Skeletal Muscle Cells Lacking the Retinoblastoma Protein Display Defects in Muscle Gene Expression and Accumulate in S and G_2 Phases of the Cell Cycle

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Abstract. Viral oncoproteins that inactivate the retinoblastoma tumor suppressor protein (pRb) family both block skeletal muscle differentiation and promote cell cycle progression. To clarify the dependence of terminal differentiation on the presence of the different pRb-related proteins, we have studied myogenesis using isogenic primary fibroblasts derived from mouse embryos individually deficient for pRb, p107, or p130. When ectopically expressed in fibroblasts lacking pRb, MyoD induces an aberrant skeletal muscle differentiation program characterized by normal expression of early differentiation markers such as myogenin and p21, but attenuated expression of late differentiation markers such as myosin heavy chain (MHC). Similar defects in MHC expression were not observed in cells lacking either p107 or p130, indicating that the defect is specific to the loss of pRb. In contrast to wild-type, p107-deficient, or p130-deficient differentiated myocytes that are permanently withdrawn from the cell cycle, differentiated myocytes lacking pRb accumulate in S and G_2 phases and express extremely high levels of cyclins A and B, cyclin-dependent kinase (Cdk2), and Cdc2, but fail to readily proceed to mitosis. Administration of caffeine, an agent that removes inhibitory phosphorylations on inactive Cdc2/cyclin B complexes, specifically induced mitotic catastrophe in pRb-deficient myocytes, consistent with the observation that the majority of pRb-deficient myocytes arrest in S and G_2. Together, these findings indicate that pRb is required for the expression of late skeletal muscle differentiation markers and for the inhibition of DNA synthesis, but that a pRb-independent mechanism restricts entry of differentiated myocytes into mitosis.

SKELETAL muscle differentiation involves the coordinated activation of muscle-specific gene expression and cessation of cell division, resulting in the formation of terminally differentiated myotubes. Studies of the past decade have demonstrated that the myogenic basic-Helix-Loop-Helix (bHLH)[1] proteins (i.e., MyoD, Myf-5, myogenin, and MRF-4) act in cooperation with the MEF2 family of transcription factors to activate muscle-specific gene expression (for reviews see Buckingham, 1992; Wright, 1992; Emerson, 1993; Sassoon, 1993; Weintraub, 1993; Lassar and Münsterberg, 1994; Olson and Klein, 1994). Myf-5 and MyoD proteins are present in proliferating myoblasts and initiate a cascade of events resulting in terminal skeletal muscle differentiation when myoblasts are deprived of mitogens (for review see Lassar et al., 1994). Several hypotheses have been proposed to explain how the activity of the myogenic bHLH proteins is restrained in proliferating myoblasts; these include inhibitory phosphorylation of the myogenic bHLH factors (Li et al., 1992; Hardy et al., 1993), inhibition of myogenic bHLH function via the Id family of dominant-negative bHLH factors (Benezra et al., 1990; Jen et al., 1992), and either direct or indirect inhibition by cyclin D–dependent kinases (Rao et al., 1994; Rao and Kohtz, 1995; Skapek et al., 1995). A role for cyclin D–dependent kinases in controlling MyoD function is supported by the finding that ectopic expression of p16, a specific inhibitor of cyclin-D/Cdk4 and Cdk6 kinases (Serrano et al., 1993), or p21, a more universal inhibitor of cyclin-dependent kinases (Cdks) (Gu et al., 1993*b*; Harper et al., 1993; Xiong et al., 1993), can activate MyoD function in serum-stimulated myoblasts (Skapek et al., 1995).

It has been well established that the D-type cyclins,

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1. *Abbreviations used in this paper*: bHLH, basic-Helix-Loop-Helix; BrdU, 5-bromo-2′-deoxyuridine; CAT, chloramphenicol acetyl transferase; Cdk, cyclin-dependent kinase; CMV, cytomegalovirus; DAPI, 4′, 6′-diamidino-2-phenylindole; MCK, muscle creatine kinase; MEFs, mouse embryonic fibroblasts; MHC, myosin heavy chain; pRb, retinoblastoma tumor suppressor protein.

when bound to Cdk4 or Cdk6, can phosphorylate and inactivate the product of the retinoblastoma tumor suppressor gene, pRb (for reviews see Sherr, 1994*a*,*b*; Weinberg, 1995). Retinoblastoma family proteins (i.e., pRb, p107, and p130) are important regulators of the cell cycle (for reviews see Sherr 1994*a*,*b*; Müller, 1995; Weinberg, 1995) and have been implicated as mediators of both cellular growth arrest and myogenic bHLH activity in skeletal muscle. The importance of this regulatory family in promoting terminal skeletal muscle differentiation was first suggested by the finding that expression of oncoproteins known to inactivate the pRb family, such as SV-40 large T antigen, polyoma virus large T antigen, and adenovirus E1A, can both block differentiation and induce DNA synthesis in normally quiescent myotubes (Fogel and Defendi, 1967; Yaffe and Gershon, 1967; Graessmann et al., 1973; Webster et al., 1988; Endo and Nadal-Ginard, 1989; Enkemann et al., 1990; Iujvidin et al., 1990; Braun et al., 1992; Mymryk et al., 1992; Cardoso et al., 1993; Caruso et al., 1993; Gu et al., 1993*a*; Taylor et al., 1993; Ohkubo et al., 1994; Crescenzi et al., 1995). Further studies have demonstrated that upon muscle differentiation, the expression of pRb increases (Coppola et al., 1990; Endo and Goto, 1992; Martelli et al., 1994), and pRb assumes a hypophosphorylated, activated state that is tightly tethered to the nucleus (Gu et al., 1993*a*; Thorburn et al., 1993). In addition, DNA binding complexes containing members of the E2F and DP transcription factor families are found associated with both pRb and p130 after muscle differentiation (Corbeil et al., 1995; Halevy et al., 1995; Kiess et al., 1995*a*; Shin et al., 1995), suggesting that both pRb and p130 may regulate E2F function in differentiated skeletal muscle. Furthermore, MyoD-mediated transactivation of muscle genes in SAOS-2 osteosarcoma cells, which lack functional pRb, requires coexpression of either exogenous wild-type pRb (Gu et al., 1993*a*) or p107 (Schneider et al., 1994), indicating that MyoD function may be regulated by members of the pRb family. Finally, pRb is required for terminal cell cycle withdrawal, as loss of pRb through targeted gene inactivation prevents the normal G_0 arrest of skeletal muscle (Schneider et al., 1994).

Whereas the above studies have suggested that pRb plays a positive role during skeletal muscle differentiation, mice specifically lacking *Rb* form grossly normal skeletal muscle (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994), suggesting that pRb-related proteins may be able to compensate for the loss of pRb in the development of skeletal muscle. To further clarify the role of the pRb family in skeletal muscle differentiation, we have examined both MyoD-mediated induction of various skeletal muscle proteins and MyoD-mediated cell cycle withdrawal in cells engineered to specifically lack either pRb, p107, or p130. We have found that pRb-deficient muscle cells can activate normal levels of "early" differentiation markers (i.e., myogenin and p21); however, expression of "late" differentiation markers (i.e., myosin heavy chain) is attenuated in these cells, and the cells fail to exit the cell cycle. These differentiation defects appeared to be specific to the loss of pRb, as MyoD was able to fully induce the muscle differentiation program and promote terminal cell cycle arrest in the absence of either p107 or p130.

Ectopic expression of pRb family members can significantly augment MyoD-mediated transactivation of a muscle creatine kinase–chloramphenicol acetyl transferase (MCK-CAT) reporter and prevent abnormal DNA synthesis in pRb-deficient cells, consistent with a role for this family in promoting the later stages of muscle differentiation. In contrast, ectopic expression of Cdk inhibitors can neither significantly augment MyoD function nor prevent S phase entry in myocytes lacking pRb, suggesting that a primary function of Cdk inhibitors in skeletal muscle may be to maintain the hypophosphorylated, activated state of pRb. Finally, we demonstrate that pRb-deficient myocytes inappropriately enter S phase but fail to readily progress into mitosis, even though these cells express extremely high levels of cyclins A and B, Cdk2, and Cdc2. Since caffeine administration, which removes inhibitory phosphorylations on inactive Cdc2/cyclin B complexes (Steinmann et al., 1991; Smythe and Newport, 1992), specifically increased the frequency of mitotic catastrophe in differentiated pRb-deficient myocytes, we propose that the majority of these cells have arrested in S and G_2 phases and contain Cdc2/cyclin B complexes that are maintained in a nonfunctional state by inhibitory phosphorylation. Thus, during the process of skeletal muscle differentiation, MyoD activates cell cycle–arrest mechanisms that block cell cycle progression in a pRb-dependent manner in G_0 and in a pRb-independent manner in M phase.

Materials and Methods

Cell Culture

Primary embryonic fibroblasts were isolated from wild-type and homozygous mutant mouse embryos at 13 d of gestation, according to the protocol of Robertson (1987). Briefly, embryos were dissected and the carcass minced in trypsin (0.25%) for 45 min before plating in a T-75 flask. The adherent fibroblasts were passaged once before freezing at a density of 3×10^6 cells per vial (Robertson, 1987). Primary embryonic fibroblast cultures were generally free of contaminating myoblasts and were used at passages 3–8. Cells were grown in DME supplemented with 10% FBS (Hyclone Laboratories, Logan, VT) for primary fibroblasts or 20% FBS for C2 cells and fibroblast cell lines stably expressing MyoD. All cells were differentiated in DME containing 2% horse serum and 10 μg/ml insulin (differentiation medium [DM]) for 2–4 d, unless otherwise noted.

Production of MyoD Retrovirus

BOSC 23 ecotropic retrovirus packaging cells (Pear et al., 1993) were generously provided by W. Pear (Massachusetts Institute of Technology) and were grown in GPT selective media (Mulligan, 1981) until the time of transfection when the media was changed to DME supplemented with 10% FBS. 2×10^6 BOSC 23 cells were seeded onto 60-mm tissue culture dishes in DME supplemented with 10% FBS and grown for ~24 h. The culture media was then replaced with 4 ml of DME supplemented with 10% FBS and 25 μM chloroquine just before transfection. Two plasmids encoding MyoD retrovirus were used, pLMDSN (Weintraub et al., 1989) and pBabe-MyoD, which is the 1.7-kb EcoRI fragment containing the mouse MyoD gene from pVZC11α (Davis et al., 1987) cloned into the EcoRI site of the vector pBabe-Puro (Morgenstern and Land, 1990). 10 μg of plasmid DNA was mixed with $CaCl_2$, added dropwise to 2× Hepes buffered saline, pH 7.05, in a final volume of 500 μl, and then added immediately to the above media. Cells were incubated for 8–10 h at 37°C, 5% CO_2, washed once with PBS, given fresh DME supplemented with 10% FBS without chloroquine, and incubated for an additional 16 h. Media was again removed and replaced with 3 ml of DME with 10% FBS per 60-mm dish. Virus-containing media was harvested from the BOSC 23 cells ~24 h later (48 h after the addition of DNA), centrifuged at 500 *g* to remove cells and debris, and then used for infection immediately or frozen at −80°C for later use.

