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# Regulation of Expression and Activity of Distinct pRB, E2F, D-Type Cyclin, and CKI Family Members during Terminal Differentiation of P19 Cells

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**The cell cycle regulatory proteins, which include cyclin-dependent kinases (cdks), cdk inhibitors (CKIs), cyclins, and the pRB, and E2F families of proteins, constitute a network of interacting factors which govern exit from or passage through the mammalian cell cycle. While the proteins within these families have similar structural characteristics, each family member exhibits distinct expression patterns during embryogenesis and distinct biological activities. In order to begin to understand the tissue-specific roles of these interacting factors, we determined the expression pattern and activity of the pRB, E2F, cyclin, cdk, and CKI families of cell cycle regulatory proteins during retinoic acid-induced (neuronal pathway) and DMSO-induced (cardiac muscle pathway) differentiation of the pluripotent murine embryonal carcinoma cell line, P19. We demonstrate here that P19 terminal differentiation causes lineage-specific changes in the expression and activity of distinct members of the E2F, pRB, cyclin, and CKI families. Furthermore, dynamic changes in the activities of these cell cycle regulatory proteins occur through several overlapping mechanisms, culminating in repression of DNA-binding activity by all of the E2F family members as cells terminally differentiate.** 

***Key Words:*** **differentiation; cell cycle; kinase activity; E2F; D cyclins.**

## INTRODUCTION

Entry into and passage through the $G_1$ phase of the cell cycle are controlled by a network of interacting molecules which regulate the activity of the E2F family of transcription factors. One important group of factors which directly modify E2F activity are the pRB family proteins, pRB, p107, and p130. These proteins bind to and repress E2F activity [1–3] through regions contiguous with those required for growth arrest [4, 5]. This phosphorylation-dependent association of the pRB family proteins with E2F [6–8] is, in turn, regulated by the activity of cyclin-dependent kinases (cdks). These serine/threonine-directed kinases are either activated, via association with the cyclins, or repressed as a consequence of complex formation with cyclin-dependent kinase inhibitors (CKIs; for reviews, see [9, 10]).

A number of observations indicate that distinct members of these different families of cell cycle regulatory proteins may have tissue-specific roles. The E2F family, for example, contains five members, E2F-1 [11], E2F-2 [12], E2F-3 [13], E2F-4 [3, 14] and E2F-5 [14, 15]. When heterodimerized with either DP1 [16], DP2 [17], or DP3 [18], these bHLH transcription factors bind to DNA encoding an E2F consensus sequence [17–19] and can generally drive growth-arrested cells into S phase [20–24]. Despite these apparent overlapping activities, the E2Fs are expressed in different cell populations during embryonic development [25, 26] and appear to regulate the expression of overlapping but distinct sets of genes [27–29]. Members of the E2F family proteins also exhibit differentiation-dependent changes in their expression and activity. For example, expression of E2F-1 is repressed during myogenic differentiation of $C_2C_{12}$ cells [30] while terminal differentiation of F9 teratocarcinoma cells causes downregulation of all E2F site-binding complexes [31–33]. That the expression and activity of the E2F family proteins must be tightly regulated has been clearly demonstrated both *in vivo* and *in vitro* where, for example, differentiation of specific cell types is blocked in the absence of the pRB family proteins [34–36].

Expression of the upstream effectors of E2F is also regulated in a developmental and differentiation-specific manner. In the case of the pRB family proteins, *in situ* analysis of developing murine embryos shows high levels of pRB transcript in specific developing structures including neural and myogenic tissues [37]. p107 is not expressed in developing skeletal or cardiac mus-

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cle but is expressed in the CNS in a pattern distinct from pRB. p130 is expressed at low levels in most tissues in the embryo [37]. These patterns of expression of pRB during embryogenesis are consistent with the tissue-specific effects of losing pRB activity in both humans and mice [38–42].

Likewise, the D-type cyclins also exhibit distinct expression patterns during embryogenesis [43–45] and exhibit distinct biological activities. For example, expression of cyclins D1 and D2 is repressed during myogenesis *in vitro* and *in vivo*, while cyclin D3 levels are induced [46–48], although the kinase activity associated with the induced cyclin D3 levels is repressed in these cells. These distinct expression patterns for the D-type cyclins are reflected by their distinct biological activities. For example, ectopic expression of cyclins D1 or D2, but not cyclin D3, blocks myogenic differentiation [49] while forced expression of cyclins D2 and D3, but not cyclin D1, blocks granulocyte differentiation [50]. Constitutive cyclin D1 expression, under the control of the CMV promoter, disrupts normal neural differentiation apparently by induction of apoptosis [51]. As expected from their tissue-specific expression pattern, failure to express specific D-type cyclins gives rise to very specific developmental defects. Germline deletion of cyclin D1 in mice results in a severe retinopathy caused by impaired development of all neural layers of the retina [45, 52] while mice nullizygous for cyclin D2 show defects in gonadal development [44]. The CKIs, $p16^{Ink4}$, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, are also required for the normal development of specific sets of tissues, demonstrated in mouse knock-out experiments [53–57] and in a number of *in vitro* models of differentiation [58–60].

Taken together, the tissue-specific pattern of expression and distinct biological activities of the members of the CKI, cyclin, pRB, and E2F families of cell cycle regulators imply a complex network of regulatory interactions governing cell cycle control and differentiation during embryonic development. In order to begin to understand the regulation of the expression and activities of these factors and their role during cellular differentiation, we employed the pluripotent embryonal carcinoma (EC) cells, P19 (for reviews, see [61, 62]). We describe here the differentiation-dependent regulation of the expression and activities of distinct members of the E2F, pRB, cyclin, and CKI family proteins. Our data show that changes in the activities of these cell cycle regulatory proteins occur through several overlapping mechanisms and culminate in repression of DNA-binding activity by all of the E2F family members.

## MATERIALS AND METHODS

*Antibodies and DNA.* The anti-cyclin D2, D3, E, p107, p130, $p27^{kip1}$, cdk2, and cdk4 polyclonal antibodies, and the appropriate blocking peptides, as well as the monoclonal anti-cyclin D1, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An anti-cyclin D1 polyclonal antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). An anti-cyclin A monoclonal was provided by J. Slingerland (Sunnybrook Hospital, Toronto, ON). The anti-pRB monoclonal (14001A) was purchased from Pharmingen (Richmond, CA). The anti-p107 monoclonal (SD15) was provided by N. Dyson (MGH Cancer Center, Boston, MA). cDNA for mouse cyclin D1 was generously provided by C. Sherr (St. Jude's Hospital, Memphis, TN), and mouse cyclins D2 and D3 from J. Hanley-Hyde (NIH, Bethesda, MD). A plasmid containing a *Bam*HI/*Pst*I 800-bp genomic fragment of the murine cyclin D1 5′ upstream region, ligated upstream of the CAT reporter gene in the 5′ to 3′ orientation, D1-10CAT, was obtained from M. Roussel (St. Jude's Hospital). Also obtained from Dr. Roussel was an 800-bp *Hin*dIII/*Pst*I fragment of the murine cyclin D2 5′ upstream region ligated in the 5′ to 3′ orientation upstream of the CAT reporter gene, D2–4CAT.

*Cell culture.* Mouse P19 embryonal carcinoma cells [62] were cultured in $\alpha$-minimum essential medium ($\alpha$MEM) containing 5% heat-treated bovine calf serum and 5% heat-treated fetal calf serum. Cells were subcultured 1:8 every 48 h to maintain exponential growth. Undifferentiated P19 cells were induced to differentiate by plating 1:8 in 300 nM retinoic acid (RA) or 0.75% dimethyl sulfoxide on bacterial grade plates for 48 h, followed by subculturing 1:4 into bacterial grade plates for an additional 48 h in RA, or 72 h in DMSO, to allow aggregates to form. Aggregates were then plated out 1:4 onto tissue culture grade plastic in the absence of RA on Day 4, or DMSO on Day 5. Media were changed at 1-day intervals for the duration of the differentiation time course. The rat $L_6$ skeletal myoblast cell line [63] was grown in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). For induction of muscle differentiation, the medium of a confluent culture was changed to DMEM containing 2% FBS.

