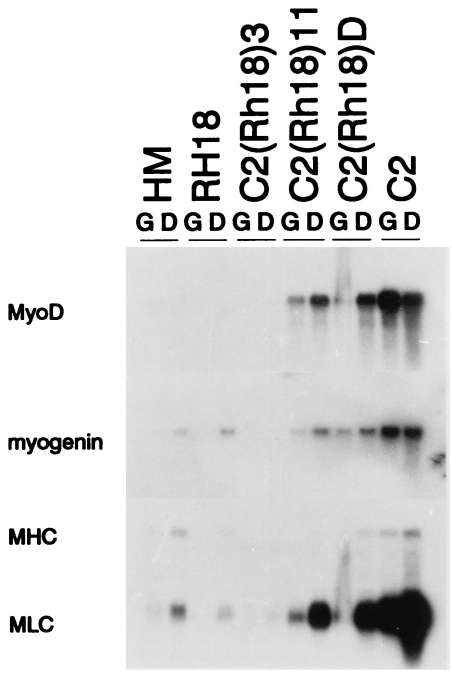

FIG. 1. Expression of muscle-specific genes in Rh18-C2C12 microcell hybrids. Northern blot analysis of 5 μg of cytoplasmic RNA extracted from cells in growth medium (G) and cells in differentiation medium (D) is shown. Northern blots were probed with cDNAs corresponding to MyoD, myogenin, MHC, and MLC. Ethidium bromide staining of the samples used for the Northern analysis showed equal loading of all samples. HM, human myoblast.

and was stopped by mixing with 200 μl of tetramethylpentadecane (TMPD)-xylene (2:1) (Sigma), and then 130 μl of the upper phase was added to the scintillation cocktail and the radioactivity was counted. Results are presented as percent activity or fold activation with error bars indicating standard deviations.

Northern (RNA) analysis. Total cytoplasmic RNA was prepared as described previously (6). Five micrograms of total cytoplasmic RNA was used for Northern analysis on 1.5% agarose gels containing 6.7% formaldehyde. RNA was transferred to GeneScreen (Dupont) by capillary transfer in 10× SSC (1× SSC is 150 mM NaCl plus 15 mM Na-citrate). RNA was cross-linked by exposure to UV followed by baking at 80°C for 2 to 4 h. Blots were prehybridized for several hours at 42°C in hybridization buffer (50% formamide, 1% bovine serum albumin [fraction V], 1 mM EDTA, 0.5M sodium phosphate [pH 7.2], 5% sodium dodecyl sulfate [SDS]). Hybridizations were for 24 h at 42°C in fresh hybridization buffer containing 10^8 cpm of a randomly primed ^{32}P-labeled DNA probe. The filters were washed in 2× SSC–0.1% SDS for 15 min at room temperature, in 0.1× SSC–0.1% SDS for 15 min at room temperature, and in two changes of 0.1× SSC–0.1% SDS at 55°C for 15 min each. The blots were stripped for reuse by being boiled for 2 min in double-distilled water.

Southern analysis. High-molecular-weight DNA (10 μg) was digested to completion with *Hind*III (New England Biolabs) and separated on 0.8% agarose gels in 0.04 M Tris acetate–2 mM EDTA. The DNA was capillary transferred in 10× SSC to GeneScreen (Dupont) membranes and UV cross-linked as described previously (3a). The blots were prehybridized and hybridized as for Northern blots. Probes containing *Alu* sequences were processed and hybridized as described by Budowle and Baechtel (2) to minimize background from repetitive sequences. The blots were stripped for reuse in 0.2 N NaOH for 30 min.

Inter-*Alu* PCR. The PCR was carried out in a total volume of 50 μl with 1 μg of DNA and 1 μM primer in 50 mM KCl–10 mM Tris HCl (pH 8.0)–1.5 mM $MgCl_2$–0.01% gelatin–200 μM dCTP, dATP, dGTP, and dTTP (Pharmacia)–1 U of *Taq* polymerase (Cetus) for 30 cycles of 94°C denaturation (1 min), 60°C annealing (30 s), and 72°C extension (30 s) in an automated thermal cycler (Cetus). The reaction was carried out in the presence of a single *Alu* primer, 517 (25).

Fluorescent in situ hybridization. DNA probes were nick translated by using standard protocols to incorporate biotin-11-dUTP or digoxigenin-dUTP. Slides of chromosomally normal male metaphase spreads were obtained from peripheral blood (48). Hybridizations were carried out on slides at 37°C for 16 h. Final probe concentrations varied from 40 to 60 ng/μl. Signal detection was carried out as described by Trask and Pinkel (44). Amplification of the biotinylated probe signal utilized alternating incubations of slides with antiavidin (Vector) and fluorescein isothiocyanate (FITC)-Extravidin (Sigma). Amplification of digoxygenated probes utilized alternating incubations of slides with FITC-tagged sheep antibodies made in rabbits and FITC-tagged rabbit antibodies made in sheep (Boehringer Mannheim). Slides were stained with propidium iodide (0.3 μg/ml), covered with coverslips, and viewed under UV fluorescence with FITC filters (Zeiss). Metaphase spreads showing a probe signal were photographed with Fuji color film (ASA 100) at ASA 400. Identifications of chromosomal loci were obtained by sequentially staining the same metaphase spreads with chromomycin A3-distamycin to produce fluorescent R-bands. R-banded metaphase spreads were then photographed with technical pan 2415 film (Kodak) at ASA 400.

RESULTS

Inhibition of muscle-specific gene expression by different Rh18 chromosomes. To study the genetic basis of the dominant inhibitory phenotype of Rh18 cells, we utilized microcell fusion to transfer chromosomes from Rh18 cells into the mouse myoblast cell line C2C12. We chose C2C12 cells as recipients because nondifferentiating variants occur at a low frequency ($\sim 10^{-6}$) (32). Rh18 chromosomes, randomly tagged by transfection with the plasmid pRSVNEO (Neo^r), were transferred into C2C12 cells by microcell fusion. Following selection in medium containing G418, we screened approximately 100 hybrid clones for expression of the muscle phenotype by visual inspection (see Fig. 2). Ten nondifferentiating clones, named the C2(Rh18) family, were isolated and expanded. All 10 clones were assayed for expression of MHC by indirect immunofluoresence. Three clones, C2(Rh18)-3, C2(Rh18)-11, and C2(Rh18)-D, continued to show greater than 90% reductions in the number of cells expressing MHC compared with parental C2C12 cells (41a) and were chosen for further analysis.