Viral Infection, Transfection, and Myogenic Conversion of Embryonic Fibroblasts

$1.5–1.75 \times 10^5$ mouse embryonic fibroblasts (MEFs) were seeded onto 60-mm dishes the day before infection or transfection. In experiments where primary cultures of MEFs were transiently infected with retroviral MyoD and muscle gene expression was analyzed by immunoblotting, 1×10^6 MEFs were seeded onto 150-mm dishes. For transient viral infection, cells were incubated for 24–48 h with a 1:1 mixture of virus-containing media from BOSC 23 cells and DME containing 20% FBS, supplemented with polybrene to a final concentration of 4 μg/ml. After infection, cells were grown in DM for 2–4 d to induce differentiation. For the generation of stably infected cell lines, cells were infected as described above, trypsinized, seeded at low culture density, and grown under selective conditions (0.4 mg/ml G418 for pLMDSN viral infections and 1.5 μg/ml puromycin for pBabe-MyoD infections) in DME supplemented with 20% FBS. Isolated colonies were then picked and propagated under selective growth conditions. For the time course experiments, 7.5×10^4 $Rb^{-/-}$ MEF were seeded into 60-mm dishes and infected the next day as described above. After 2 d, the viral mixture was removed and changed to DME containing 0.1% FBS and 10 μg/ml insulin, and cells were maintained in this media for another 3 d. Cultures were then stimulated with DME containing 20% FBS, and cells were fixed at the indicated time points. For the 0 time point, 5-bromo-2′-deoxyuridine (BrdU) was added to the low-serum differentiation media and maintained on the cells for 6 h.

For MyoD transactivation experiments, 0.5 μg of pCSA-MyoD plasmid (Skapek et al., 1995) was mixed with 0.5 μg of p-3300-MCK-CAT (Jaynes et al., 1988) or p4x(MEF1)-TK-CAT (Weintraub et al., 1990) reporter plasmids and 1.0 μg of cytomegalovirus (CMV) expression vehicles encoding either pRb (Qin et al., 1992), p107 (Zhu et al., 1993), p130 (Vairo et al., 1995), p16 (Skapek et al., 1995), p21 (Harper et al., 1993), or an empty CMV vehicle. Plasmids were mixed in DME with 10 μl of lipofectamine reagent (GIBCO BRL, Gaithersburg, MD) and incubated with the cells for 2–3 h, according to the manufacturer's instructions. All transfections were done in duplicate. DM was added ~48 h after the addition of DNA to the cells, and cells were maintained under these conditions for an additional 2–4 d. Cells were then harvested for CAT assay, normalized to protein content, as previously described (Skapek et al., 1995). Transient transfection assays involving BrdU uptake were performed essentially as described above.

Immunostaining of Converted Fibroblasts

After differentiation, cells were washed three times with PBS, fixed in 2% paraformaldehyde in PBS for 15 min, and permeabilized with 0.25% Triton X-100 in PBS for an additional 15 min. MyoD was then detected using rabbit antiserum (Tapscott et al., 1988) at a dilution of 1:250 in a blocking solution containing 5% goat serum, 1% horse serum, and 0.1% Triton X-100 in PBS and visualized using rhodamine-conjugated donkey anti–rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:250. Myosin heavy chain (MHC) was detected using a 1:10 dilution of hybridoma-conditioned media containing the mouse monoclonal IgG_{2b} antibody MF-20 (kindly provided by D. Fischman, Cornell University Medical College, New York; Bader et al., 1982) and a 1:250 dilution of fluoroscein-conjugated donkey anti–mouse IgG antibodies (Jackson ImmunoResearch Laboratories). The following additional antibodies and dilutions were used: cyclin A, a 1:500 dilution of affinity purified rabbit antibodies (kindly provided by J. Roberts, Fred Hutchinson Cancer Center); cyclin B, a 1:500 dilution of affinity purified rabbit antibodies (kindly provided by C. McGowan, The Scripps Research Institute, La Jolla, CA), or a 1:100 dilution of commercially available affinity purified rabbit antibodies (H-433; Santa Cruz Biotechnology Inc., Santa Cruz, CA); cyclin E, a 1:1,000 dilution of rabbit antiserum (kindly provided by J. Roberts); Cdk2, a 1:50 dilution of affinity purified rabbit antibodies (M2; Santa Cruz Biotechnology, Inc.); Cdc2, a 1:200 dilution of affinity purified rabbit antibodies (GIBCO BRL). Stained cells were examined and photographed using a Zeiss Axiovert microscope (Thornwood, NY) equipped with a 40× water-immersion objective.

Western Analysis

Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, and 20% glycerol with 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin and pepstatin [10 μg/ml each], aprotinin [20 μg/ml], and phosphatase inhibitors [10 mM $NaPPO_4$, 1 mM NaF, and 0.1 mM Na_3VO_4]). Cell extracts were sonicated using a Branson sonicator (Branson Ultrasonics Corp., Danbury, CT), clarified by centrifugation, and frozen at −80°C. Extracts were normalized to DNA content by DNA fluorometry (Hoefer Scientific Instrs., San Francisco, CA), and proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. The following antibodies and dilutions were used for immunoblotting: MyoD, a 1:500 dilution of purified monoclonal anti-MyoD antibody 5.8A (kindly provided by P. Dias and P. Houghton, St. Jude Children's Research Hospital); myogenin, a 1:10 dilution of hybridoma-conditioned medium containing the monoclonal antimyogenin antibody F5D (kindly provided by W. Wright, University of Texas Southwestern Medical Center, Houston, TX); MHC, a 1:10 dilution of hybridoma-conditioned medium containing the monoclonal anti-MHC antibody MF20; p21, a 1:1,000 dilution of rabbit antiserum to mouse p21 (Halevy et al., 1995); p27, a 1:2,000 dilution of rabbit antiserum to mouse p27 (kindly provided by J. Roberts); pRb, a 1:1,000 dilution of affinity purified monoclonal antibody to pRb (PMG3-245; PharMingen, San Diego, CA); p107, a 1:20 dilution of hybridoma-conditioned medium containing the monoclonal anti-p107 antibody SD9 (kindly provided by N. Dyson, Massachusetts General Hospital Cancer Center); p130, a 1:200 dilution of affinity purified rabbit antibodies to p130 (C-20; Santa Cruz Biotechnology, Inc.). Proteins were visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

DNA Synthesis Assay

After differentiation, cells were stimulated with DME containing 20% FBS and 10 μM BrdU (Boehringer Mannheim Corp., Indianapolis, IN). Cells were washed three times with PBS, fixed for 10 min in ice-cold AFA (70% ethanol, 3.7% formaldehyde, 5% glacial acetic acid), and then washed several times more with PBS. MHC was detected using a 1:10 dilution of the anti-MHC antibody MF-20 and a 1:250 dilution of rhodamine-conjugated donkey anti–mouse IgG antibodies (Jackson ImmunoResearch Laboratories). After the secondary antibody incubation step, immune complexes were fixed for 10 min with 2% paraformaldehyde, and DNA was then denatured by incubation of the cells in 2 N HCl, 0.2% Triton X-100 for 10 min. Cells were washed with PBS, incubated with 50 mM glycine in PBS for 5 min, and washed again with PBS. BrdU was detected using either a 1:30 dilution of a FITC-conjugated monoclonal antibody against BrdU or a 1:2,000 dilution of a mouse monoclonal IgG_1 antibody to BrdU (G3G4) and a 1:200 dilution of biotinylated-goat anti–mouse IgG_1-specific antibodies (Southern Biotechnology Associates, Inc. Birmingham, AL) followed by incubation with a 1:250 dilution of fluorescently labeled avidin D (Vector Laboratories, Inc., Burlingame, CA) and 1 μg/ml 4′, 6′-diamidino-2-phenylindole (DAPI) in PBS.

Flow Cytometry

Myogenic cell lines were differentiated for 2–3 d in low-calcium differentiation medium consisting of $CaCl_2$-free DME (GIBCO BRL) reconstituted with $CaCl_2$ to a final concentration of 50 μM and supplemented with 2% dialyzed horse serum and 10 μg/ml insulin. Under these culture conditions, ~98% of C2 muscle cells expressing MHC remained mononucleated. Cultures were then trypsinized and resuspended in PBS containing 1% horse serum, centrifuged at 800 *g* for 5 min at 4°C, and washed several times with PBS. Cells were fixed in PBS containing 2% paraformaldehyde for 15 min on ice, incubated with 50 mM glycine for an additional 5 min, permeabilized in 0.1% Triton X-100 in PBS for 15 min, washed several times with PBS, and then stained for MHC as described above. After antibody staining, cell pellets were resuspended in a propidium iodide solution (69 μM propidium iodide, 38 mM sodium citrate) to a concentration of approximately 1×10^6 cells/ml, RNase A was added to a final concentration of 10 μg/ml, and samples were incubated for 30 min at 37°C. Cells were then examined for fluoroscein and propidium iodide fluorescence using a Becton-Dickinson FACScan® flow cytometer (Becton-Dickinson Immunocytometry Sys., Mountain View, CA) and analyzed using Lysis II and CellFit software (Becton-Dickinson Immunocytometry Sys.). Negative controls included nonmyogenic cell lines and primary embryonic fibroblasts processed as described above and myogenic cell lines stained with the secondary antibodies alone.

Caffeine Treatment

Established myogenic cell lines were serum-starved for 3 d in DME containing 0.1% FBS plus 10 μg/ml insulin. Cells were then stimulated with high-serum medium (DME containing 20% FBS) for 12 h and then grown

for an additional 6 h in either the presence or absence of 5 mM caffeine in DME containing 20% FBS. Cells were fixed in 2% paraformaldehyde in PBS and stained for MHC and DAPI as described above.

Results

pRb Is Necessary for High-Level Expression of a Subset of Muscle Genes

To examine the requirement of pRb family members for skeletal muscle differentiation, we infected primary MEFs obtained from wild-type mouse embryos (*WT*) or embryos lacking both copies of either the *Rb* gene ($Rb^{-/-}$; Jacks et al., 1992), the p107 gene ($p107^{-/-}$), or the p130 gene ($p130^{-/-}$), with a retrovirus encoding MyoD. After infection, the cells were challenged to differentiate by serum removal, and both MyoD expression and muscle differentiation in the various infected fibroblast populations were examined by immunocytochemistry. Ectopic expression of MyoD induced expression of MHC in each of the fibroblast genotypes tested (Fig. 1). However, we consistently observed intense MHC staining with wild-type, $p107^{-/-}$, and $p130^{-/-}$ fibroblasts (Fig. 1, *B*, *H*, and *K*) and relatively weak MHC staining in cells lacking pRb (Fig. 1 *E*), even though the expression levels of MyoD were comparable in these cell populations (Fig. 1 *A*, *D*, *G*, and *J*). In addition, the resultant pRb-deficient myocytes formed smaller myotubes that predominantly contained one or two nuclei in comparison with wild-type, $p107^{-/-}$, or $p130^{-/-}$ myocytes, which frequently contained several nuclei, suggesting that myocyte fusion may also be impaired in the absence of pRb.