*Glutathione S-transferase (GST)-fusion proteins.* Bacterially produced, recombinant human full-length pRB ($rhRB^{FL}$) was provided by J. Slingerland (Sunnybrook Health Sciences Centre, Toronto, Ontario). GST-pRB fusion constructs in the pGEX-2T vector were provided by R. Bremner (Eye Research Institute of Canada, Toronto, ON). Full-length cDNA of human p130 (P. Whyte, McMaster University, Hamilton, ON) was cloned in frame with GST into the pGEX-2TK vector (Pharmacia Biotech Inc.). GST-fusion proteins were expressed in bacteria and purified on glutathione Sepharose 4B beads (Pharmacia Biotech Inc.) according to the manufacturer's instructions.

*Electrophoretic mobility shift assays (EMSA).* Nuclear lysates were prepared as previously described [64]. An end-labeled ($^{32}$P $\gamma$-ATP) double-stranded oligonucleotide containing a single E2F-binding site (5′-GGATTTAAGTTTCGCGCCCTTTCTCAA-3′) was used as template. Of nuclear lysates 1.5 $\mu$g was used in 15-$\mu$l reactions containing $2 \times 10^4$ cpm of labeled DNA (approximately 0.2 ng), 120 mM NaCl, 4% Ficoll, 20 mM Hepes, pH 7.9, 50 ng/$\mu$l salmon sperm DNA, 2.5 mM $MgCl_2$, 40 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 250 ng/$\mu$l BSA. For supershift EMSAs or EMSAs with added GST-fusion proteins, reaction mixtures were incubated at room temperature for 10 min, followed by addition of 1 $\mu$l of antibody and/or the indicated amounts of GST-fusion proteins. These reactions were allowed to incubate for an additional 15–20 min. The DNA-protein complexes were then resolved on a 4.5% polyacrylamide gel in 0.25X TBE at 20 V/cm and the dried gel was exposed to film for 8 to 48 h.

*Transfections and assays.* Using the calcium phosphate method, 10 $\mu$g of CAT reporter constructs was cotransfected with 2 $\mu$g of pgk-puro, which contains the gene for puromycin resistance under the control of the constitutive pgk promoter. The recipient cells were undifferentiated P19 cells plated 24 h previously at 5% confluency. Puromycin was added at a concentration of 2.0 $\mu$g/ml 24 h after removal of the precipitate. Cells were selected for stable integration of transfected DNA in puromycin for a 1-week period and all resis-

tant clones were combined. Pools were then differentiated with RA and cells were harvested at 1-day intervals and lysed by a freeze/thaw procedure. Lysates were assayed for CAT activity using the quantitative ethyl acetate extraction procedure [65]. CAT activities were normalized to total protein and results are presented as a ratio of CAT activity, divided by total protein.

*Western blots.* Total protein lysates were isolated by NP-40 lysis (0.5% NP-40, 120 mM NaCl, 50 mM Tris, pH 8.0), while nuclear protein lysates were obtained from both undifferentiated and RA-treated P19 cells as previously described [64]. Of each lysate 20 $\mu$g was run on 6% SDS-PAGE gels and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked overnight (O/N) at 4°C in PBS/0.1% Tween 20 containing 5% skim milk powder, followed by incubation at RT for 2 h in a 1:1000 dilution of primary antibody, followed by a 1-h incubation with a 1:8000 dilution of goat anti-rabbit, or anti-mouse, HRP-conjugated 2° antibody (Bio-Rad, Richmond, CA). Blots were developed with the ECL fluorescent detection kit according to the manufacturers instructions (Amersham Canada, Oakville, ON).

*Northern blots.* Total cellular RNA was isolated from undifferentiated P19, as well as RA- and DMSO-differentiated P19 cells at 1-day intervals [66], and 3 $\mu$g of glyoxal-denatured total RNA was run on a 1% agarose gel and transferred to Genescreen Plus membrane overnight in 25 mM sodium phosphate, pH 7.0. RNA was crosslinked with 245 nm ultraviolet irradiation and the blots were prehybridized for 1 h at 65°C in 500 mM salt, in the absence of formamide. Random-primed cDNA probes (Pharmacia Oligolabelling kit) were added in fresh hybridization solution at approximately $5 \times 10^6$ cpm/ml and hybridized overnight at 65°C. Blots underwent two 65°C washes in 400 mM $NaPO_4$ followed by one 65°C wash in 100 mM salt. Signal intensity was determined for cyclins D1, D2, and D3, as well as for GAPDH using an LKB Laserscan XL laser densitometer, and the D cyclin levels were normalized to GAPDH.

*Immunoprecipitations.* Undifferentiated, as well as RA- and DMSO-treated, P19 cells were labeled for 5 h with 200 $\mu$Ci *trans*-$^{35}$S label (ICN) in labeling media (methionine and glutamine-free RPMI 1640 (GIBCO), supplemented with 5% dialyzed fetal bovine serum (GIBCO) and 2 mM glutamine (GIBCO)). The cells were washed twice with ice cold PBS and total protein was obtained by lysing cells on ice for 20 min in NP-40 lysis buffer (0.5% NP-40, 120 mM NaCl, 50 mM Tris, pH 8.0) containing protease inhibitors. Following centrifugation the supernatant was precleared with 10 $\mu$l protein A-Sepharose beads (Pharmacia) preincubated with 10 $\mu$l rabbit serum (Gibco) followed by incubation of $2 \times 10^7$ incorporated counts with 0.5 $\mu$g polyclonal antibody at 4°C for 1.5 h. Immune complexes are then incubated with 25 $\mu$l protein A-Sepharose for 45 min at 4°C, followed by three washes in 750 $\mu$l lysis buffer minus inhibitors. Proteins were eluted by boiling 2 min in 1X SDS sample loading buffer and analyzed on a 13% SDS-PAGE gel.

*Immunoprecipitaion:Westerns.* Immunoprecipitations were performed as described above with the following exceptions; unlabeled lysates (400 $\mu$g) were used, and preclear beads were preincubated with each 10 $\mu$l rabbit serum, 10 $\mu$l mouse serum, and 10 $\mu$l goat serum. Immune complexes were run on 13% SDS-PAGE gels and transferred to nitrocellulose membrane (Schleicher & Schuell). Blots were blocked O/N at 4°C in PBS/0.1% Tween 20 containing 5% skim milk powder. Blots were incubated at 4°C O/N in a 1:1000 dilution of primary antibody, followed by a 1:8000 dilution of the appropriate goat anti-IgG HRP conjugated 2° antibody. Blots were developed with the ECL fluorescent detection kit (Amersham Canada) and following exposure, blots were stripped according to the ECL protocol (TechTip No. 22) with 2% SDS, 100 mM mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, for 30 min at 70°C. This was followed by reprobing with a subsequent primary antibody.

*Kinase assays.* Lysate for pRB kinase assays was obtained by resuspending undifferentiated, as well as RA- and DMSO-treated, cells in kinase assay lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol) and sonicating with three, 10 s, low energy pulses. Immunoprecipitations were performed with 100 $\mu$g lysate and 0.5 $\mu$g polyclonal antibody, for 1.5 h at 4°C. Protein A-Sepharose beads were added for 1 h, and complexes were washed two times with kinase assay lysis buffer (minus glycerol), and two times with 50 mM Hepes, pH 7.5, 1 mM DTT. Of kinase mix 30 $\mu$l was added to each immunoprecipitation (0.2 $\mu$g GST-pRB(392–928) fusion protein, 50 mM Hepes, pH 7.5, 20 $\mu$M ATP, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 1 mM $NaVO_3$, 1 mM NaF, 2 $\mu$Ci $\gamma$-ATP), and incubated at 30°C for 30 min. Reaction mixes were analyzed on 10% SDS-PAGE gels, and exposed at −80°C with a Lightning Plus fluorescent screen (Dupont) for 24 to 48 h.