The expression of MyoD, myogenin, MHC, and myosin light chain 1/3 (MLC) was assayed by Northern blot hybridization. Figure 1 shows that clone C2(Rh18)-3 does not express detectable levels of the myogenic regulatory genes for MyoD and myogenin or the differentiation-specific mRNAs for MHC or MLC. Clones C2(Rh18)-11 and C2(Rh18)-D continued to express MyoD and myogenin, albeit at a reduced level, compared with parental C2C12 cells and expressed low but detectable levels of MHC and MLC. These results indicate that the transfer of Rh18 chromosomes into C2C12 cells can result in at least two different phenotypes, presumably because of retention of different Rh18 chromosomes. One phenotype is characterized by a complete lack of expression of MyoD and myogenin, and a second is characterized by continued expression of MyoD and myogenin; both result in a greater than 90% reduction in the number of cells expressing muscle differentiation-specific genes.

Characterization of loci responsible for the nonmuscle phenotype. Since microcell fusion can result in the transfer of more than one chromosome, we next determined the number of human chromosomes retained in the C2(Rh18) hybrids. We used total human DNA to specifically "paint" human chromosomes in metaphase spreads from each of the three primary microcell hybrid clones. All three hybrids retain two or three human chromosomes (42a). Therefore, in order to determine whether the Neo^r insertions are located in the chromosomes that contain the dominant inhibitory loci and to determine the chromosomal map positions of these loci, we conducted a second round of microcell fusions. Each primary microcell hybrid, C2(Rh18)-3, C2(Rh18)-11, and C2(Rh18)-D, was utilized as a donor in microcell fusions with C2C12 cells. Secondary microcell hybrids were generated by selecting for transfer of Neo^r by growth in medium containing G418 and were assayed for expression of the muscle phenotype. Initially, the secondary microcell hybrids were scored for the ability to form myotubes under differentiation-inducing conditions by visual inspection. From this analysis, it was obvious that C2(Rh18)-11

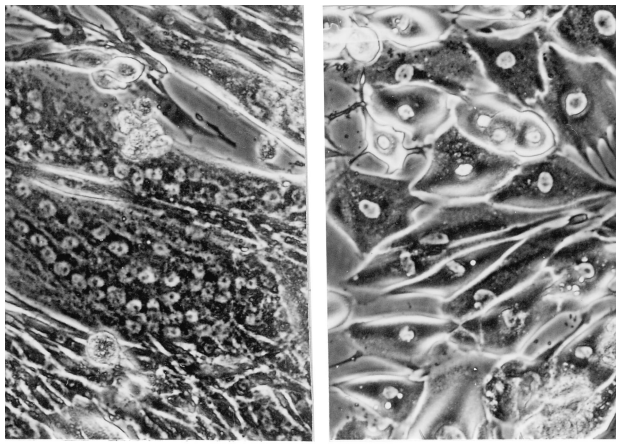

FIG. 2. Lack of myotube formation in C2Rh1811-6. Phase-contrast micrographs of myotube formation of mouse myoblast C2C12 cells and the secondary microcell hybrid C2Rh1811-6 are shown. Cells were grown to confluence and then placed in differentiation medium for 48 h. The multinucleated syncitium is representative of myotube formation.

transmitted the nonmuscle phenotype with the Neo^r marker, since five of six clones, named the C2Rh1811 family, did not display myotube formation. Figure 2 shows a photomicrograph of the secondary microcell hybrid C2Rh1811-6 compared with parental C2C12 cells under differentiation-inducing conditions. The nondifferentiating phenotype of the secondary microcell hybrids was confirmed by Northern blot hybridizations and is similar to that of C2(Rh18)-11 (6a). In contrast, all of the secondary microcell hybrid clones (at least 10 clones of each) generated from C2(Rh18)-3 and C2(Rh18)-D displayed extensive cell fusion and myotube formation; in addition, Northern blot hybridizations showed that these secondary clones expressed MLC and MHC mRNAs at parental C2C12 levels. Therefore, it is unlikely that the Neo^r insertions in these two primary microcell hybrids are linked to inhibitory loci. Characterization of the loci involved in inhibition of myogenesis in C2(Rh18)-3 and C2(Rh18)-D awaits the isolation of additional primary clones with different Neo^r insertions.

To determine the human DNA content in the C2Rh1811 microcell hybrids, total human DNA was labeled and applied to chromosome spreads of C2Rh1811-6. Hybridization to a single human chromosome was detected (40a) (Fig. 3). G-banding of metaphase spreads indicates that this chromosome is a derivative chromosome 14 containing a large homogeneous staining region (HSR) (Fig. 3a and g). To determine whether only chromosome 14 sequences are present on this chromosome, we utilized fluorescent in situ hybridization (FISH) with a chromosome 14-specific "paint" as a probe. Figure 3b shows that chromosome 14 sequences are present in the centromeric and telomeric regions of this chromosome but are excluded from the HSR. Therefore, the HSR is likely to contain sequences from elsewhere in the genome. To identify the chromosomal origin of the HSR, we isolated human-specific probes from the derivative chromosome 14.

Inter-*Alu* PCR was developed to isolate human-specific sequences directly from somatic cell hybrids by using PCR with primers directed at the human *Alu* repeat element (20, 24, 25). Figure 4 shows the inter-*Alu* PCR products generated from DNA isolated from the three primary microcell hybrids C2(Rh18)-3, C2(Rh18)-11, and C2(Rh18)-D compared with those generated from Rh18 and C2C12 DNAs. The smear of PCR products from Rh18 DNA is expected and is derived from amplification products from all chromosomes. Inter-*Alu* PCR products are easily detected from C2(Rh18)-11 DNA but not from C2(Rh18)-3 or C2(Rh18)-D DNA even though the last two retain two or three human chromosomes. The intensity of the ethidium bromide-stained bands of the inter-*Alu* PCR products from C2(Rh18)-11, even after a relatively low number of cycles, suggests that the derivative chromosome 14 contains amplified DNA. Furthermore, increasing the number of cycles during the PCR results in amplification of inter-*Alu* PCR products from C2(Rh18)-3 and C2(Rh18)-D (42a).