To better characterize the defects in pRb-deficient myocytes, we generated cell lines stably expressing MyoD. Western analysis of a representative cell line lacking pRb ($Rb^{-/-}$-MyoD) demonstrated that MyoD protein accumulated to high levels in these cells and could induce expression of myogenin, an early marker of the muscle differentiation program (Hollenberg et al., 1993; Andrés and Walsh, 1996), to a level comparable to that in a pRb-positive 10T1/2 fibroblast cell line expressing retroviral MyoD (10T1/2-MyoD) (Fig. 2 *A*, compare lanes *2* and *4*). The Cdk inhibitor p21, another early marker of the skeletal muscle differentiation program (Guo et al., 1995; Halevy et al., 1995; Missero et al., 1995; Parker et al., 1995; Andrés and Walsh, 1996), was similarly induced to wild-type levels in the absence of pRb (Fig. 2 *A*). In contrast to the normal levels of expression of these early markers of muscle differentiation, expression of MHC, which is induced at relatively late times in the skeletal muscle differentiation program (Gunning et al., 1987; Lin et al., 1994; Andrés and Walsh, 1996; see also Ohkubo et al., 1994), was dramatically reduced in cells lacking pRb (Figs. 1 and 2). To confirm that these findings were not specific to these individual cell lines, we transiently infected both *WT* and $Rb^{-/-}$ MEFs with a MyoD retrovirus and analyzed the relative expression levels of muscle differentiation markers in these polyclonal populations. MyoD activated the expression of both myogenin and p21 in pRb-deficient MEFs to levels that were equal to or even greater than that observed in *WT* MEFs, but failed to induce high level MHC expression specifically in the pRb-deficient cells (Fig. 2 *B*). Together, these findings indicate that while pRb is not essential for either the initiation of the skeletal muscle differentiation program or the induction of early muscle proteins such as myogenin and p21, pRb is necessary to promote high-level expression of some end-stage differentiated muscle proteins such as MHC.

Since the pRb-related proteins p107 and p130 may also be involved in the regulation of muscle differentiation, we examined the expression of these proteins in myogenic cell lines that either contained or lacked pRb. In pRb-positive 10T1/2-MyoD cells, p107 protein levels appeared to decline slightly under differentiation conditions, whereas p107 increased in the comparable pRb-deficient muscle cell line (Fig. 2 *A*; see also Schneider et al., 1994). Consistent with its increased presence in E2F complexes in differentiated muscle cells (Corbeil et al., 1995; Halevy et al., 1995; Kiess et al., 1995*a*; Shin et al., 1995), total protein levels of p130 increased upon serum starvation in both the pRb-positive and -negative muscle cell lines (Fig. 2 *A*, compare lanes *1* and *2*; lanes *3* and *4*). However, we also observed an increase in p107 and p130 expression in pRb-deficient fibroblasts that lacked MyoD expression (data not shown), suggesting that the increases in p107 and p130 protein are not specific to muscle differentiation. In summary, although p107 and p130 levels are increased in pRb-deficient muscle cell cultures, the reduced MHC expression in these cells indicates a specific role for pRb in the regulation of the myogenic differentiation program.

pRb Family Proteins but Not Cdk Inhibitors Augment the Activity of MyoD

As an independent means of evaluating the requirement of pRb for MyoD function, we transiently cotransfected pRb-deficient MEFs with plasmids encoding MyoD under the control of the cytomegalovirus promoter (CMV-MyoD) and a reporter construct containing the regulatory sequences of the muscle creatine kinase gene driving expression of chloramphenicol acetyl transferase (MCK-CAT). When transfected into pRb-deficient fibroblasts, CMV-MyoD did not significantly activate expression of MCK-CAT (Fig. 3 *A*, lane *3*), even when coexpressed with CMV-driven E12, a bHLH binding partner of MyoD (Fig. 3 *A*, lane *4*). In contrast, expression of MCK-CAT was increased 23–35-fold when a pRb expression vehicle (CMV-pRb) was cotransfected with MyoD and E12 (Fig. 3 *A*, lane *5*). In the absence of MyoD, pRb did not activate MCK-CAT expression in these cells (data not shown).

MCK gene expression requires binding sites for both myogenic bHLH proteins and MEF2 family members (Cserjesi and Olson, 1991; Amacher et al., 1993); therefore, it was unclear from these results whether pRb was affecting the function of MyoD or other ancillary transcription factors such as MEF2. To ascertain whether pRb directly affected the activity of MyoD, we assayed the ability of MyoD to induce the expression of a simplified muscle reporter construct containing four reiterated MyoD binding sites (MEF1 sites) upstream of a minimal thymidine kinase promoter driving CAT (4×[MEF1]-TK-CAT) in pRb-deficient cells (Fig. 3 *A*). In either the absence or presence of ectopic E protein, MyoD activated this reporter poorly in cells lacking pRb (Fig. 3 *A*, lanes *8* and *9*). However, cotransfection of MyoD and E12 with CMV-

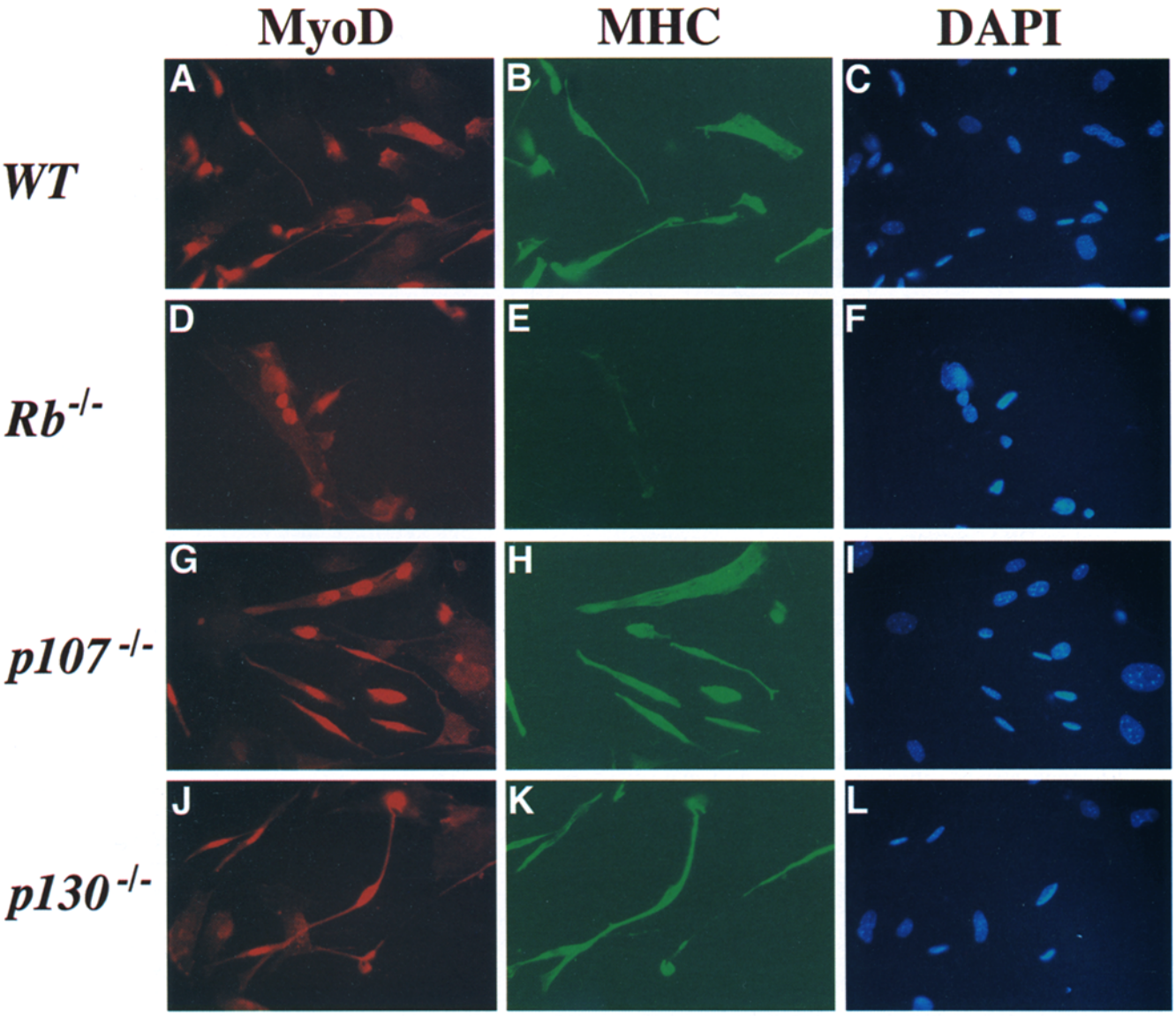

Figure 1. Attenuated expression of myosin heavy chain in differentiated myocytes lacking pRb. MEFs were infected with an ecotropic retrovirus encoding MyoD driven by a constitutive viral promoter and then differentiated for 3 d. After differentiation, cells were fixed, permeabilized, and immunostained for MyoD (*A*, *D*, *G*, and *J*) and MHC (*B*, *E*, *H*, and *K*). Nuclei were visualized by staining with DAPI (*C*, *F*, *I*, and *L*).

pRb led to a 3–10-fold increase in 4×(MEF1)-TK-CAT expression in $Rb^{-/-}$ cells (Fig. 3 *A*, lane *10*). Since cotransfected CMV-pRb only slightly enhanced the activity of CMV-CAT (approximately twofold; data not shown) and does not greatly affect MyoD protein levels (Skapek, S., and A.B. Lassar, data not shown), the dependency on ectopic pRb to promote high-level gene expression in $Rb^{-/-}$ cells appears to be specific to MCK-CAT, 4×(MEF1)-TK-CAT and other muscle-specific reporter constructs (data not shown). At present, it is unclear why pRb augments expression of all transfected muscle reporter constructs yet is required for high level expression of only late markers of the endogenous muscle differentiation program.

Because p107 and p130 have several biochemical properties in common with pRb (for reviews see Müller, 1995; Vairo et al., 1995; Weinberg, 1995; Zalvide and DeCaprio, 1995), we tested whether higher-level expression of these proteins could substitute for pRb in supporting MyoD function. Both CMV-driven p107 and p130 were able to augment MyoD-mediated transactivation of MCK-CAT, although in both cases the level of MCK-CAT expression was significantly below that observed following cotransfection with CMV-pRb (Fig. 3 *B*, compare lanes *4* and *5* with lane *3*). Since ectopic expression of Cdk inhibitors can promote MyoD function in pRb-positive C2 myoblasts and pRb-positive 10T1/2 fibroblasts (Skapek et al., 1995), we evaluated whether reduction of Cdk activity could increase MyoD activity in the absence of pRb. In pRb-deficient cells, neither cotransfection of CMV-driven p16 nor CMV-p21 significantly augmented MyoD transactivation of MCK-CAT; however, in the presence of ectopic pRb, cotransfection with either Cdk inhibitor boosted MyoD function in these cells (Fig. 3 *C*). Together, these data indicate that MyoD-mediated transactivation of muscle pro-

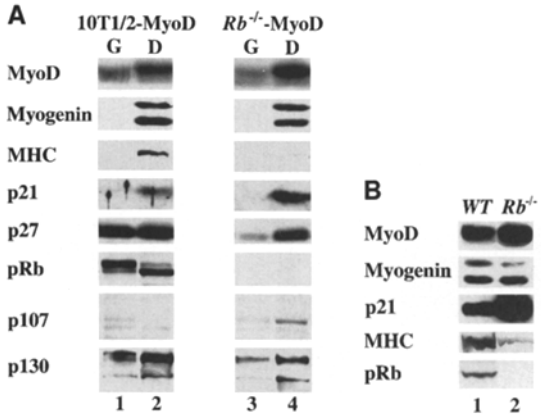

Figure 2. Differential expression of early versus late differentiation markers in pRb-deficient myocytes. (*A*) pRb-positive 10T1/2 fibroblasts and $Rb^{-/-}$ MEFs were stably infected with a retrovirus encoding MyoD driven by a constitutive viral promoter. Protein lysates were then made from cultures that were either exponentially growing (*G*) or differentiated for 3 d (*D*). Lysates, normalized to DNA content, were subjected to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated antibodies. (*B*) *WT* and $Rb^{-/-}$ MEFs were transiently infected with a MyoD retrovirus, allowed to differentiate, and subjected to immunoblotting analysis as in *A*.