## RESULTS

### *E2F Site Binding Complexes Decrease during P19 Neuronal Differentiation*

Terminal differentiation of P19 cells into postmitotic neurons or cardiac muscle requires repression of activated transcription mediated by the E2F family proteins [34, 67]. In order to understand the mechanisms giving rise to this repression, we determined the expression and activity the E2F family proteins and the upstream effectors of E2F activity which include the cyclins, cdks, CKIs, and pRB family proteins. We first evaluated the DNA-binding activity of E2F-containing complexes during terminal differentiation of P19 cells using electrophoretic mobility shift assays (Fig. 1A). Using a double-stranded oligonucleotide containing a single E2F site, two predominant complexes, A2 and A4, and three relatively weaker complexes, A1, A3, and C1, are detected using nuclear lysates prepared from undifferentiated P19 cells. During retinoic acid-induced differentiation of these cells, the intensities of complexes A1–4 are decreased greater than 10-fold while the slower migrating complex, C1, is reduced less than 2-fold. The single C1 complex seen in undifferentiated cells is replaced by two complexes, C1 and C2, in the RA-treated cells (see Fig. 1B). The faster migrating of the two complexes, C1, contains p107, determined by its supershift following addition of the $\alpha$-p107 antibody, SD15 (Fig. 1B, middle panel). As the supershift assay in the right panel of Fig. 1B depicts, the new C2 complex contains p130. In all of these supershift assays, complexes A1, A2, A3, or A4 were unaffected by the addition of antibodies to the pRB family proteins, including antibodies directed against pRB (data not shown), indicating that these E2F site-binding complexes were not associated with any pRB family proteins (referred to as “free” E2F).

A more variable pattern of E2F site-binding complexes is observed for the DMSO-induced differentiation. Like the RA-treated cells, the levels of E2F complexes are decreased by Day 5 of the differentiation. However, by Day 8, these complexes return to their previous levels, due likely to the increasing proportion

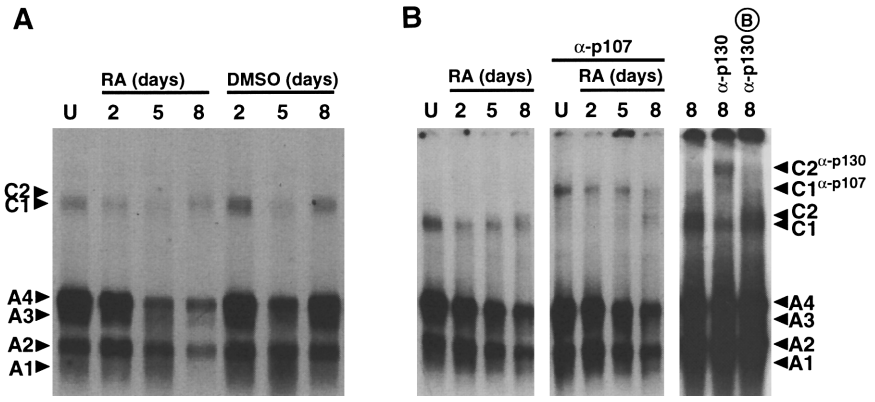


**FIG. 1.** E2F transcription complexes are reduced in differentiating P19 cells. (A) Nuclear lysate from undifferentiated, as well as RA- and DMSO-treated, P19 cells was incubated at RT for 10 min with a $^{32}$P end-labeled double-stranded oligonucleotide probe containing a single E2F-binding site, before resolving on a 4.5% nondenaturing acrylamide gel. Four specific bands migrate quickly and relatively close together, termed A1, A2, A3, A4, and the slowest migrating specific complex is termed C1. Bands A1 and A3 are very faint and are difficult to identify due to the intensity of A2 and A4. Complexes A2 and A4 decrease approximately 10-fold during the course of neuronal differentiation. In addition, by Day 8 following RA treatment, a new, slower migrating complex, C2, is observed. (B) Nuclear lysates were incubated with a $^{32}$P end-labeled double-stranded oligonucleotide probe containing a single E2F-binding site as described above. A total of 100 ng of either p107 monoclonal, or p130 polyclonal, antibody was then added and the reactions were allowed to continue for an additional 10 min at RT before resolving on a 4.5% nondenaturing acrylamide gel. C1 complex shifted by the p107 monoclonal is labeled C1$^{\alpha\text{-p107}}$, while C2, shifted by the p130 polyclonal, is labeled C2$^{\alpha\text{-p130}}$. Note that addition of a p130 blocking peptide (last lane) abolished the shift of C2.

of cycling cells (noncardiac muscle) which continue to proliferate in these cultures.

We expected that the changes in the levels of the different E2F site-binding complexes were due, in part, to changes in the levels and/or activities of the E2F and pRB family proteins. Figure 2A illustrates the differential expression pattern of the E2F family proteins during both RA- and DMSO-induced P19 cell differentiation. Low but detectable levels of E2F-1 and E2F-3 are seen in nuclear lysates of undifferentiated cells, while E2F-2 and E2F-4 are easily observed. As cells differentiate, E2F-1 and E2F-3 levels transiently increase and are subsequently reduced to low or undetectable levels. E2F-4, whose levels are generally high in quiescent and/or postmitotic cells [68], is downregulated during terminal differentiation of P19 cells. Translocation of E2F-4 between the nuclear and cytoplasmic compartments has been reported [69, 70]. We determined that for P19 cells, E2F-4 appears to be distributed in both compartments and that kinetics of its loss during differentiation are identical for these two fractions (data not shown). In contrast to E2F-1, -3, and -4, we observe little or no fluctuation in E2F-2 protein levels. Thus, while structurally similar to each other, expression of the E2F-family proteins is regulated in a differential manner during neuronal and cardiac muscle differentiation of P19 cells.

The pRB family proteins also undergo differential changes in their expression during differentiation, as we and others have reported previously (Fig. 2B) [71–74]. p130 is induced during differentiation, consistent with the appearance of p130-containing E2F site-binding complexes in RA-induced cells. A weak but reproducible increase in p130 levels is also seen in DMSO-treated cells. pRB levels increase in these cells during differentiation despite the failure to detect pRB-containing E2F site-binding complexes in the EMSAs. The shift of pRB exclusively to its active, hypophosphorylated form is also evident after Day 5 in RA-treated cultures, this being the point where terminal differentiation of the neurons has occurred. Finally, p107 is detected throughout both RA- and DMSO-induced differentiation, with a weak peak of expression on Days 4 and 5.

Despite persistent expression of E2F-2 and active (hypophosphorylated) pRB in terminally differentiated P19 cells, pRB-containing E2F site-binding complexes could not be detected in these cells. Since pRB binding to E2F alters its ability to bend DNA encoding an E2F-binding site [75, 76], we hypothesized that pRB might also weaken the affinity of E2F for DNA. This hypothesis was tested by titrating a recombinant, full-length version of pRB or GST-pRB fusion proteins into nuclear lysates prepared from undifferentiated P19 cells (Fig. 3A). Addition of GST-pRB$^{LP}$, which is deleted for the N-terminal domain, reduces the levels of the A3/A4 complexes and, to a lesser degree, the A1/A2 complexes. This reduction in free E2F site-binding complexes is coincident with the appearance of a new, slower migrating complex (B$^{pRB}$) which can be supershifted with either an $\alpha$-GST or $\alpha$-pRB antibody (data not shown). Specificity of these effects was shown where GST-pRB$^{N}$, the N-terminal domain alone, failed to affect any of the free E2F complexes. Likewise, GST-