To determine whether the inter-*Alu* PCR products obtained from C2(Rh18)-11 are derived from amplified DNA, we performed Southern blot hybridizations under conditions that suppress hybridization of *Alu* repeats (2). Inter-*Alu* PCR products were size fractionated on an agarose gel and isolated as pools of fragments representing fragments with high (1,000 to 2,000 bp), middle (500 to 1,000 bp), and low (200 to 500 bp) molecular sizes. Figure 5A and B show that both the high- and middle-molecular-size probes detect amplified bands in Rh18, C2(Rh18)-11, and the secondary microcell hybrid C2Rh1811-1 compared with primary human skin fibroblast (HSF) DNA. In addition, the middle probe detects an unamplified band which serves as an internal control for both the quantity and quality of the HSF DNA. By utilizing different *Alu* primers and varying the PCR conditions used to amplify the products, we have been able to isolate 28 different inter-*Alu* PCR products that detect amplified DNA in Rh18 and the derivative chromosome 14 microcell hybrids. We have mapped the inter-*Alu* PCR products, which detect amplified DNA, to normal chromosome 12 by utilizing Southern blot hybridizations to a somatic cell hybrid mapping panel (6a). In addition, chromosome 14-specific probes (*TGFB3*, *MAX*, and *FOS*) do not detect amplified DNA. Figure 5C shows that *TGFB3* is not amplified in Rh18 DNA and is present in the microcell hybrid C2(Rh18)-11 in a single copy. Similar results were obtained with *MAX* and *FOS* probes (6a). Furthermore, utilizing FISH and a chromosome 12-specific "paint," we showed that the DNA in the derivative chromosome 14 HSR originated from chromosome 12 (Fig. 3c). Therefore, it seems likely that the derivative chromosome 14 consists of chromosome 14 sequences present in a single copy and chromosome 12 sequences that have been amplified. As shown below, the gene amplification is responsible for the nonmuscle phenotype of C2(Rh18)-11.

To obtain a larger number of probes for the DNA amplification, a cosmid library was constructed from the secondary microcell hybrid C2Rh1811-6. The cosmid library was screened with labeled human DNA to identify *Alu*-containing clones. Approximately 3% of the total clones represented within the C2Rh1811-6 cosmid library hybridize with the human-specific probe; the remaining 97% presumably are derived from C2C12 DNA and represent mouse sequences. Twenty human-specific cosmids were isolated and used as probes for Southern blot hybridizations. Ten of 20 human-specific cosmids detected amplified DNA present on the derivative chromosome 14 (6a). Thus, 1 to 2% of the cosmid clones in this library are derived from the DNA amplification. Figure 3d to f show FISH of three cosmid clones hybridizing to the derivative chromosome 14 HSR. These three cosmids were also localized by FISH to normal 46XY chromosome spreads and were found to map to 12q13-15 (40a).

Identification of amplified genes. Amplification of cellular proto-oncogenes has been described for a number of different

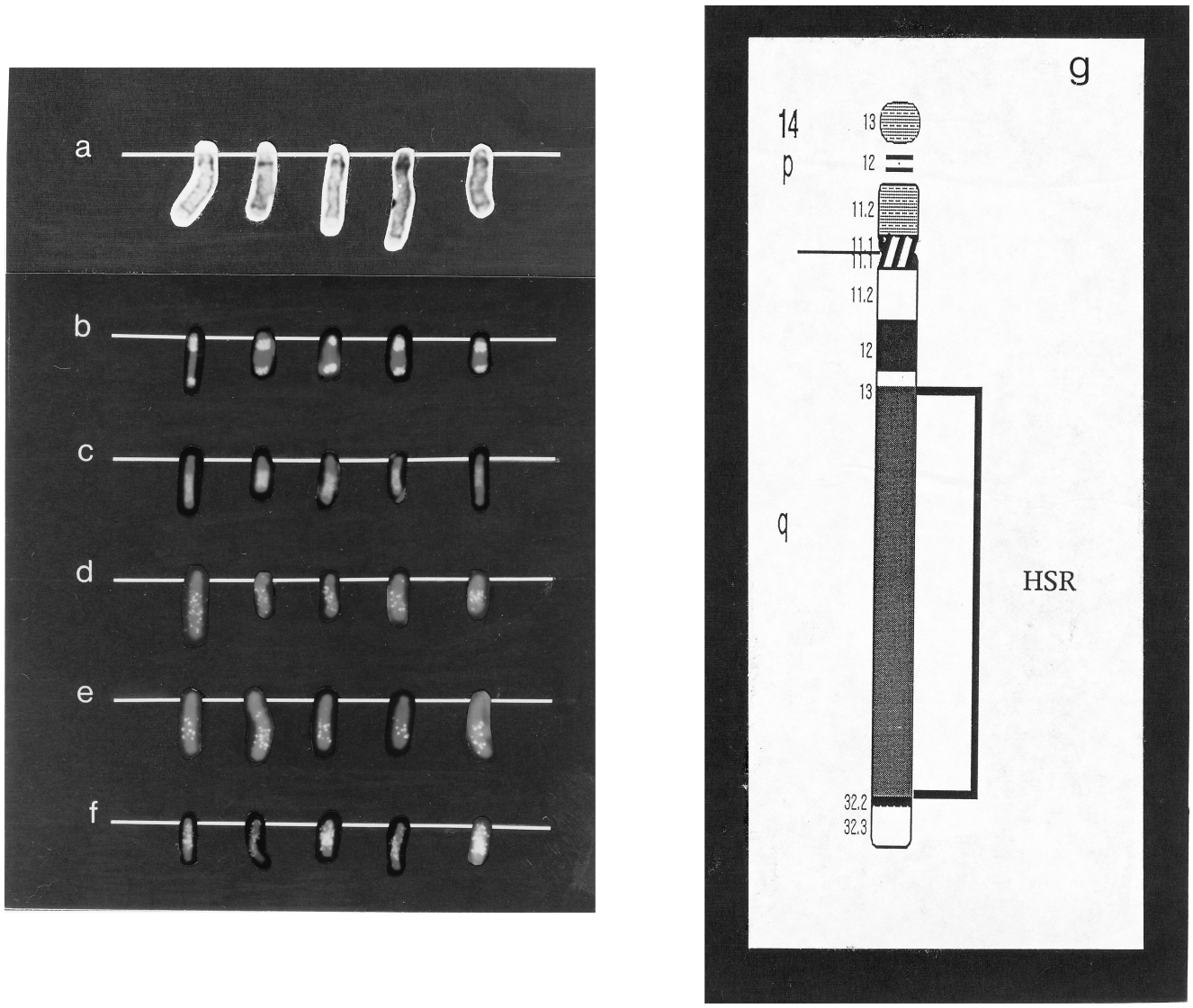

FIG. 3. Cytogenetic characterization of the derivative chromosome 14. (a) G-banded preparations of the derivative chromosome 14 present in the secondary microcell hybrid C2Rh1811-6. (b) FISH with the derivative chromosome 14 probed with a chromosome 14-specific paint (Oncor). (c) FISH with the derivative chromosome 14 probed with a chromosome 12-specific paint (Oncor). (d to f) FISH with the derivative chromosome 14 probed with human cosmid clones Cospil-4, Cos60, and Cos85, respectively. These human cosmid clones detect single-copy DNA in normal human DNA and amplified DNA in Rh18 and C2Rh1811-6 DNAs as assayed by Southern analysis (6a). (g) Schematic diagram of the derivative chromosome 14, showing the location of the HSR.