moters is compromised in cells lacking pRb. Overexpression of p107 and p130 can partially restore MyoD activity, raising the possibility that a critical mass of any member of the pRb family may be sufficient to ensure normal muscle differentiation; nevertheless, the endogenous levels of p107 and p130 fail to compensate for the loss of pRb. Finally, the enhancement of MyoD activity by p16 and p21 is dependent on the presence of pRb, supporting a role for these proteins in maintaining the functional, hypophosphorylated state of pRb in differentiated skeletal muscle.

pRb Alone Is Necessary for Permanent Growth Arrest

Previous studies have demonstrated that infection of already differentiated skeletal muscle cultures with DNA tumor viruses, or induction of the SV-40 large T antigen within myotubes, can induce DNA synthesis and mitosis (Fogel and Defendi, 1967; Yaffe and Gershon, 1967; Graessmann et al., 1973; Endo and Nadal-Ginard, 1989; Iujvidin et al., 1990; Cardoso et al., 1993; Gu et al., 1993*a*; Ohkubo et al., 1994; Crescenzi et al., 1995). Since viral oncoproteins inactivate the entire pRb family, induce the expression of c-jun, Cdks, and cyclins (Endo, 1992; Ohkubo et al., 1994), and extinguish expression of the myogenic bHLH factors (Endo, 1992), it was unclear whether all or only a subset of these induced cellular responses was necessary to permit DNA synthesis in differentiated myocytes. To specifically examine the role of pRb family members in this process, wild-type (*WT*), $Rb^{-/-}$, $p107^{-/-}$, and $p130^{-/-}$ MEFs were transiently infected with a retrovirus encoding MyoD and challenged to differentiate under low-serum conditions. After 2 d of differentiation, the cells were refed with medium containing both high serum (i.e., 20% FBS) and the thymidine analogue BrdU and then fixed 36 h later for evaluation of BrdU uptake. Differentiated, MHC-expressing myocytes containing pRb failed to synthesize DNA after serum stimulation, as evidenced by the absence of BrdU incorporation in these cells, even when the cells lacked either p107 or p130 (Fig. 4, *A*, *C*, and *D*). In marked contrast, 75–85% of the pRb-deficient, MHC-positive myocytes had incorporated BrdU after 36 h in high-serum media (examples are displayed in Fig. 4 *B*). These results are consistent with the findings of others (Schneider et al., 1994) and indicate that pRb alone is required to inhibit DNA synthesis and maintain G_0 arrest in differentiated myocytes.

We next evaluated whether ectopic expression of other pRb family members or Cdk inhibitors could restore terminal G_0 arrest to pRb-deficient myocytes. MEF cultures were transiently cotransfected with CMV-MyoD and either CMV-driven pRb, p107, p130, p16, or p21. After transfection, the cells were induced to differentiate in low-serum medium for 2 d and then stimulated with medium containing 20% FBS supplemented with BrdU (Fig. 5). Expression of exogenous pRb, p107, p130, and p21 was confirmed by immunostaining dishes transfected in parallel (data not shown). Under these conditions, ~82% of pRb-deficient MHC-positive cells cotransfected with an empty CMV expression vehicle incorporated BrdU. Cotransfection of CMV-MyoD with CMV-pRb reduced BrdU uptake to ~5% in MHC-positive cells (Fig. 5, lane *2*). Cotransfection of CMV-MyoD with CMV-p107 or CMV-p130 also reduced BrdU uptake in MHC-positive cells, in these cases to ~22 and 42%, respectively (Fig. 5, lanes *3* and *4*). In contrast to the reduction in BrdU incorporation after cotransfection of MyoD with pRb family members, cotransfection with either p16 or p21 failed to significantly prevent DNA synthesis in pRb-deficient myocytes (Fig. 5, lanes *5* and *6*). Together, these results demonstrate that ectopic expression of any pRb family member can maintain the G_0 arrest of skeletal muscle to some extent. In contrast, neither endogenous levels of the Cdk inhibitors p21 and p27 (Fig. 2), nor ectopic p16 or p21 (Fig. 5) are capable of preventing S phase entry in differentiated myocytes in the absence of pRb.

pRb-deficient Myocytes Accumulate in S and G_2 Phases

Having established that pRb-deficient differentiated myocytes display aberrant BrdU incorporation, we examined the DNA content of these cells to verify that incorporation of BrdU signified entry into S phase versus DNA repair. To monitor DNA content, we performed flow cytometry on cultures of myocytes that either contained or lacked pRb and quantitated the amount of propidium iodide fluorescence in either MHC-positive or -negative cells in the population. During the process of skeletal muscle differentiation, normal, pRb-containing myocytes fuse to form multinucleate myotubes. Thus, we could not easily evaluate nuclear DNA content in whole cells under normal differentiation conditions because of multinucleation of myotubes. To minimize this problem, we used culture conditions in which muscle differentiation and terminal cell cycle arrest could occur in the absence of cell fusion, based on the sensitivity of fusion to the concentration of calcium in the media (for example see Adamo et al., 1973). Under low-calcium differentiation conditions (50 μM $CaCl_2$), pRb-positive 10T1/2-MyoD cells failed to form multinucleated

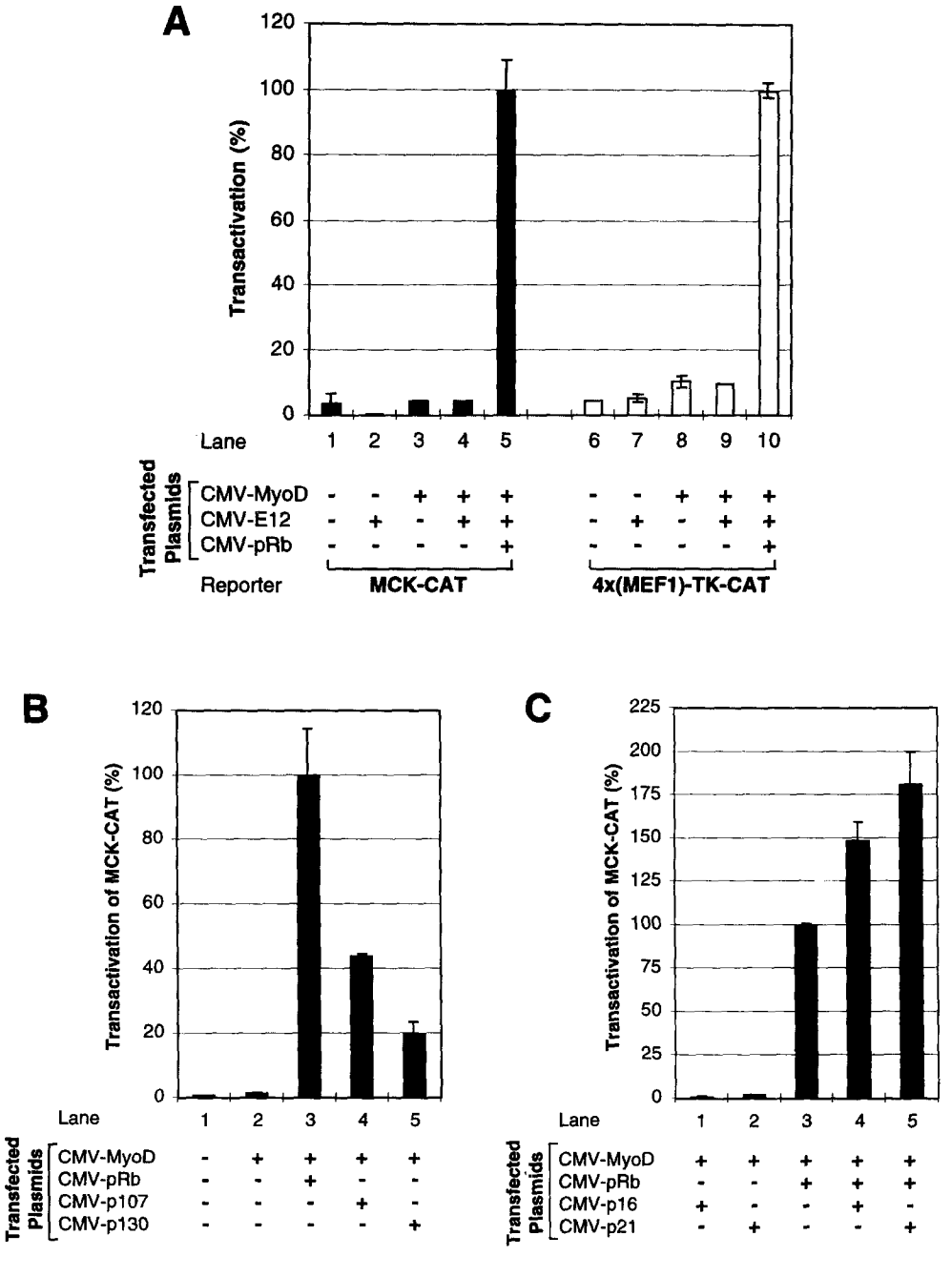

Figure 3. MyoD transactivation of exogenous muscle genes requires pRb family members. (*A*) Effects of CMV-pRb on CMV-MyoD transactivation of a MCK-CAT reporter construct (lanes *1–5*, *filled bars*), or a simplified muscle-specific reporter containing four reiterated MyoD binding sites upstream of a minimal thymidine kinase promoter driving the CAT gene (4× [MEF1]-CAT) (lanes *6–10*, *open bars*). pRb-deficient MEFs were cotransfected with CMV-MyoD (lanes *3–5*; *8–10*), CMV-E12 (lanes *2*, *4*, *5*, *7*, *9*, and *10*), and CMV-pRb (lanes *5* and *10*). CAT activity relative to lanes *5* and *10* are displayed, respectively. Error bars represent duplicate plates from a representative experiment. (*B*) Augmentation of MCK-CAT expression in pRb-deficient fibroblasts after cotransfection of CMV-MyoD (lanes *2–5*) with CMV-pRb (lane *3*), p107 (lane *4*), and p130 (lane *5*). CAT activity relative to lane *3* is displayed. (*C*) Enhancement of MyoD activity by Cdk inhibitors requires the presence of cotransfected pRb. pRb-deficient fibroblasts were cotransfected with CMV-MyoD (lanes *1–5*), CMV-pRb (lanes *3–5*), CMV-p16 (lanes *1* and *4*), and CMV-p21 (lanes *2* and *5*). CAT activity relative to lane *3* is displayed.

myotubes, and the majority of the MHC-positive cells (~86%) had accumulated in G_1/G_0, as measured by propidium iodide fluorescence (Fig. 6 *A*). In these pRb-containing cells, the frequency of cells with an S phase DNA content was reduced to 3% in the MHC-positive portion of the population compared to 17% in the MHC-negative portion, while the proportion of cells with a G_2+M DNA content was the same (11%) in both portions (Fig. 6, *A* and *B*). Identical results were seen with pRb-positive C2 muscle cells (data not shown). In pRb-deficient cells, the frequency of MHC-positive cells with an S phase DNA content was 20% and that with a G2+M DNA content was 24%. In contrast, only 12 and 13% of the MHC-negative cells in this population displayed either an S or G2+M DNA content, respectively (Fig. 6, *C* and *D*). Thus, whereas differentiated pRb-positive myocytes or nondifferentiated pRb-deficient cells maintain predominantly a 2 N DNA content in low serum conditions, pRb-deficient myocytes display a relative tendency to accumulate a >2 N DNA content under identical conditions.