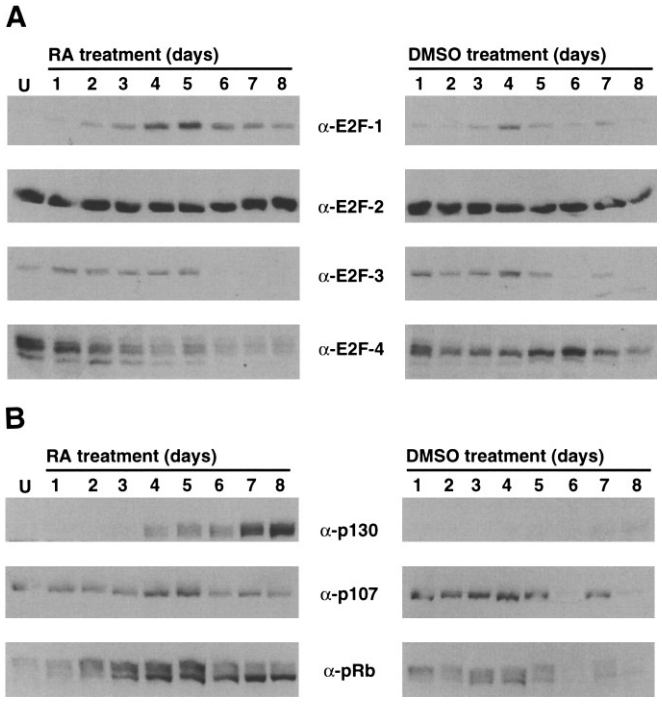


**FIG. 2.** E2F and pRB family member protein levels during P19 differentiation. (A) Of nuclear lysate 20 $\mu$g was isolated from undifferentiated as well as RA- and DMSO-treated P19 cells at 1-day intervals and run on 10% SDS-PAGE gels. Protein was transferred to nitrocellulose and probed versus $\alpha$-E2F-1, $\alpha$-E2F-2, $\alpha$-E2F-3, and $\alpha$-E2F-4 polyclonal antibodies. Blots were developed with the ECL fluorescence kit and exposed for 30 s to 2 min. Equal loading in all lanes was verified by probing the identical blots for cdk4 (data not shown). (B) Nuclear lysate was isolated from undifferentiated and RA- and DMSO-treated P19 cells at 1-day intervals, and 20 $\mu$g was run on 8% SDS-PAGE gels and Western-blotted versus $\alpha$-pRB monoclonal and $\alpha$-p107 and $\alpha$-p130 polyclonal antibodies. Blots were developed with the ECL fluorescence kit and exposed between 10 s and 5 min.

$pRB^{SP}$, which is deleted for both the N- and C-terminal domains, had only a weak effect of these free complexes confirming the requirement for the C-terminus of pRB for strong E2F binding. When the full-length recombinant pRB ($rhRB^{FL}$) was used in the same assay, however, new pRB-containing complexes were not detectable despite the ability of this protein to reduce the levels of all free E2Fs. This result was confirmed using nuclear lysates from rat $L_6$ myoblast cells in which we have identified most of the free E2F complexes (M. Gill, unpublished data; see Fig. 3B). GST-$pRB^{LP}$ preferentially titrates the E2F-1, -2, and -3 complexes and results in the appearance of a new E2F site-binding complex. Addition of GST-p130 demonstrates its preferential association with E2F-4 and E2F-5. Using the identical lysates but adding the $rhRB^{FL}$ protein, preferential inhibition of E2F-1, -2, and -3 DNA-binding activity is again observed. However, as was seen for $rhRB^{FL}$ when added to P19 nuclear lysates, new E2F site-binding complexes containing pRB are not detected when added to $L_6$ nuclear lysates. We conclude, therefore, that full-length pRB alters the affinity of E2F for DNA. Furthermore, at the concentrations of endogenous pRB bound to E2F in lysates prepared from differentiated P19 cells, the weakened binding of these complexes to a single E2F site precludes their detection under the conditions used in these EMSAs.

### *Differentiation-Dependent Transcriptional Regulation of the D-Type Cyclins*

pRB family-mediated regulation of E2F activity is essential for P19 terminal differentiation since blocking this interaction with E1a leads to apoptotic cell death in this system [34]. Furthermore, changes in the phosphorylation state of pRB imply that $G_1$ cyclin-associated kinase activity is also regulated during P19 differentiation. Thus, changes in the levels and associated kinase activities of the $G_1$ cyclins during P19 cell differentiation were determined.

Immunoprecipitations of cyclins D1, D2, and D3 and their catalytic subunits, cdk2 and cdk4, were performed on $^{35}S$-labeled extracts in order to quantify alterations in their synthesis (Fig. 4A). A relatively constant level of cyclin D3 protein was observed throughout differentiation of RA- and DMSO-stimulated P19 cells. However, both cyclins D1 and D2 were strongly induced in RA-treated cells but only weakly in DMSO-treated cells. For these $G_1$ cyclins, a peak of expression is seen on Day 5 and Day 6, respectively, in RA-treated cells. The change in cyclin D1 synthesis was reflected by changes in its steady-state protein level, determined by Western analysis (Fig. 8A). The lower two panels in Fig. 4A show further that cdk2 and cdk4 are present at constant levels throughout the differentiation.

The levels of cyclins A and E were also determined by Western analysis (Fig. 4B). For both RA- and DMSO-treated cells, cyclin A shows a gradual decrease in its level as cells reach a terminally differentiated state. In RA-treated cells, a small, transient increase in cyclin E is reproducibly observed and its expression persists in terminally differentiated cells.

### *D Cyclins Are Transcriptionally Regulated during Differentiation of P19 Cells*

The basis for the changes in expression of the distinct D-type cyclins during P19 cell differentiation was determined by Northern analysis and in promoter assays. First, the levels of cyclin D1, D2, and D3 transcript were determined by Northern analysis (Fig. 5A). Changes in the levels of the D-type cyclin transcripts resembled changes in protein synthesis (see Fig. 4A), with cyclins D1 and D2 showing peaks of expression on Day 5 in RA-treated cells. Weak induction of cyclin D1