tumor types, including rhabdomyosarcomas (8, 9, 22, 35). Since the HSR present on the derivative chromosome 14 contains chromosome 12 sequences and since a gene (*GLI*) known to reside on chromosome 12, located at 12q13-14.3 (17), has been shown to be amplified in 1 of 13 rhabdomyosarcomas (35), we next determined whether genes located on chromosome 12 were present on the derivative chromosome 14. We have determined that *GLI*, *SAS*, *CHOP*, *LALB-A*, *RARG1*, *VDR*, *COL2A*, *CDK4*, and *MDM2* are present on the derivative chromosome 14 and show various levels of amplification. Figure 6 shows a representative Southern blot with *MDM2* as a probe. In addition, the following chromosome 12 genes do not reside on the derivative chromosome 14: *MYF-5*, *TEL*, *RAP1B*, *CDK-2*, *CCND2*, *SP1*, *HOXC5*, *WNT1*, *A2MR*, *ATF-1*, *BTG*, *ASCL1*, *PRPH*, and *ERBB-3*.

The results presented above indicate that a genetic locus present on the derivative chromosome 14 is capable of inhibiting muscle differentiation when transferred into C2C12 cells. One method for characterizing genes identified by purely genetic approaches is to analyze candidate genes that map to the same chromosomal position. Since a number of studies have demonstrated amplification and overexpression of cellular oncogenes in the DNA of tumors and since overexpression of a number of different oncogenes inhibits myogenesis (18, 29), we considered oncogenes present in the amplified region to be candidates for the inhibitory locus.

***MDM2* inhibits MyoD-dependent transcription.** To determine whether the failure to differentiate observed in C2(Rh18)-11 and its secondary microcell hybrids was due to an inability of MyoD to activate transcription, as well as to establish a simple assay system to test candidate genes, we assayed muscle-specific gene expression by transient transfection. The muscle-specific reporter constructs consisted of the CAT gene driven by either the muscle creatine kinase enhancer (MCKCAT) or four mul-

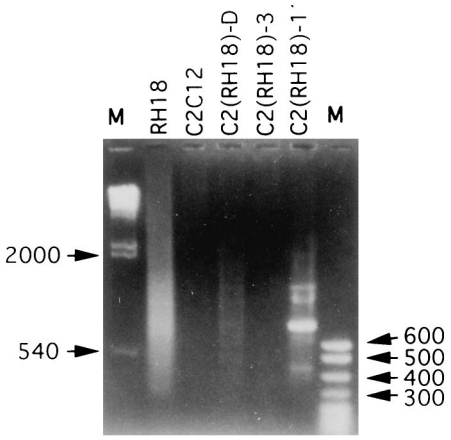

FIG. 4. Inter-*Alu* PCR products from the primary nonmuscle microcell hybrids. PCR was carried out on 1 μg of genomic DNA with a single *Alu* primer. The PCR products were separated by electrophoresis through a 1% agarose gel and visualized by ethidium bromide staining. Increasing the cycle number results in the generation of inter-*Alu* PCR products from C2(Rh18)-3 and C2(Rh18)-D (not shown). M, molecular size markers; numbers indicate base pairs.

timerized MyoD binding sites that were cloned upstream of the herpes simplex virus thymidine kinase promoter (4RCAT). Figure 7A shows that the secondary microcell hybrid, C2Rh1811-6, contains significantly less MCKCAT and 4RCAT activity than parental C2C12 cells, while the expression of RSVCAT in the two cell lines was equivalent. Therefore, the derivative chromosome 14 inhibits muscle differentiation and decreases muscle-specific gene expression.

To test the candidate genes for the ability to inhibit muscle differentiation, we have utilized the transient-transfection system. To date, we have assayed *CDK4*, *CHOP*, and *MDM2* for the ability to inhibit expression of MCKCAT and 4RCAT. We have not observed any significant effect of *CDK4* or *CHOP* in this assay (6a). In contrast, transfection of C2C12 cells with an *MDM2* expression vector (27) and various reporter constructs demonstrates that *MDM2* is capable of inhibiting expression of both MCKCAT and 4RCAT but has little or no effect on RSVCAT (Fig. 7B). To test whether *MDM2* inhibition of muscle-specific transcription is due to inhibition of MyoD, a second series of transient transfections was performed. 10T1/2 cells lack the ability to initiate transcription from muscle-specific promoters in the absence of exogenously added MyoD. Therefore, we assayed MyoD-dependent transactivation in 10T1/2 cells cotransfected with a MyoD expression vector and an *MDM2* expression vector. Figure 7C shows that *MDM2* represses MyoD-dependent transactivation of the 4RCAT and the MCKCAT constructs. Figure 7D shows that cotransfection of 10T1/2 cells with increasing amounts of the *MDM2* expression vector does not affect the level of MyoD RNA expressed from the MyoD expression vector. These results indicate that *MDM2* is a potent inhibitor of MyoD function.

Antisense *MDM2* restores MyoD-dependent transcription. One criterion for a candidate inhibitory gene is that it be expressed in the Rh18 cells, as well as in hybrids that retain the inhibitory locus. We assayed expression of *MDM2* in RNA isolated from Rh18 and the derivative chromosome 14 micro-