Interestingly, a number of MHC-positive cells lacking pRb appeared to have a >4 N DNA content, suggesting that these cells had undergone multiple rounds of DNA replication after differentiation. Indeed, pRb-deficient myocytes frequently had enlarged nuclei with increased DAPI fluorescence (data not shown). To exclude the possibility that incubation specifically under low-calcium differentiation conditions was altering the DNA content of pRb-deficient myocytes, we repeated our analysis under normal-calcium differentiation conditions and analyzed DNA content in individual nuclei isolated from differentiated myotubes by performing flow cytometric analysis us-

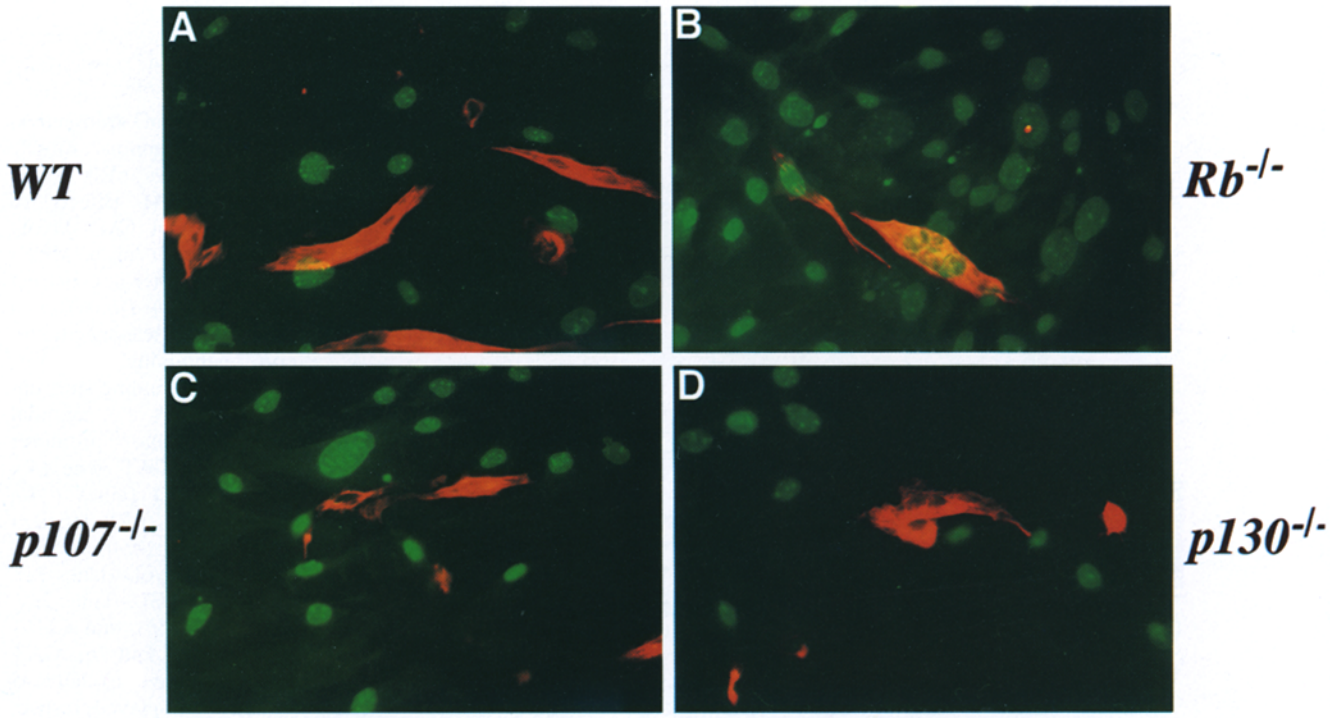

Figure 4. pRb is required to inhibit DNA synthesis in differentiated myocytes. *WT* (*A*), $Rb^{-/-}$ (*B*), $p107^{-/-}$ (*C*), and $p130^{-/-}$ (*D*) MEFs were infected with a MyoD retrovirus, differentiated, and then incubated in media containing 20% FBS and 10 μM BrdU for 36 h. Cells were then fixed, permeabilized, and stained for MHC (*red*) and BrdU (*green*). Note the specific uptake of BrdU in the nuclei of the $Rb^{-/-}$ MHC-positive cells (*B*).

ing myogenin as a nuclear differentiation marker. With this approach, we found that 69% of myogenin-positive nuclei isolated from the pRb-deficient myocytes had a >2 N DNA content (as compared to 28% in the nondifferentiated cells in this population) and that a significant portion of these differentiated nuclei had a >4 N DNA content, although in this case nuclear clumping prevented a thorough statistical evaluation of this phenomenon (data not shown).

pRb-deficient Myocytes Enter S Phase but Do Not Readily Progress to Mitosis

To examine the kinetics of cell cycle progression in pRb-deficient myocytes, we infected pRb-deficient MEFs with a MyoD retrovirus, induced the cells to differentiate in mitogen-poor medium for 3 d, and followed progression of these cells through the cell cycle following serum stimulation (Fig. 7). Under low-mitogen conditions, nearly 50% of the cells incorporated BrdU into their nuclei during the last 6 h of culture in this medium, regardless of MHC expression, demonstrating the low serum requirement of MEFs lacking pRb (Fig. 7 *A*). After serum stimulation, 75% of MHC-negative cells in the population incorporated BrdU by 12 h, and nearly all of the MHC-negative cells took up BrdU by 24 h. In contrast, the percent of the MHC-positive cells incorporating BrdU did not increase above the 50% base line during the first 12 h after serum stimulation, and this frequency only slowly increased to ~70% after 48 h. These data indicate a kinetic difference in cell cycle reentry after serum stimulation, with MHC-expressing cells entering S phase at a slower rate than their MHC-negative counterparts.

As a marker for the G_1 to S transition, we also examined the expression of cyclin A, which normally peaks during this stage of the cell cycle and likely participates in S phase entry and progression (Pagano et al., 1992). Cyclin A expression was observed in 22% of the MHC-negative cells maintained in low-serum conditions, and this frequency of expression slightly increased following serum stimulation to a peak of 35% after 24 h (Fig. 7 *B*). In contrast to the low level and frequency of cyclin A expression in cells lacking MHC staining, cyclin A protein, which is normally absent in MHC-positive myocytes containing pRb (data not shown; see also Ohkubo et al., 1994; Rao et al., 1994; Guo et al., 1995), was present in 67% of the MHC-positive cells maintained in the low-serum conditions, and the intensity of staining was dramatically enhanced over that seen in MHC-negative cells (Fig. 8 *A–C*). 48 h after serum stimulation, the percentage of MHC-positive cells expressing high levels of cyclin A steadily declined to 55%. We also evaluated the expression of cyclin E and Cdk2, which are normally present in MHC-positive myocytes containing pRb (data not shown; see also Jahn et al., 1994; Guo et al., 1995; Kiess et al., 1995b), and found that these proteins were also elevated in MHC-expressing myocytes lacking pRb (Fig. 8, *G–I*, *J–L*). These findings suggest that up to 50% of pRb-deficient, MHC-positive myocytes are already in S phase while maintained under low serum conditions, and that mitogen stimulation can induce an additional percentage of these cells to progress into S phase with kinetics that are slow compared to the MHC-negative cells in the population.

To evaluate further progression of pRb-deficient myocytes through the cell cycle, we monitored the expression

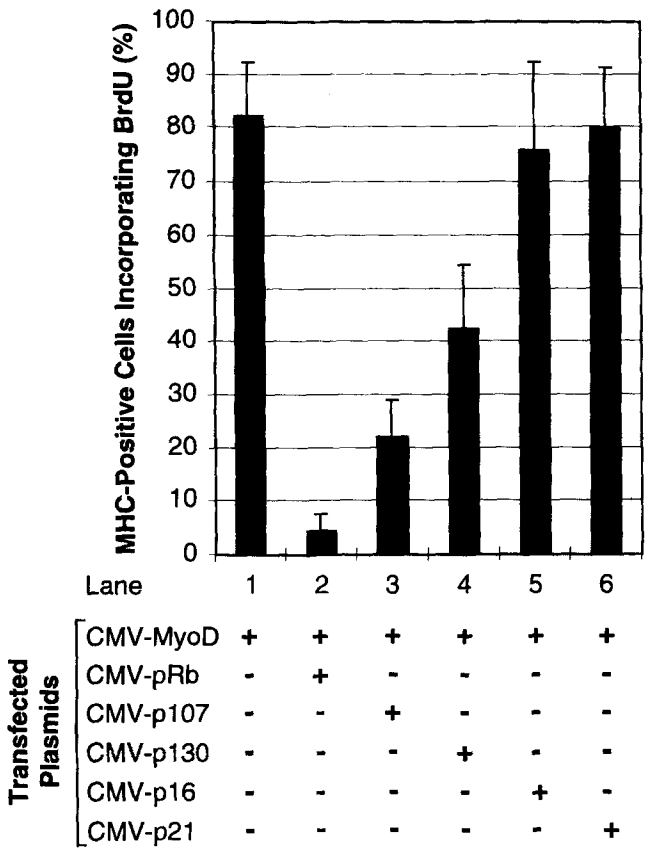

Figure 5. Suppression of BrdU uptake in differentiated pRb-deficient myocytes by pRb family members, but not by Cdk inhibitors. $Rb^{-/-}$ fibroblasts were cotransfected with CMV-MyoD and either an empty CMV expression vehicle (lane *1*), CMV-pRb (lane *2*), CMV-p107 (lane *3*), CMV-p130 (lane *4*), CMV-p16 (lane *5*), or CMV-p21 (lane *6*). Cells were differentiated for 2 d under low-serum conditions, stimulated with high-serum media containing 10 μM BrdU for 24–48 h, fixed, and immunostained for both MHC and BrdU. The displayed results are the average percentage of MHC-positive cells that incorporated BrdU derived from five independent experiments. Error bars depict variation between the experiments. The total number of MHC-positive cells counted for each plasmid combination were: empty CMV vehicle (n = 959), CMV-pRb (n = 1118), CMV-p107 (n = 836), CMV-p130 (n = 361), CMV-p16 (n = 528), and CMV-p21 (n = 530).