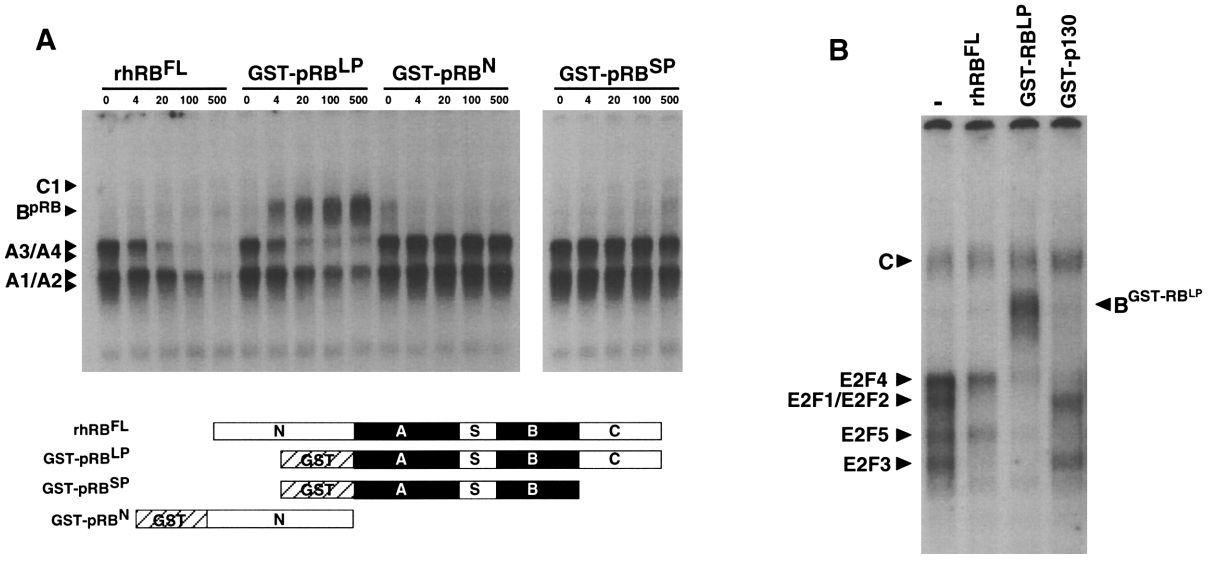


**FIG. 3.** Titration of GST-RB or GST-p130 into E2F-DNA complexes. (A) Of GST-pRB$^{LP}$, GST-pRB$^{SP}$, GST-pRB$^{N}$, or purified recombinant full-length pRB (rhRB$^{FL}$), 0, 4, 20, 100, or 500 ng was titrated into reactions containing 1.5 $\mu$g of undifferentiated P19 cell lysate and EMSAs were performed. GST-RB$^{SP}$ and GST-RB$^{N}$ had little effect on free E2F complexes, while both GST-pRB$^{LP}$ and rhRB$^{FL}$ significantly reduced levels of free complexes. rhRB$^{FL}$, unlike GST-pRB$^{LP}$, did not produce a supershifted pRB-containing complex upon binding to E2F complexes. (B) Nuclear lysates from rat $L_6$ myoblasts were incubated with the E2F-site probe. Incubations were allowed to continue for 10 min before addition of 8 ng of rhRB$^{FL}$, GST-RB$^{LP}$, or GST-p130 and a further 10 min incubation followed by resolution on a 4.5% native acrylamide gel. Five free E2F complexes are seen in these lysates. The levels of E2F-3 and E2F-1/E2F-2 complexes are reduced by both rhRB$^{FL}$ and GST-RB$^{LP}$, while GST-p130 reduces the levels of complexes E2F-4 and E2F-5.

and D2 message was also observed during DMSO-induced differentiation, corresponding with increased cyclin D1 steady-state protein levels (Fig. 8A).

We determined next if changes in the levels of cyclin D1 and D2 transcripts were due to changes in the activity of their promoters. Of the murine cyclin D1 or cyclin D2 promoters, 800 bp ligated upstream of a CAT reporter gene were stably transfected into undifferentiated P19 cells and pools of resistant clones isolated. Clones were then induced to differentiate using RA and cell lysates assayed for CAT activity (Fig. 4B). Both promoters had very little activity in undifferentiated cells. However upon differentiation, an induction in promoter activity is observed on Day 3, which increases to a peak on Day 5. The kinetics of induction of cyclin D1 and D2 promoter activity corresponds closely to the relative levels of steady-state transcript and increases in protein levels. Thus, cyclin D1 and D2 expression appears to be controlled during differentiation of P19, at least in part, by changes in the activities of their respective promoters.

### *Kinase Activity Associated with the D-Type Cyclins during P19 Differentiation*

Switching of pRB to its active, hypophosphorylated form occurs at a point during P19 cell differentiation when high levels of cyclins D1 and D2 persist. This discordance predicts that alterations in the kinase activity associated with the $G_1$ cyclins occur independently of their regulated expression. This prediction was confirmed in kinase assays following immunoprecipitation of cdk2, cdk4, cyclins D1, D2, and D3 (Figs. 6A and 6C). Despite having relatively steady levels of expression throughout RA- and DMSO-induced differentiation, it is clear that the kinase activity associated with cdk2 and cdk4 is strongly regulated in these cells. Specifically, cdk2 and cdk4 showed detectable levels of kinase activity in undifferentiated cells. Following RA treatment, both kinases exhibited an increase in activity on Day 5 followed by reduction of activity as cells terminally differentiate by Day 8. During DMSO-induced cardiac muscle differentiation, there is a reduction in activity by Day 5, followed by a slight recovery on Day 8.

Kinase activity associated with cyclins D1 and D2 in RA-treated cells generally followed the expression pattern of these proteins (Fig. 6C). Little or no kinase activity is observed in undifferentiated and in Day 2 RA-treated P19 cells, while a peak of activity is seen at Day 5. Activity is substantially reduced by Day 8, when most of the cells are terminally differentiated. However at this point detectable levels of cyclin D2 are still seen (Fig. 4A). The kinase activity of cyclin D3 also indicates that mechanisms other than protein levels govern alterations in its associated kinase activity. Specifically, relatively constant levels of cyclin D3 pro-

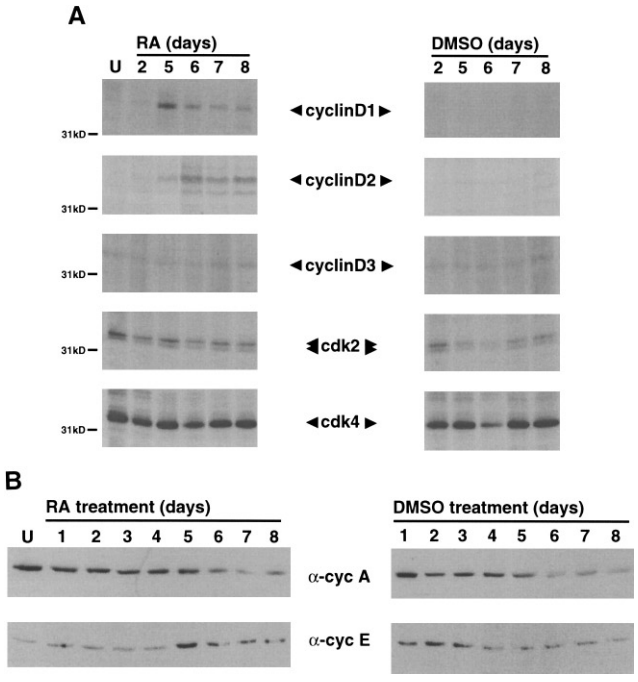


**FIG. 4.** Expression of $G_1$ cyclins and cdks in P19 neuronal and cardiac muscle differentiation. (A) Total protein was obtained from undifferentiated and RA- and DMSO-treated P19 cells labeled for 5 h with 200 $\mu$Ci *trans*-$^{35}$S label. Lysates were precleared and $2 \times 10^7$ incorporated counts were immunoprecipitated with 0.5 $\mu$g $\alpha$-cdk2, $\alpha$-cdk4, $\alpha$-cyclin D1, $\alpha$-D2, or $\alpha$-D3 polyclonal antibodies for 1.5 h at 4°C. Following a 45-min incubation with protein A-Sepharose beads, proteins were eluted by boiling 2 min in 1X SDS sample loading buffer and analyzed on 13% SDS-PAGE gels. Gels were enhanced with 1 M sodium salicylate and exposed for 3 to 6 days at −80°C with a Dupont NEN Lightning Plus enhancement screen. (B) Nuclear lysate was isolated from undifferentiated and RA- and DMSO-treated P19 cells at 1-day intervals, and 20 $\mu$g was run on 8% SDS-PAGE gels and Western-blotted versus monoclonal $\alpha$-cyclin A or polyclonal $\alpha$-cyclin E.

tein are observed throughout RA-induced differentiation despite a significant decrease in its associated kinase activity by Day 8. In DMSO-treated cells, regulation of cyclin D3-associated kinase activity is also evident. While there is little change in cyclin D3 protein levels, kinase activity peaks on Day 2, followed by a reduction to relatively low levels on Day 5. Another increase in cyclin D3 kinase activity is observed by Day 8, again likely due to expansion of a noncardiac muscle cells in these plates.

### *p27, but Not p21 or p16, Is Associated with Decreased Cyclin D Kinase Activity*

We next determined if changes in the kinase activity associated with the D-type cyclins were due to alterations in associated cdk and/or CKI family members. The identity of the kinases associated with the D-type cyclins during P19 cell differentiation was determined by immunoprecipitations of each of the D-type cyclins followed by Western blotting to detect associated cdk2 and cdk4 (Fig. 7A). While signals from 10 $\mu$g of a COS-1 cell lysate or from cdk2 immunoprecipitated from Day 8 RA-treated P19 cells were easily detected, no trace of cdk2 could be detected coimmunoprecipitating with any of the D-type cyclins. In contrast, when the identical blots were probed for cdk4, coimmunoprecipitation of cdk4 can be detected with cyclins D1, D2, and D3.