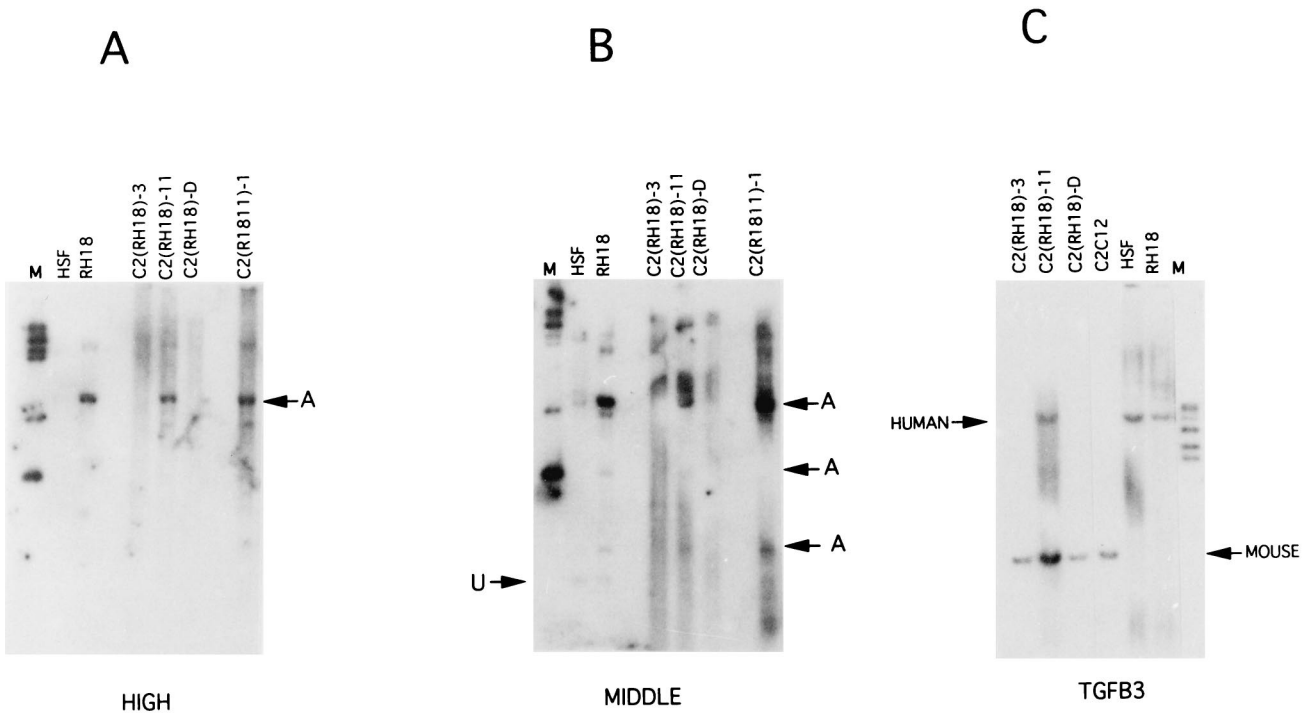

FIG. 5. Inter-*Alu* PCR products detect amplified DNA sequences. Southern blot hybridizations with 10 μg of genomic DNA are shown. Hybridizations were carried out in hybridization buffer overnight at 65°C. Probes were random prime labeled by using [^{32}P]dCTP. Arrow A, amplified DNA sequences detected by the probe; arrow U, unamplified DNA sequence detected by the probe. (A) Hybridization with pooled inter-*Alu* PCR products representing high-molecular-size weight products (1,000 to 2,000 bp). DNAs were extracted from primary HSFs; Rh18; the primary microcell hybrids C2(Rh18)-3, -11, and -D; and the secondary microcell hybrid C2R1811-1 [C2(Rh1811)-1]. (B) Hybridization with pooled inter-*Alu* PCR products representing middle-molecular-size products (500 to 1,000 bp). (C) Hybridization with *TGFB-3* as a probe. *TGFB-3* detects both mouse and human sequences. *TGFB-3* does not detect amplified DNA in Rh18 or in the microcell hybrid C2(Rh18)-11. Lanes M, molecular size marker.

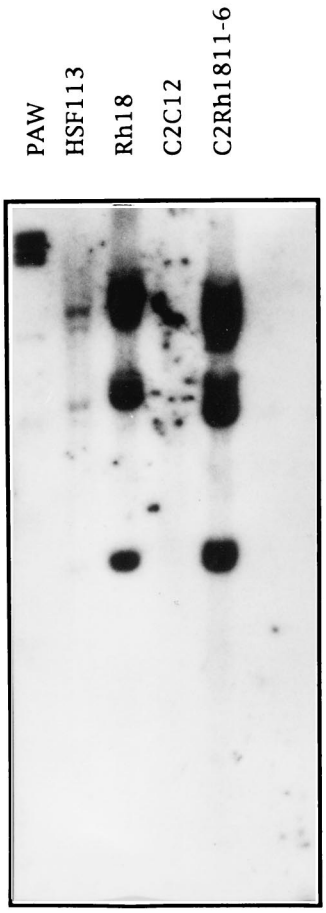

FIG. 6. *MDM2* is amplified in Rh18 and in the C2(Rh18)-11 microcell hybrids. Southern blot hybridizations with *MDM2* cDNA as a probe are shown. Ten micrograms of genomic DNA was extracted from primary HSFs (HSF113), Rh18, C2C12, and the secondary microcell hybrid C2Rh1811-6. PAW, molecular weight marker.

cell hybrids by Northern blot hybridization. Figure 8 shows that Rh18 and the secondary microcell hybrids C2Rh1811-6 and C2hiRh1811-0 express significantly more *MDM2* mRNA than control primary human myoblasts and primary HSFs. In contrast, the rhabdomyosarcomas Rh30, Rh28, RD, and RhJT, which display a recessive nondifferentiating phenotype (42), do not express high levels of *MDM2* mRNA. Furthermore, the recessive rhabdomyosarcomas do not contain *MDM2* DNA amplifications (6a, 16). In addition, a secondary microcell hybrid that retained myogenic potential, C2hiRh1811-E, does not retain *MDM2* amplification and does not express high levels of *MDM2* mRNA. Thus, overexpression of *MDM2* correlates with the presence of the inhibitory locus in Rh18 and in the microcell hybrids that fail to differentiate into muscle.

To further test whether *MDM2* is responsible for inhibition of MyoD function in our hybrid system, we tested whether expression of antisense *MDM2* could restore muscle-specific gene expression in the derivative chromosome 14 microcell hybrids. The *MDM2* cDNA was cloned in the antisense orientation under control of the cytomegalovirus promoter. This construct was cotransfected with either MCKCAT, 4RCAT, or RSVCAT into the secondary microcell hybrid C2Rh1811-6. Muscle-specific gene expression is restored when increasing amounts of antisense *MDM2* are transfected into these cells (Fig. 9). In addition, since the 4RCAT construct is activated by antisense *MDM2*, MyoD-dependent transcription is restored by antisense *MDM2*. We conclude that amplification and overexpression of *MDM2* inhibit MyoD function, resulting in a dominant nondifferentiating phenotype.

DISCUSSION

One obvious phenotype of tumor cells is a lack of terminal differentiation. Previously, we classified rhabdomyosarcoma cell lines as having either a recessive or a dominant nondifferentiating phenotype (42). To study the genetic basis of the dominant nondifferentiating phenotype of Rh18 cells, we utilized microcell fusion to transfer chromosomes from Rh18 cells into the mouse myoblast cell line C2C12. Rh18 chromosomes were tagged with a selectable marker and transferred into C2C12 cells. Two different nonmuscle phenotypes were obtained: one was characterized by a complete lack of expression of MyoD and myogenin, and another was characterized by continued expression of MyoD and myogenin. Karyotypic analysis indicates that a derivative chromosome 14 is responsible for the MyoD- and myogenin-positive nondifferentiating phenotype. The derivative chromosome 14 contains an amplification of chromosome 12 DNA sequences. *MDM2* is amplified and overexpressed in Rh18 and in the nondifferentiating microcell hybrids that retain the derivative chromosome 14. Forced expression of *MDM2* in C2C12 cells, and in 10T1/2 cells transfected with a MyoD expression vector, results in repression of MyoD-dependent transcription. In addition, expression of antisense *MDM2* in C2C12 hybrids containing the derivative chromosome 14 results in restoration of MyoD-dependent transcriptional activity and therefore in restoration of muscle-specific gene expression. We conclude that amplification and overexpression of *MDM2* in rhabdomyosarcomas lead to inhibition of MyoD-dependent transcription, resulting in a dominant nondifferentiating phenotype.