of cyclin B in these cells as a marker for the G_2/M transition. Cyclin B normally accumulates in the cytoplasm of proliferating cells during S and G_2 and then translocates to the nucleus just before mitosis (Pines and Hunter, 1991). Surprisingly, cyclin B protein, which is absent from pRb-containing myocytes (data not shown; see also Ohkubo et al., 1994; Rao et al., 1994), accumulated in the nuclei of differentiated pRb-deficient myocytes (Fig. 8, *D–F*) and was observed in 17% of the MHC-positive cells under low-mitogen conditions. This frequency steadily increased to ~25% after serum stimulation (Fig. 7 *C*), suggesting that a subset of pRb-deficient myocytes are capable of reaching the earliest stages of mitosis. In contrast, cyclin B expression in the MHC-negative population slightly increased during the first 12 h of serum stimulation (Fig. 7 *C*), and then sharply declined by 24 h, coinciding with a peak in mitosis (Fig. 7 *D*). Whereas a wave of mitosis occurred in the MHC-negative cells in the population at 24 h after serum stimulation, mitosis was not evident in MHC-expressing cells until the latest time point (48 h after serum addition), where a very small number of MHC-positive cells in mitosis was noted (Fig. 7 *D*). Together, the absence of a decline in cyclin B levels and relative infrequency of mitoses in pRb-deficient myocytes suggest that these cells can traverse S phase and enter G_2 but fail to fully progress into mitosis.

pRb-deficient Myocytes Express Both Cdc2 and Cyclin B and Can Be Driven into Mitosis Prematurely by Caffeine Treatment

To clarify the mechanism of the apparent mitotic blockade in pRb-deficient myocytes, we examined whether Cdc2, the catalytic partner for cyclin B, was present in these cells. Interestingly, Cdc2 was specifically elevated in differentiated pRb-deficient myocytes as visualized by immunostaining (Fig. 8, *M–O*) and absent from pRb-containing muscle cells cultured in parallel (data not shown; see also Cardoso et al., 1993; Jahn et al., 1994; Ohkubo et al., 1994; Rao et al., 1994; Guo et al., 1995; Wang and Nadal-Ginard 1995). Since both Cdc2 and cyclin B were expressed at extremely high levels in at least a fraction of pRb-deficient myocytes, it seemed paradoxical that the mitotic frequency was quite low in these cells. We reasoned that the absence of mitosis could result from a failure to activate the Cdc2/cyclin B complex. This complex is negatively regulated by inhibitory phosphorylations on Cdc2 at threonine 14 and tyrosine 15, thought to be mediated by the wee1/mik1 kinase family, and positively regulated by the removal of these inhibitory phosphorylations by the cdc25 phosphatase family (for reviews see Coleman and Dunphy, 1994; King et al., 1994).

As MHC-expressing cells constitute only a small fraction of cells in pRb-deficient myogenic cell cultures, we were unable to biochemically evaluate the phosphorylation status of Cdc2 specifically in the MHC-positive portion of the population. To address whether Cdc2 accumulated in an inactive, phosphorylated form in pRb-deficient myocytes, we examined the effects of caffeine administration on these cells. Caffeine treatment has previously been shown to trigger premature mitosis (i.e., "mitotic catastrophe") in S phase–arrested cells containing Cdc2/cyclin B complexes (Schlegel and Pardee, 1986; Steinmann et al., 1991) by removing the inhibitory phosphorylations on Cdc2 (Steinmann et al., 1991; Smythe and Newport, 1992). Thus, cells containing incompletely replicated DNA and expressing both Cdc2 and cyclin B should display aberrant mitosis (i.e., mitotic catastrophe) in response to caffeine administration. pRb-deficient myocytes were induced to differentiate under low-mitogen conditions, stimulated with high levels of serum for 12 h, and subsequently treated with 5 mM caffeine. The frequency of normal and abnormal mitoses was then quantitated 6 h after caffeine administration in both cells containing and lacking MHC expression. Caffeine treatment of the MHC-expressing cells led to a 10-fold increase in the frequency of abnormal mitoses, characterized by a "pulverized" nuclear appearance (Fig. 9, compare *B* and *D*). This pulverized nuclear phenotype is reminiscent of the mitotic catastrophe in-

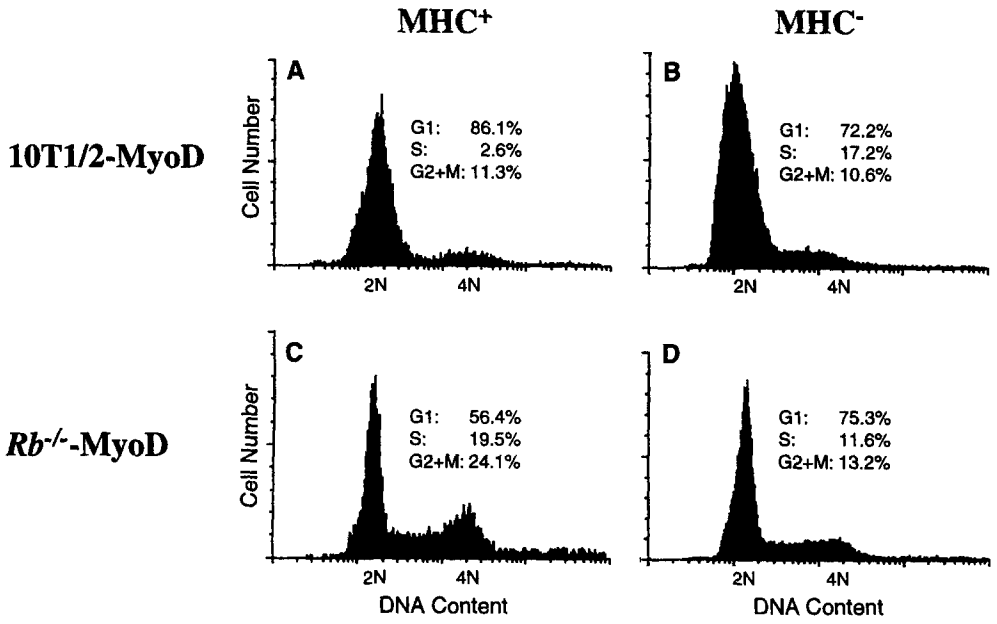

Figure 6. Differentiated pRb-deficient myocytes accumulate an S or G_2+M phase DNA content. pRb-positive 10T1/2-MyoD cells (*A* and *B*) and a pRb-deficient cell line stably infected with MyoD retrovirus ($Rb^{-/-}$-MyoD) (*C* and *D*) were differentiated for 3 d in media containing low amounts of calcium to inhibit myocyte fusion. Whole cells were detached from dishes, washed, fixed, stained for MHC (using a monoclonal antibody to MHC) and for DNA (using propidium iodide), and analyzed using flow cytometry. The DNA content of MHC-positive cells (*A* and *C*) and MHC-negative cells (*B* and *D*) are shown.

duced by overexpression of the Cdc2 activator cdc25 in the presence of both Cdc2 and cyclin B (Heald et al., 1993) and was only observed in the MHC-positive cells in the population (summarized in Table I). In contrast to the increased frequency of pulverized nuclei in MHC-expressing cells, caffeine treatment only modestly increased the frequency of normal mitosis in the MHC-positive cells from ~0.8 to 1.2% and had little effect on the number of normal mitosis in MHC-negative cells (Table I). The effects of caffeine were also specific to myocytes lacking pRb and did not alter the nuclear morphology of pRb-containing muscle cells cultured in parallel (data not shown). In summary, these experiments indicate that caffeine treatment specifically increases the frequency of mitotic catastrophe in differentiated pRb-deficient myocytes, consistent with an arrest of these cells in S and G_2 phases with an accumulation of Cdc2/cyclin B complexes that are maintained in a nonfunctional state by inhibitory phosphorylation.

Discussion

It has been very well documented that DNA tumor viruses such as SV-40, polyoma, and adenovirus can inhibit differentiation and reactivate the mitotic cell cycle in already formed myotubes. Several studies have further demonstrated that the oncoproteins harbored in these viruses are alone capable of blocking differentiation and initiating DNA synthesis. However, interpretation of these studies has been complicated, since these viral oncoproteins sequester numerous proteins (including the pRb family, the p300 family, and p53; Moran, 1993), induce cell cycle regulators (Endo, 1992; Ohkubo et al., 1994), and extinguish myogenic bHLH gene expression (Endo, 1992). In this study, we have analyzed the role of pRb family proteins in muscle gene activation and in terminal cell cycle withdrawal. In agreement with the results of Nadal-Ginard and colleagues who examined muscle differentiation in an ES cell–derived myogenic cell line lacking pRb (Schneider et al., 1994) and with analysis of pRb-deficient mice that develop a grossly normal musculature (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992, 1994), we have observed that at least some aspects of skeletal muscle differentiation can proceed in the absence of pRb (summarized in Fig. 10).

pRb Is Necessary for High-Level Expression of Late Markers of the Skeletal Muscle Differentiation Program

Whereas pRb-deficient myocytes express normal levels of early muscle differentiation markers such as myogenin and p21, high-level expression of genes activated in the end stages of muscle differentiation, such as MHC and MCK, apparently require pRb. These findings are consistent with the observation that inhibition of C2 myoblast differentiation by SV-40 T antigen apparently occurs at a step after the induction of myogenin (Tedesco et al., 1995). Once the muscle differentiation program is triggered, a positive feedback loop is established as MyoD induces the Cdk inhibitor p21 (Guo et al., 1995; Halevy et al., 1995; Missero et al., 1995; Parker et al., 1995). This may primarily serve to ensure that pRb maintains its active, hypophosphorylated state, which is critical for later stages of differentiation. Indeed, cotransfection of both p16 and p21 can augment MyoD function only in the presence of pRb. Other Cdk inhibitors that increase upon muscle differentiation, such as p27 (Halevy et al., 1995), or that are highly expressed in differentiated skeletal muscle, such as p18 (Guan et al., 1994) and p57 (Lee et al., 1995; Matsuoka et al., 1995), may also contribute to this process.

Although pRb-deficient myocytes express other pRb family members (i.e., p107 and p130), the endogenous levels of these pRb-related proteins are apparently unable to compensate for the loss of pRb in promoting MHC expression. Indeed, a unique role for pRb in allowing high-level expression of late muscle differentiation markers is consistent with the finding that MyoD activates apparently normal levels of MHC expression in MEFs engineered to specifically lack either p107 or p130 but induces signifi-