The reciprocal experiment was performed for cyclin D1 in order to confirm these results. Specifically, P19 lysates were immunoprecipitated using antibodies against cdk4 and cdk2 and associated cyclin D1 detected by Western analysis (Fig. 7B). Cyclin D1 is clearly detected co-immunoprecipitating with cdk4 on Day 5 of RA treatment, but only weakly on Days 2 and 8, and not at all in undifferentiated cells. There are weak, but detectable, levels of cdk4-associated cyclin D1 in DMSO-treated Day 5 and Day 8 lysates. However, cyclin D1 cannot be detected coimmunoprecipitating with cdk2 at any point in differentiation, indicating that the induced cyclin D1 contributes to cdk4 activation but not cdk2.

The expression pattern and cyclin/cdk complex formation of the CKIs, p16, p21, and/or p27, was also determined (Fig. 8). Little or no expression of p16 or p21 in undifferentiated or differentiating P19 cells was observed, determined both by Western and Northern analyses (data not shown). However, p27 levels are strongly increased during P19 cell differentiation (Fig. 8A). Specifically, p27 is almost undetectable in undifferentiated cells, but is highly expressed on Days 5 and 8 of neuronal differentiation. p27 is also induced by Day 8 of DMSO treatment.

Since p27 was the only CKI whose levels were significantly altered during P19 differentiation, its association with complexes containing cdk2, cdk4, and cyclin D1 was determined. Lysates from undifferentiated, RA-treated, and DMSO-treated P19 cells were immunoprecipitated with antibodies against p27 (panel B), cdk2 (panel C), or cdk4 (panel D) and associated cdk2, cdk4, p27, or cyclin D1 determined by Western analysis. The $\alpha$-p27 Western seen in panel B confirms the increased levels of p27 during both RA- and DMSO-induced differentiation. As expected, the level of cdk2 associated with p27 increases proportionally in these cells. However, the cdk4 associated with p27 peaks on Day 5 and decreases by Day 8. Since p27 binds simultaneously to the cyclin and the cdk [77, 78], the peak of cdk4 associated with p27 on Day 5 predicts that cyclin D1 would show a similar peak of association with p27. The immunoprecipitation/Western analysis in the upper portion of panel B, where a peak of cyclin D1 coimmunoprecipitated with p27 on Day 5 of RA treatment, confirmed this prediction. In the case of the

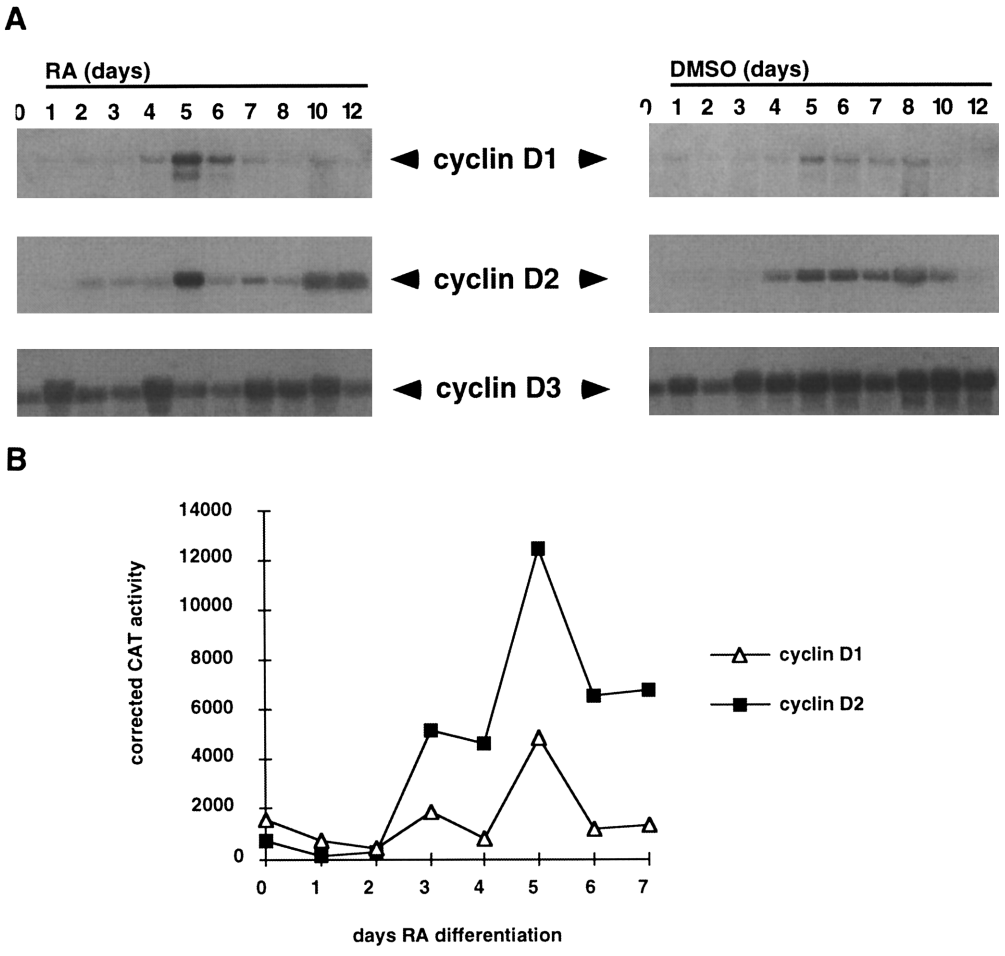


**FIG. 5.** Cyclins D1 and D2 show a peak of expression during neuronal differentiation due to an increase in transcription. Undifferentiated P19 cells were induced to differentiate with either RA or DMSO. Medium was changed at 1-day intervals for the duration of the differentiation time course. (A) Total cellular RNA was isolated from both RA- and DMSO-differentiated P19 cells and Northern-blotted. Blots were probed, stripped, and reprobed with murine cyclin D1, D2, and D3, as well GAPDH cDNA radiolabeled probes. (B) A total of 10.0 $\mu$g of CAT reporter constructs D1–10 and D2–4, containing 800 bp of the murine cyclin D1 and D2 promoters, respectively, was cotransfected with 2.0 $\mu$g of pgk-puro into undifferentiated P19 cells plated 24 h previously at 5% confluency. Cells were selected for stable integration of transfected DNA in 2.0 $\mu$g/ml puromycin for 1 week and resistant clones were combined and differentiated with RA. Cells were harvested at 1-day intervals and assayed for CAT activity using the quantitative ethyl acetate extraction procedure. Activities were normalized to total protein and results are presented as a ratio of CAT activity, divided by total protein.

DMSO-induced P19 cell differentiation, associated cdk2, cdk4, and cyclin D1 are seen only after Day 5 where the levels of p27 have increased to significant levels.

The increased levels of p27 suggested that altered stoichiometry of this CKI in cdk2-containing (panel C) and cdk4-containing (panel D) complexes would be seen. The lower portion of each panel again shows that cdk2 and cdk4 levels are relatively unaltered during P19 differentiation. As expected, cdk2-associated cyclin D1 was not detectable in these lysates while a peak of p27 associated with cdk2 was seen in Day 8 lysates in both RA- and DMSO-treated cells, reflecting sustained levels of cyclin A and E in these cells (see Fig. 4B). This peak of associated p27 coincides with the reduction in cdk2-associated kinase activity. In the case of cdk4, an increase of associated p27 is detectable by Day 2 of RA-induced differentiation (Fig. 4D). The amount of p27 coimmunoprecipitating with cdk4 is then maintained at high levels throughout the remainder of the differentiation. However, the inverse immunoprecipitation showed that despite high levels of p27, only a low amount of cdk4 coimmunoprecipitated with this CKI on Day 8 of RA differentiation. This result could indicate that in terminally differentiated cells, the cdk4 associated with p27 is bound by multiple p27 molecules.