Gene amplification. Gene amplifications are easily detected in tumors and transformed cell lines, while normal diploid cells lack detectable gene amplifications (43). Gene amplifications are often responsible for drug resistance in cultured mammalian cells, and amplification of cellular oncogenes is often observed in tumors. In both cases, rare clones are selected because overexpression of genes through increased copy number is thought to confer a selective advantage (41). Detailed mapping studies of amplified DNA indicate that regions far larger than the selected gene are always amplified. The size of the amplification units can vary from a few hundred kilobases to as many as 10 Mb (41). In addition, other chromosomal abnormalities, such as inversions, translocations, and deletions, are often observed in conjunction with amplification. Given these observations, perhaps it is not surprising that the DNA amplification described here involves multiple genes encompassing a large region of genomic DNA, as well as involving two different chromosomes, 12 and 14.

Amplification of cellular proto-oncogenes has been described for a number of different tumor types, including rhabdomyosarcomas (8, 9, 22, 35). A high frequency of *MDM2* gene amplification has been observed in a variety of tumors, including bone and soft tissue sarcomas (27) and gliomas (34). We have shown here that a number of genes from chromosome 12, including *MDM2*, *GLI*, and *CDK4*, are coamplified in the rhabdomyosarcoma cell line Rh18. While it is not presently known how often *MDM2* becomes amplified in primary rhabdomyosarcomas, at least one other rhabdomyosarcoma cell line retains amplified *MDM2* (14a). Regardless, *MDM2* amplification is a common event in soft tissue sarcomas (16, 27) and therefore represents a frequent genetic alteration in the generation

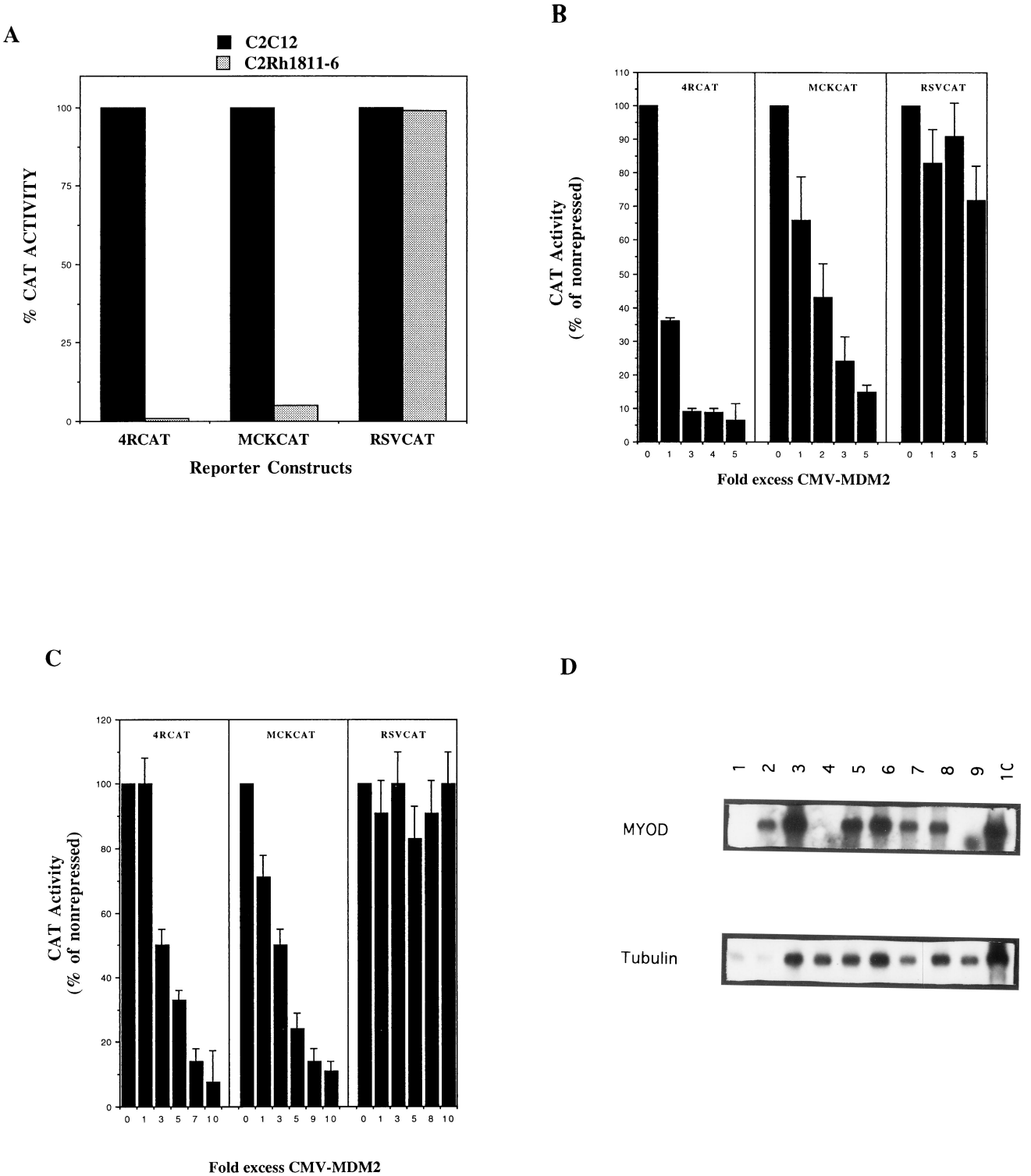

FIG. 7. *MDM2* represses MyoD-dependent transcription. (A) Parental C2C12 and the microcell hybrid C2Rh1811-6 were transfected with the muscle-specific reporter constructs MCKCAT and 4RCAT and the control plasmid RSVCAT. Cells were harvested after 72 h, and CAT assays were performed. The CAT activity expressed in C2C12 cells was set at 100%. C2Rh1811-6 cells expressed approximately 1% of the CAT activity from the 4RCAT construct and approximately 5% of the CAT activity from the MCKCAT construct. The CAT activity expressed from the control RSVCAT construct was similar in the two cell lines. The graph shows results of a representative experiment, with each experiment containing duplicate dishes. Duplicate dishes showed less than 10% difference. (B) C2C12 cells were cotransfected with an *MDM2* expression vector and the indicated reporter constructs. Increasing amounts of *MDM2*, designated as fold excesses with respect to the reporter construct, were added. Values represent the percentages of CAT activity obtained in the absence of *MDM2*. All transfections were kept at a total of 6 μg of DNA by substituting an empty expression vector for the *MDM2* expression vector. The values represent the averages from three separate experiments, with each experiment containing duplicate dishes. (C) 10T1/2 cells were transfected with 0.5 μg of a MyoD expression vector, 0.5 μg of the indicated reporter constructs, and increasing amounts of *MDM2*. MyoD induced CAT activity 60-fold on 4RCAT and 10-fold on MCKCAT compared with control transfections in the absence of MyoD. Increasing amounts of *MDM2*, designated as fold excesses with respect to the reporter construct, were added. Values represent the percentages of CAT activity obtained in the absence