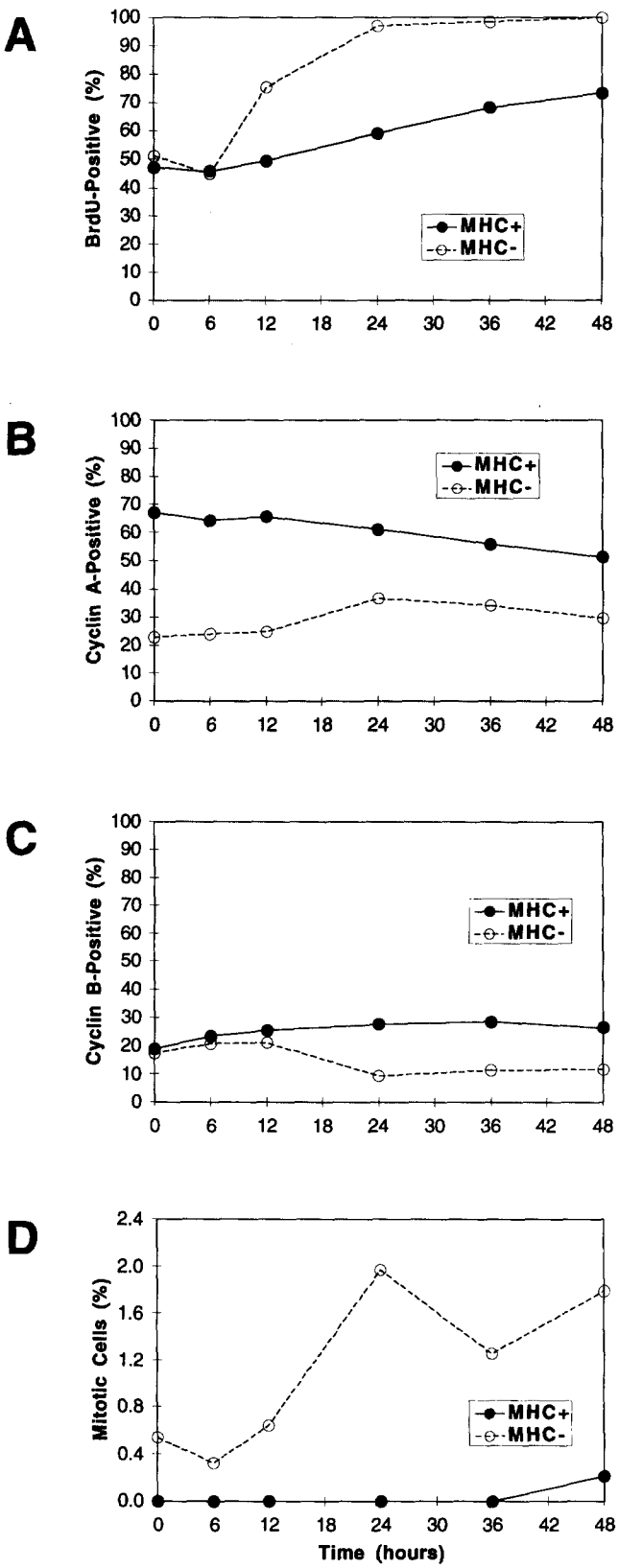

Figure 7. Differentiated pRb-deficient myocytes display a delayed entry into S phase after growth factor stimulation and do not readily progress into mitosis. $Rb^{-/-}$ MEFs were infected with a retrovirus containing MyoD and differentiated for 3 d in media containing 0.1% FBS plus 10 μg/ml insulin. After differentiation, cultures were stimulated with media containing 20% FBS, fixed at the indicated time points, and simultaneously immunostained for MHC and the indicated proteins. At least 300 cells of each MHC-staining class were evaluated, and multinucleated structures were counted as single cells. (*A*) 10 μM BrdU was added to the culture media, and cells were double stained for MHC and BrdU. The percentage of MHC-positive (●) and negative (○) cells containing BrdU staining is indicated. For time zero, cells were maintained for an extra 6 h in the 0.1% FBS + insulin media before fixation. (*B*) Frequency of cyclin A in MHC-positive (●) and -negative (○) cells. (*C*) Frequency of cyclin B in MHC-positive (●) and -negative (○) cells. (*D*) Mitotic activity of MHC-positive (●) and -negative (○) cells. Mitotic cells were characterized by condensed chromosomes and formation of a mitotic spindle. At least 1,000 cells of each MHC-staining class were evaluated for mitosis.

cantly decreased levels of this late differentiation marker in pRb-deficient myocytes. On the other hand, transfection of pRb-deficient cells with high levels of ectopic p107 or p130 can to some extent augment MyoD-mediated transactivation of MCK-CAT, suggesting that all members of the pRb family may share properties that promote muscle gene expression. Although all members of the pRb family are known to interact with E2F transcription factor complexes, it is unclear whether this or other properties of these proteins are necessary to induce high-level muscle gene expression (see Gu et al., 1993*a*; Schneider et al., 1994).

It is unclear why only some muscle genes require pRb for normal levels of expression. We have compared the ability of myogenic bHLH protein complexes from cells containing or lacking pRb to specifically bind DNA in electrophoretic mobility-shift assays (EMSA) and have found that myogenic bHLH complexes from both cell types have similar affinity for DNA and identical mobility in EMSA (Novitch, B.G., and A.B. Lassar, data not shown). The differences in pRb sensitivity in early versus late differentiation markers may reflect either dissimilarities in the promoters of these two classes of muscle genes which render the former immune to the loss of pRb, or the activation of late differentiation markers by pRb-dependent myogenic regulators that are themselves expressed at later times during the muscle differentiation program (i.e., myogenin, MRF-4, and MEF2). It may be relevant in this regard that the pRb-mediated boost in transactivation of a MCK-CAT reporter (which contains both an E box and MEF2 site) was much greater than that observed with an artificial reporter containing four reiterated E boxes (a 23–35-fold versus a 3–10-fold increase in activity, respectively). It is interesting to note that a role for pRb in specifically promoting the later stages of differentiation programs has also been observed during erythropoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), neuronal differentiation (Lee et al., 1994), and in lens fiber cell formation (Morgenbesser et al., 1994) and that pRb has also been shown to activate the transcription factor NF-IL6, which is involved in macrophage differentiation (Chen et al., 1996).

pRb Is Required to Maintain Terminal G_0 Arrest in Differentiated Myocytes

It is clear from the results of the present study and from that of others (Schneider et al., 1994) that pRb plays a critical role in preventing aberrant DNA synthesis in termi-

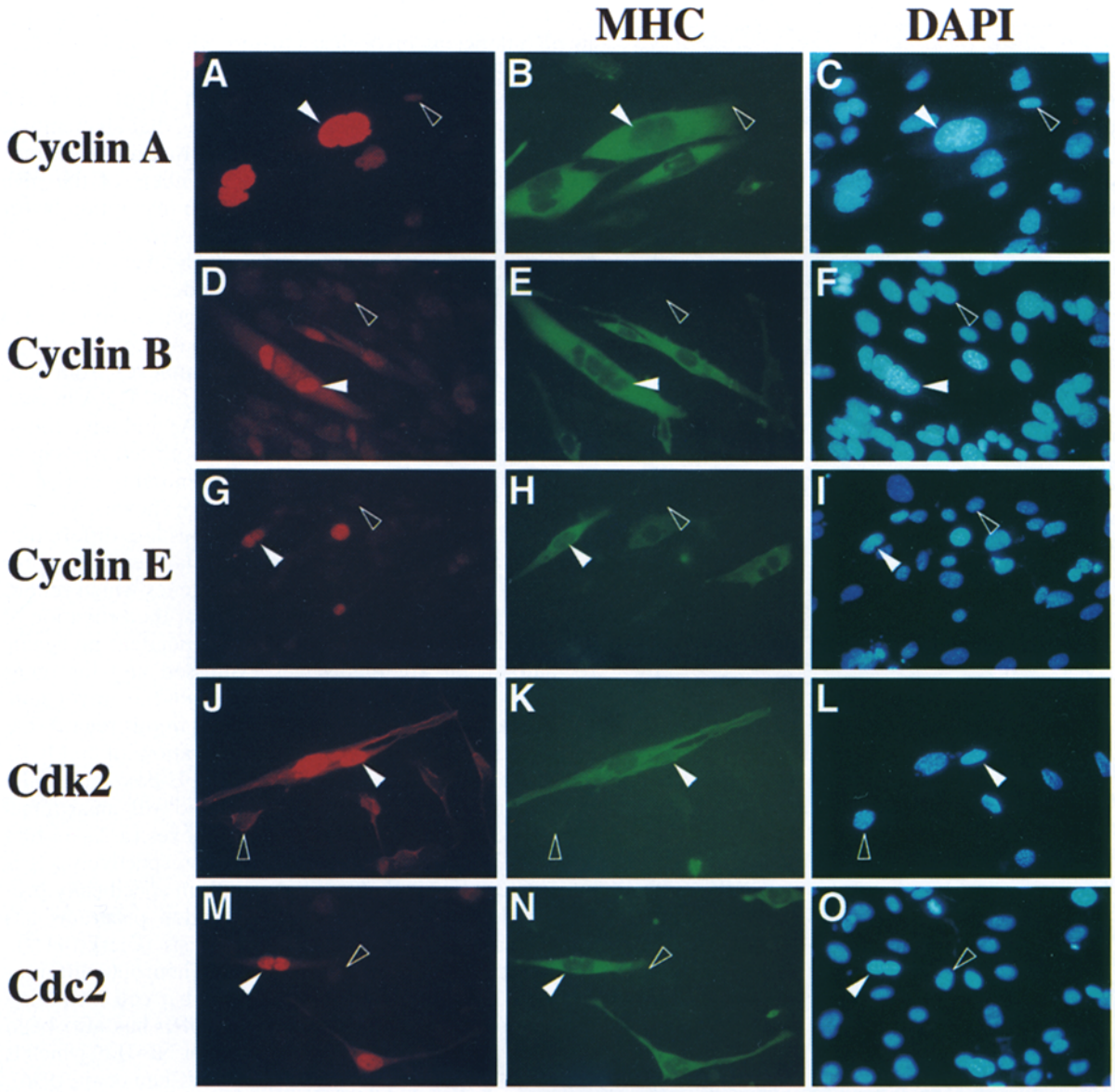

Figure 8. Differentiated pRb-deficient myocytes over-express both cyclins and Cdks. pRb-deficient MEFs infected with a retrovirus containing MyoD were differentiated for 3–4 d in media containing 0.1% FBS plus 10 μg/ml insulin, serum-stimulated with media containing 20% FBS for 12–24 h, fixed in 2% paraformaldehyde, and double stained for MHC (*B*, *E*, *H*, *K*, and *N*) and the indicated proteins. (*A*) Cyclin A, (*D*) cyclin B, (*G*) cyclin E, (*J*) Cdk2, and (*M*) Cdc2. Filled and open arrows indicate representative MHC-positive and -negative cells, respectively, in each field.

nally differentiated myocytes. In contrast to pRb-deficient myocytes that continue to synthesize DNA, myocytes lacking either p107 or p130 display normal G_0 arrest, indicating that pRb can maintain terminal cell cycle withdrawal in muscle cells in the absence of either p107 or p130. Whereas ectopic expression of either pRb or other pRb family members can inhibit DNA synthesis in pRb-deficient myocytes, neither endogenous expression of p21 and p27 nor ectopic expression of p16 and p21 is sufficient to prevent DNA synthesis in these cells. Recent studies have similarly found that the p16 family of Cdk inhibitors lack growth inhibitory function in cells lacking pRb (Guan et al., 1994; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). In contrast, p21, p27, and p57 have been shown to promote G_1 accumulation, even in SAOS-2 cells which lack pRb (Harper et al., 1993; Toyoshima and Hunter 1994; Matsuoka et al., 1995). We surmise that the ability of p21 to induce G_1 accumulation in SAOS-2 cells but not inhibit DNA synthesis in differentiated pRb-deficient myocytes may be due to either methodological differences in assay conditions (i.e., flow cytometry versus BrdU uptake measurement) or inherent differences in the cell types. Furthermore, the absence of G_0 arrest in differentiated pRb-deficient myocytes is consistent with findings that overexpression of E2F-1 alone can promote DNA synthesis in serum-starved cells (Johnson et al., 1993; Qin et al., 1994; Shan and Lee 1994; DeGregori et al., 1995*b*). Presumably by binding E2F/DP complexes, pRb silences transcription of genes required for entry into S phase, and therefore DNA synthesis is promoted under