Taken together, these data demonstrate that the network of factors regulating passage through $G_1$, including the CKI, cyclin, pRB, and E2F family proteins,

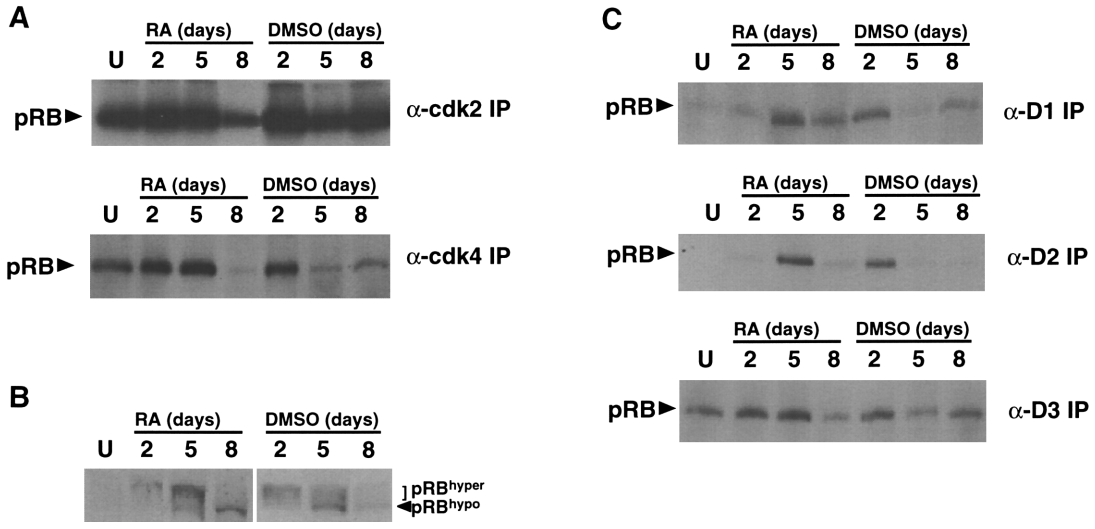


**FIG. 6.** Kinase activity associated with the D cyclins corresponds to the phosphorylation state of pRB during terminal differentiation. (A) Cdk2 and cdk4 were immunoprecipitated from both undifferentiated, and Days 2, 5, and 8 of RA and DMSO differentiation as described above. Complexes coupled to protein A-Sepharose beads were then incubated in the presence of $^{32}$P-γ-ATP with 200 ng of a bacterially produced GST-pRB(392–928) fusion protein. Phosphorylated proteins were then resolved on 10% SDS-PAGE gels and exposed for 1 to 2 days at −80°C with a Dupont NEN Lightning Plus enhancement screen. (B) Nuclear lysate was isolated from undifferentiated and RA- and DMSO-treated P19 cells at the indicated time points, run on 8% SDS-PAGE gels and Western-blotted versus pRB. (C) Cyclins D1, D2, and D3 were immunoprecipitated with polyclonal antibodies as described above. Complexes coupled to protein A-Sepharose beads were then incubated in the presence of $^{32}$P-γ-ATP with 200 ng of a bacterially produced GST-pRB(392–928) fusion protein. Phosphorylated proteins were then resolved on 10% SDS-PAGE gels and exposed for 1 to 2 days at −80°C with a Dupont NEN Lightning Plus enhancement screen.

is differentially regulated as P19 cells exit the cell cycle and differentiate into neurons or cardiac muscle.

## DISCUSSION

The pluripotent EC line, P19, offers a unique system in which to study the factors which are involved in the differentiation of stem cells. Specific differentiation pathways can be induced in culture conditions, cells ultimately reaching a terminally differentiated, quiescent state as neurons (RA stimulation) or as cardiac, smooth, and immature skeletal muscle (DMSO stimulation). While many factors have been characterized which are specifically expressed in each lineage, considerably less information about the role of the cell cycle machinery during stem cell differentiation is available. In this report we have described the changes in expression and activity of the pRB and E2F families as well as the D-type cyclins, their associated kinases, and kinase inhibitors.

Free E2F complexes are present at a high level in undifferentiated P19 embryonal carcinoma cells consistent with their nearly nonexistent $G_1$ phase of 2 h [79]. In agreement with previous reports [31, 32, 80] we demonstrated that these free E2F site-binding complexes are lost when P19 cells differentiate into postmitotic neurons. Loss of some of these complexes is clearly due to repression of expression of some of the E2F family proteins, specifically E2F-1, -3, and -4. However, E2F-2 is expressed at significant levels in the terminally differentiated cells, suggesting that additional mechanisms reduce the levels of free E2F-2 complexes. One mechanism involves the induction of both pRB and p130 during the course of differentiation and the transition of these pRB family proteins to their active, hypophosphorylated state as cells terminally differentiate. pRB protein is expressed relatively early following RA stimulation and, presumably, must be maintained in its inactive, hyperphosphorylated state until cells exit the cell cycle (>Day 5). Our data demonstrate that maintenance of pRB in this inactive state occurs coincidently with the induction of cyclins D1 and D2 and a significant increase in the kinase activity associated with these cyclins. As cells begin to terminally differentiate and form postmitotic neurons, pRB becomes exclusively hypophosphorylated coincident with reduction of the kinase activity associated with the D-type cyclins. Thus, the induction of pRB (and p130) and the coordinate transition to its unphosphorylated form as repression of the kinase activity associated with the cyclin D/cdk4 complexes occurs are expected to repress the activity of the only E2F family protein whose levels are not repressed during differentiation, E2F-2. We suggest, further, that expression of hypophosphorylated pRB also accounts, in part, for the reduction in E2F-1 and E2F-3 levels via an autorepression mechanism [81].