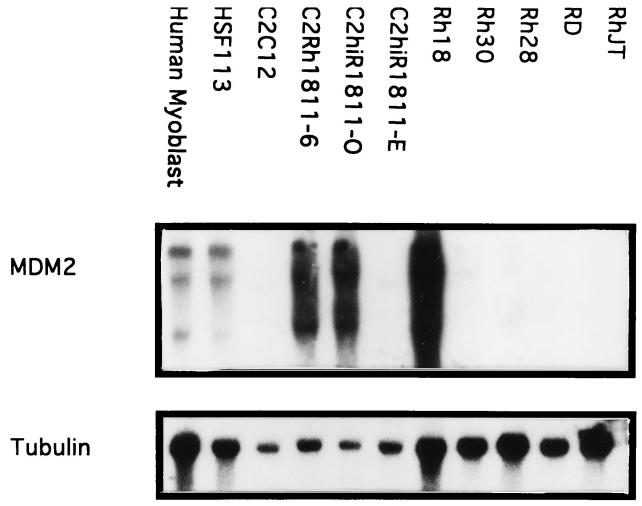

FIG. 8. *MDM2* is overexpressed in Rh18 and the derivative chromosome 14 microcell hybrids. Northern blot hybridizations to 5 μg of cytoplasmic RNAs extracted from primary human myoblasts; primary HSFs (HSF113); C2C12; the secondary microcell hybrids C2Rh1811-6, C2hiR1811-0, and C2hiR1811-E, Rh18; and the recessive rhabdomyosarcomas Rh30, Rh28, RD, and RhJT are shown. The probes were a full-length *MDM2* cDNA or α-tubulin. *MDM2* transcripts are detected from all cell lines following longer exposure (not shown).

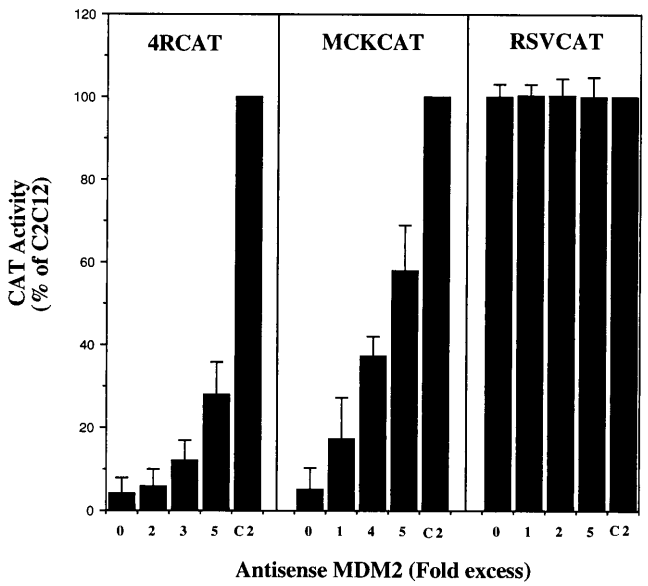

FIG. 9. Expression of antisense *MDM2* relieves repression of MyoD function. The microcell hybrid C2Rh1811-6 was transfected with increasing amounts of an antisense *MDM2* expression vector and 1 μg of the indicated reporter construct. Values represent fold activation by antisense *MDM2* compared with that with an empty expression vector. All transfections were kept at a total of 6 μg of DNA by substituting an empty expression vector for the antisense *MDM2* expression vector. The values represent the averages from three separate experiments, with each experiment containing duplicate dishes.

of tumors. Interestingly, a similar DNA amplification is present in the recessive rhabdomyosarcoma cell line Rh30. In contrast to Rh18 cells, however, Rh30 cells contain amplified *GLI* and *CDK4* but not *MDM2* (6a, 16). In addition, C2C12 microcell hybrids that retain the Rh30 *GLI* and *CDK4* amplified region continue to differentiate into muscle (42b). This result is consistent with our observation that *MDM2* amplification results in a dominant nondifferentiating phenotype expressed in Rh18 cells, while Rh30 cells display a recessive phenotype and lack amplification of *MDM2*. Thus, amplification of *GLI* and *CDK4* in Rh30 cells does not result in dominant inhibition of MyoD function. However, the functional consequences of *GLI* and *CDK4* amplification for other tumor phenotypes, such as genomic instability or altered growth rate, can be assayed by using the system described here.

***MDM2*, MyoD, and the cell cycle.** In addition to promoting tumor formation, anchorage-independent growth, and cellular immortalization, expression of transforming oncogenes inhibits cellular differentiation in several different cell lineages. In muscle cells, expression of oncogenic tyrosine kinases (v-src and v-fps), growth factor receptors (v-erbB), nuclear oncogene products (v-myc, c-myc, v-erbA, and E1A), and the activated forms of signal-transducing G proteins (H-ras and N-ras) can inhibit terminal differentiation to various extents (5, 7, 10, 14, 30, 31, 37, 45). We previously demonstrated that ras and fos prevent myogenesis by inhibiting expression of MyoD (19). Identification of the specific pathway by which each of these oncogenes and growth factors inhibits myogenesis may provide clues as to how MyoD integrates information coming from many aspects of cellular function.

The oncogenic properties of *MDM2* have been postulated to result from direct interaction with a number of cell cycle-regulatory proteins. *MDM2* interacts directly with p53 (27) and blocks p53-mediated transactivation by inhibiting the activation domain of p53 (3, 12, 23, 28, 46, 49). In addition, *MDM2* has been shown to interact directly with pRB, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of the pRB growth-regulatory function (47). Furthermore, *MDM2* interacts with the activation domain of E2F1, resulting in stimulation of E2F1/DP1 transcriptional activity (21). Taken together, these results suggest that *MDM2* not only relieves the proliferative block mediated by either p53 or pRB but also promotes proliferation by stimulating the S-phase-inducing transcriptional activity of E2F/DP1.