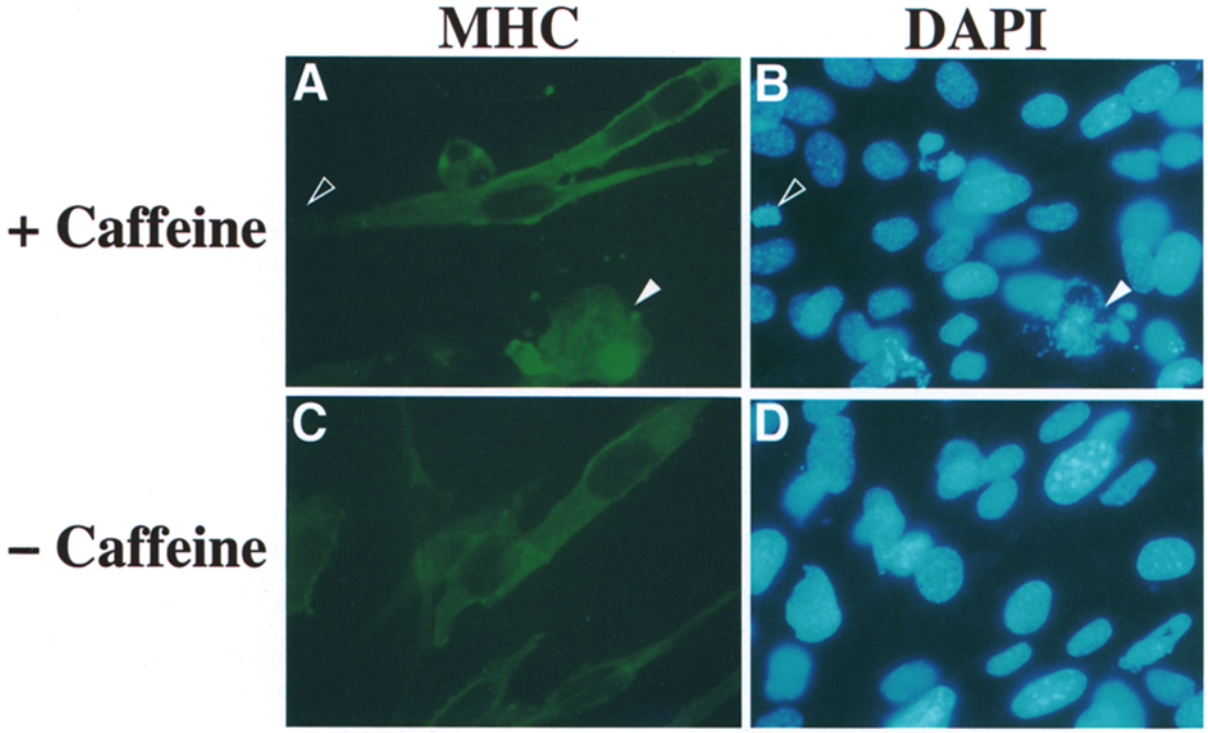

Figure 9. Caffeine administration induces mitotic catastrophe in differentiated pRb-deficient myocytes. $Rb^{-/-}$-MyoD cells were differentiated for 3 d in media containing 0.1% FBS plus 10 μg/ml insulin and then stimulated for 12 h with media containing 20% FBS. Cells were subsequently incubated for 6 additional h in 20% FBS media containing (*A* and *B*) or lacking (*C* and *D*) 5 mM caffeine, fixed in 2% paraformaldehyde, and stained with MHC antibodies and DAPI. The filled arrow indicates a "pulverized" nucleus seen in MHC-staining cells that were treated with caffeine, and the open arrow indicates a nondifferentiated cell undergoing normal mitosis.

normal G_0 conditions by either loss of pRb or overexpression of E2F-1.

pRb Loss in Differentiated Myocytes Leads to Abnormal S and G_2 Phase Accumulation

Although DNA synthesis was frequently observed in pRb-deficient myocytes, the kinetics of entry into S phase were attenuated in these cells in comparison to MHC-negative cells in the population. The slowed entry of MHC-positive, pRb-deficient myocytes into S phase may be due to high-level expression of p21 and p27 in these cells, which may act to dampen Cdk-dependent DNA synthesis. After serum stimulation, the majority of pRb-deficient myocytes remained in S and G_2 phase and did not readily progress into mitosis, as evidenced by the accumulation of excessively high amounts of cyclins A and E, Cdk2, enlarged nuclei, and a DNA content of >2 N. The appearance of a >4 N DNA content in a subset of differentiated myocytes lacking pRb raises the possibility that the failure to progress through the cell cycle may allow these cells to undergo multiple rounds of replication. The lack of mitosis was not due to a failure of the cells to express Cdc2 or cyclin B, as both proteins were present at elevated levels in pRb-deficient myocytes. Interestingly, the mitotic blockade in pRb-deficient myocytes could be overcome by caffeine treatment, which is known to remove the inhibitory phosphorylations from Cdc2 and thereby promote mitosis (Steinmann et al., 1991; Smythe and Newport, 1992). Caffeine treatment of the differentiated pRb-deficient myocytes led to a slight increase in the frequency of normal mitosis and dramatically increased the frequency of mitotic catastrophe. Previous studies have shown that caffeine promotes premature mitosis specifically in cells arrested in S that have accumulated both Cdc2 and cyclin B (Schlegel and Pardee, 1986; Steinmann et al., 1991); thus, the caffeine sensitivity of pRb-deficient myocytes is consistent with their accumulation in S and G_2 phases.

Table I. Promotion of Abnormal Mitosis in pRb-deficient Myocytes by Caffeine Treatment

	Normal mitosis	Nuclear pulverization
	%	%
MHC-positive		
− Caffeine	0.8	0.4
+ Caffeine	1.2	4.3
MHC-negative		
− Caffeine	1.4	0.0
+ Caffeine	1.3	0.0

$Rb^{-/-}$-MyoD cells were differentiated for 4 d in media containing 0.1% FBS + insulin, followed by serum stimulation with media containing 20% FBS for 12 h. High-serum media containing or lacking 5 mM caffeine was then added, and cells were grown for an additional 6 h before fixation. Cells were stained for MHC and DNA using antibodies and DAPI, respectively, and the number of MHC-positive and -negative cells containing mitotic nuclei was evaluated. See Fig. 9 for examples of the aberrant mitosis observed. At least 500 MHC-positive and -negative cells were counted for each culture condition.

These findings suggest that, in the process of promoting muscle differentiation, MyoD may positively regulate an activity that either phosphorylates Cdc2 or negatively regulates genes necessary for mitosis, such as the cdc25 phos-

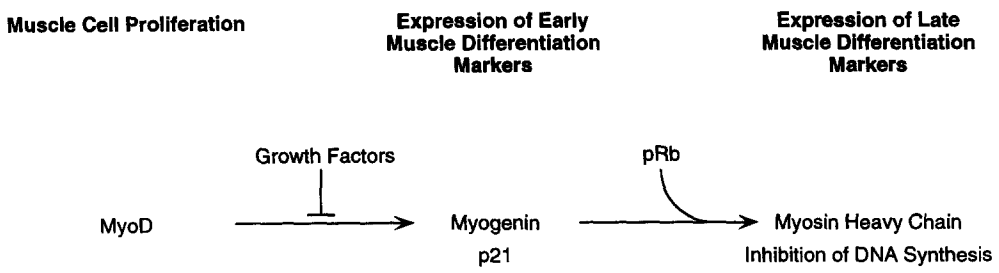

Figure 10. pRb is required for the expression of late markers of the skeletal muscle differentiation program and for permanent withdrawal from the cell cycle. This model depicts the pRb dependence of individual steps of the skeletal muscle differentiation program. Myoblasts expressing MyoD are kept in a proliferating, nondifferentiated state by the actions of growth factors. After serum removal, MyoD becomes active and induces the expression of the early muscle differentiation markers myogenin and p21, in a manner that does not require pRb. In contrast, pRb is necessary for both high level expression of late muscle differentiation markers, such as myosin heavy chain, and the inhibition of DNA synthesis in differentiated myocytes.

phatases, and thereby maintains the nonmitotic state. Although incomplete DNA replication and DNA damage are both known to induce inhibitory phosphorylation of Cdc2 (for review see Nurse, 1994), it is unclear whether inactivation of this kinase in pRb-deficient myocytes is due to incomplete DNA replication or some other physiological signal in these cells. It is interesting to note that cyclin A ablation has been shown to activate Cdc2/cyclin B complexes and that introduction of cyclin A mRNA or protein can delay Cdc2-associated kinase activation (Walker and Maller, 1991). It is therefore possible that the markedly elevated cyclin A protein levels in differentiated pRb-deficient myocytes may be related to the absence of mitosis. Another plausible explanation for the failure to progress to mitosis is the ability of MyoD to induce p21 in the absence of pRb. In cycling *Xenopus* egg extracts, the addition of recombinant p21 inhibits the kinase activity of Cdk2, blocks mitosis, and leads to an accumulation of inactive Cdc2/cyclin B complexes (Guadagno and Newport, 1996), similar to the phenotype of pRb-deficient myocytes. Moreover, expression of SV-40 T antigen has been found to induce DNA synthesis but not mitosis in senescent cells (Gorman and Cristofalo, 1985), which similarly express high levels of p21 (Noda et al., 1994).

The striking over-expression of cyclins A and E, Cdk2, and Cdc2 concomitant with S phase arrest in differentiated pRb-deficient myocytes may reflect a defect in E2F–1/DP autoregulation in these cells. In the absence of pRb, E2F–1/DP complexes should be able to activate target genes, which include cyclin A, cyclin E, Cdc2, and other cell cycle–regulated gene products (DeGregori, et al., 1995*a*; Ohtani et al., 1995). During normal procession through S phase, E2F–1/DP activity is suppressed by association of E2F–1 with cyclin A/Cdk2 and consequent phosphorylation of the DP subunit which disrupts the ability of E2F–1/DP complexes to bind DNA (Krek et al., 1994). Failure of cyclin A/Cdk2 to inhibit E2F–1/DP activity has recently been shown to result in S phase arrest and subsequent apoptosis of cells (Krek et al., 1995). We speculate that the increased levels of Cdk inhibitors like p21 and p27 in differentiated myocytes lacking pRb may interfere with this regulatory loop by decreasing cyclin A/Cdk2 kinase activity, which would thereby stabilize E2F–1/DP activity, leading to both increased expression of E2F-1–regulated proteins and S phase arrest. Since we have not observed significant levels of apoptosis in differentiated pRb-deficient myocytes (Novitch, B.G., and A.B. Lassar, data not shown), we surmise that hyperactive E2F complexes may only induce S phase arrest and not promote apoptosis in this unique cellular background.

In summary, our findings and those of previous studies (Gu et al., 1993*a*; Schneider et al., 1994) indicate that pRb plays a critical role in both promoting high-level expression of end-stage markers of the muscle differentiation program and in maintaining the G_0-arrested state of myocytes. Moreover, a pRb-independent mechanism acts to ensure that differentiated myotubes which enter S phase in the absence of pRb fail to readily progress into mitosis. It is interesting to note that in contrast to the relative absence of mitosis in myocytes specifically lacking pRb, expression of viral oncoproteins that sequester the entire pRb family and other proteins efficiently promotes mitosis in differentiated myotubes (Fogel and Defendi, 1967; Yaffe and Gershon, 1967; Graessmann et al., 1973; Iujvidin et al., 1990; Cardoso et al., 1993; Ohkubo et al., 1994; Crescenzi et al., 1995). Taken together, these findings suggest that other viral oncoprotein–associated proteins (i.e., p300, p107, and p130) may have regulatory functions in controlling S phase progression and mitosis, and that these viral oncoproteins can either negate these functions or extinguish MyoD expression and thereby produce a mitotic cell cycle.

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