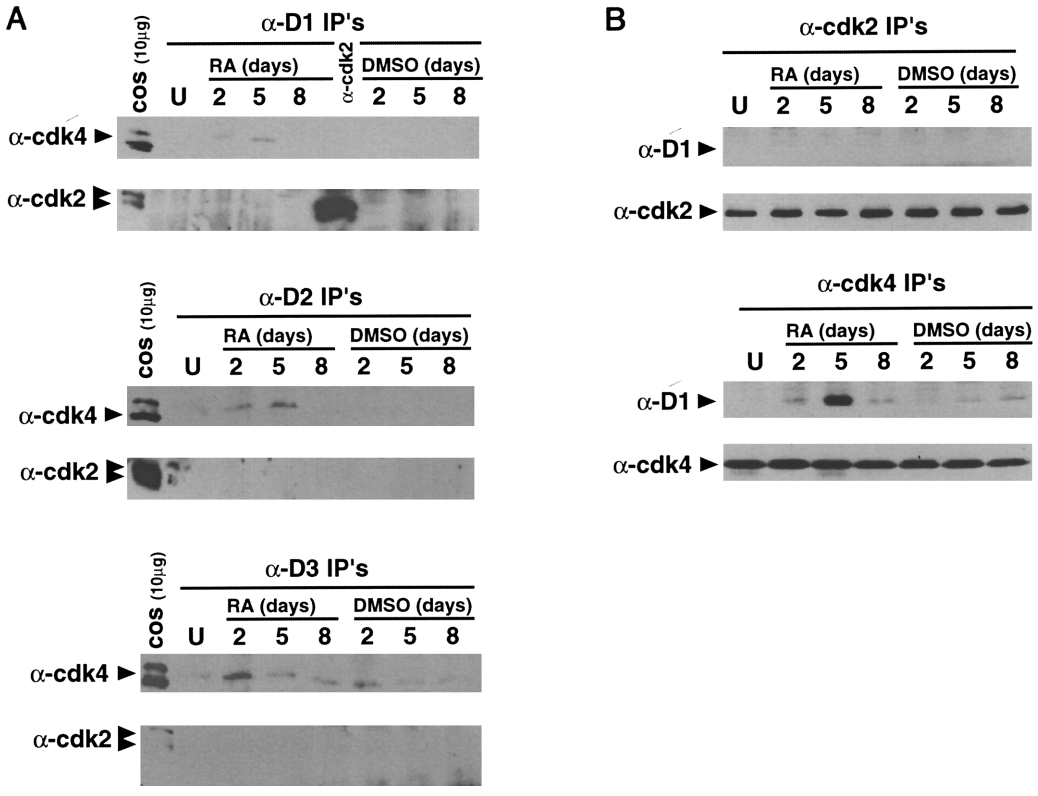


**FIG. 7.** cdk4 but not cdk2 is associating with the D cyclins. (A) Total protein was obtained from undifferentiated, as well as RA- and DMSO-treated Days 2, 5, and 8, P19 cells, and 40 $\mu$g each lysate was precleared and immunoprecipitated with $\alpha$-cyclin D1, $\alpha$-D2, or $\alpha$-D3 polyclonal antibodies for 1.5 h at 4°C. Immune complexes were run on 13% SDS-PAGE gels, along with 20 $\mu$g of straight COS cell lysate, and transferred to nitrocellulose membrane. Blots were probed with $\alpha$-cdk4 polyclonal antibody, followed by a 1:8000 dilution of goat $\alpha$-rabbit HRP-conjugated 2° antibody. Blots were developed with the ECL fluorescent detection kit and, following exposure, were stripped and reprobed with $\alpha$-cdk2 polyclonal antibody. Controls include an $\alpha$-cdk2 immunoprecipitation on the cyclin D1 blot. (B) Of kinase assay lysate 400 $\mu$g was precleared and immunoprecipitated with $\alpha$-cdk2, and $\alpha$-cdk4 polyclonal antibodies. Immune complexes were run on 13% SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with $\alpha$-cyclin D1 monoclonal antibody, followed by a 1:8000 dilution of goat $\alpha$-rabbit HRP-conjugated 2° antibody. Blots were developed with the ECL fluorescent detection kit and, following exposure, were stripped and reprobed with $\alpha$-cdk2 and $\alpha$-cdk4 polyclonal antibodies.

Despite the persistence of E2F-2 and active pRB in RA-treated cells, we and others [73, 82, 83] could not detect E2F site-binding complexes containing pRB in EMSAs using murine cell lysates. We propose that failure to detect this complex is due to the reduced affinity of E2F for DNA when E2F is complexed with pRB. This model is supported by our observation that when a full-length version of pRB is added to nuclear lysates from undifferentiated P19 cells, free E2F complexes disappear but new, pRB-containing complexes are not detected. Additionally, pRB strongly reduces the ability of E2F to bend DNA fragments encoding an E2F-binding site [76]. Thus, under the conditions used in the EMSAs and at the concentration of E2F and pRB present in these cells, the affinity of the pRB-E2F complex for a single E2F site may be insufficient to observe significant levels of DNA-bound complex. This model predicts that in cells where higher concentrations of pRB/E2F complexes are present, binding to DNA may be observed. Indeed, we can easily detect pRB-E2F complexes bound to DNA in EMSAs using nuclear lysates from CEM cells, these cells having considerably greater levels of pRB (>20-fold) relative to P19 cells (R.M.G., data not shown). It remains unclear, however, why pRB mutants which do not contain the N-terminal domain are not able to reduce the affinity of E2F for its DNA element.

While cyclin D1 and D2 transcriptional induction would account for the rise in pRB-directed kinase activity on Day 5 of neuronal differentiation, the subsequent drop in activity is unexpected since protein levels do not decrease precipitously as differentiation proceeds. Similarly the pattern of D cyclin kinase activity during cardiac myogenesis cannot be correlated with changes in the levels of the D-type cyclins. The altered kinase activities associated with the D-type cyclins do change in accordance with the induction of the CKI, p27, however. Low levels of p27 apparently promote the association of the D cyclins with cdk4 while high levels p27 repress cdk4/D cyclin

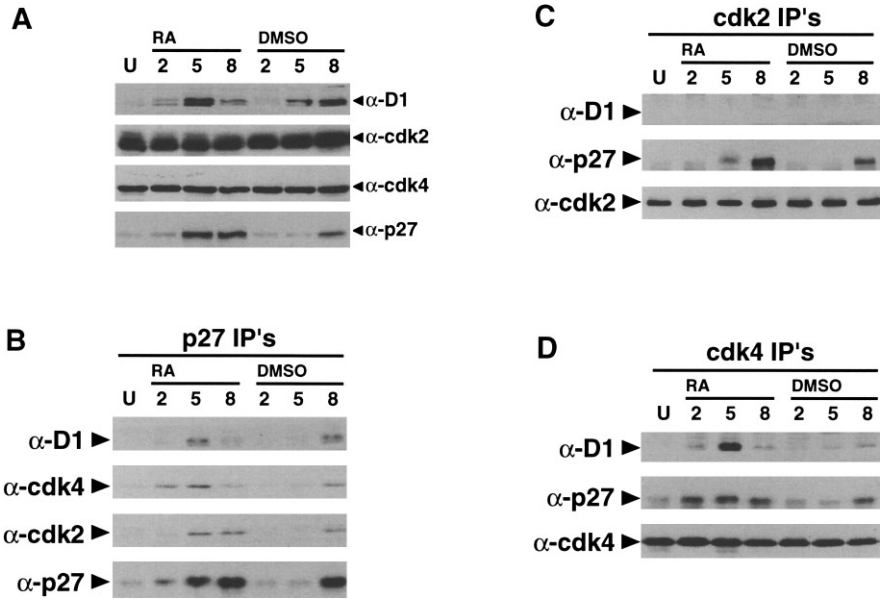


**FIG. 8.** p27$^{kip1}$ association with cdk4 and cdk2. (A) Total kinase assay lysate was isolated from undifferentiated as well as Days 2, 5, and 8 RA- and DMSO-treated P19 cells. A total of 20 $\mu$g lysate was run on 10% SDS-PAGE gels, protein was transferred to nitrocellulose and probed versus $\alpha$-D1 monoclonal, as well as $\alpha$-cdk2, $\alpha$-cdk4, and $\alpha$-p27 polyclonal antibodies. Blots were developed with the ECL fluorescence kit and exposed for 30 s to 2 min. (B) Kinase assay lysate was isolated from undifferentiated as well as Days 2, 5, and 8 RA- and DMSO-treated P19 cells. Lysates were precleared and 400 $\mu$g of lysate was immunoprecipitated overnight at 4°C with $\alpha$-p27 polyclonal antibody. Immune complexes were run on 13% SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with $\alpha$-cyclin D1 monoclonal antibody or $\alpha$-p27 and $\alpha$-cdk4 polyclonal antibodies and developed with the ECL fluorescent detection kit. Following exposure, blots were stripped and reprobed with $\alpha$-cdk2 polyclonal antibody. (C) Kinase assay lysate lysates were precleared and 400 $\mu$g of lysate was immunoprecipitated overnight at 4°C with $\alpha$-cdk2 polyclonal antibody. Immune complexes were run on 13% SDS-PAGE gels and transferred to nitrocellulose membrane. Blots were probed with $\alpha$-cyclin D1 monoclonal antibody or $\alpha$-p27, and $\alpha$-cdk2 polyclonal antibodies and developed with the ECL fluorescent detection kit. (D) Kinase assay lysate was isolated and immunoprecipitated with $\alpha$-cdk4 polyclonal antibody as described above. Immune complexes were run on 13% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were probed with $\alpha$-cyclin D1 monoclonal antibody or $\alpha$-p27 polyclonal antibody and developed with the ECL fluorescent detection kit. Following exposure, blots were stripped and reprobed with $\alpha$-cdk4 polyclonal antibody.

kinase activity [77, 84–87]. Early during P19 differentiation, low levels of p27 are expressed and the relative ratio of p27 in cdk4-containing complexes is also low. After Day 5, cyclin D1 and D2 protein levels fall and there is a relatively low level of D-cyclin-associated cdk4. However on Day 8 of RA treatment, cdk-4 coimmunoprecipitates a significant amount p27 (see Fig. 8D), suggesting that the low level of cyclin-associated cdk-4 complexes is bound by a high stoichiometric ratio of p27, resulting in inhibition of kinase activity.

Thus, our data demonstrate the differential regulation of expression of distinct members of the cyclin, CKI, pRB, and E2F family proteins. These changes culminate in the repression of cyclin-associated kinase activity, transition of the pRB family proteins to their active, hypophosphorylated state, and, ultimately, repression of E2F DNA-binding activity as cells exit the cell cycle during terminal differentiation.

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