Differentiating muscle cells fuse to form multinucleated myotubes and permanently withdraw from the cell cycle. This process is controlled by regulatory interactions involving MyoD family members and various cell cycle proteins (18, 29). MyoD transactivation of muscle-specific genes requires wild-type pRB (11) or high levels of the pRB-related protein p107 (36). In addition, transcriptional activation by MyoD has been shown to be inhibited by high levels of the G_1-phase cyclin, cyclin D1 (33, 40). Furthermore, cyclin D1 interacts with and promotes phosphorylation of pRB, resulting in stimulation of E2F/DP1 activity (15). While the mechanism by which *MDM2* inhibits MyoD function remains unknown, it seems reasonable to speculate that *MDM2* either directly interferes with MyoD transactivation, as it does for p53, or inhibits MyoD indirectly

of *MDM2*. All transfections were kept at a total of 6 μg of DNA by substituting an empty expression vector for the *MDM2* expression vector. The values represent the averages from three separate experiments, with each experiment containing duplicate dishes. (D) 10T1/2 cells were cotransfected as described for panel C, and RNA was extracted and processed for Northern blot hybridization. The probes were a full-length MyoD cDNA or α-tubulin. Lanes: 1, 4RCAT; 2, 4RCAT and MyoD; 3, 4RCAT and MyoD; 4, MCKCAT; 5, MCKCAT and MyoD; 6, MCKCAT, MyoD, and 0.5 μg of *MDM2*; 7, MCKCAT, MyoD, and 2.5 μg of *MDM2*; 8, MCKCAT, MyoD, and 4.0 μg of *MDM2*; 9, pSPORT2; 10, control C2C12 RNA (5.0 μg).

by stimulating S-phase-promoting factors such as E2F/DP1 and/or cyclin D1 or by blocking pRB mediated MyoD transactivation. Regardless, the system described here, which combines somatic cell and molecular genetics, should allow for the characterization of the molecular mechanisms functioning in rhabdomyosarcomas to inhibit differentiation.

ACKNOWLEDGMENTS

We thank P. Houghton for providing rhabdomyosarcoma cell lines and B. Vogelstein for the *MDM2* expression vector. We also thank D. Goodman, R. M. Liskay, and E. Lewis for critical reviews of the manuscript.

M.J.T. was supported by grants from the American Cancer Society and the Life and Health Insurance Medical Research Fund, and S.J.T. was supported by grants from the American Cancer Society and the Markey Molecular Medicine Center at the University of Washington.

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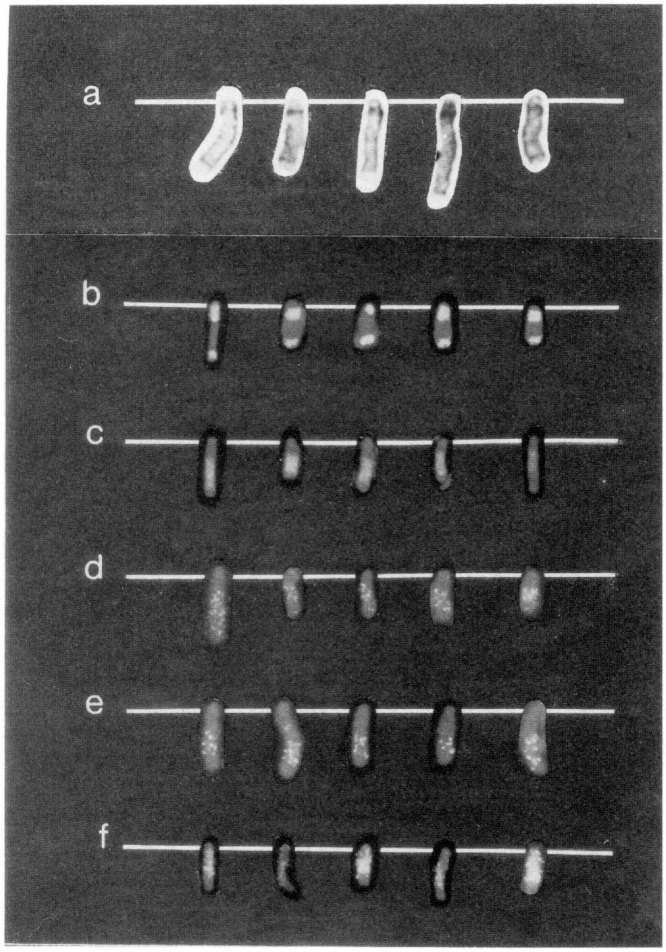
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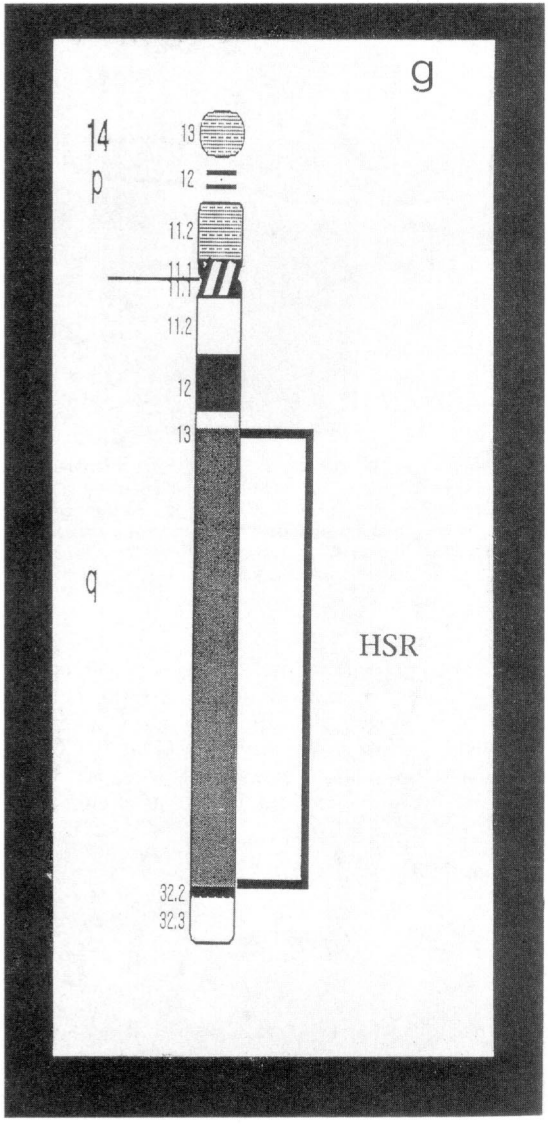
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