Cells Differentiating into Neuroectoderm Undergo Apoptosis in the Absence of Functional Retinoblastoma Family Proteins

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Abstract. The retinoblastoma (RB) protein is present at low levels in early mouse embryos and in pluripotent P19 embryonal carcinoma cells; however, the levels of RB rise dramatically in neuroectoderm formed both in embryos and in differentiating cultures of P19 cells. To investigate the effect of inactivating RB and related proteins p107 and p130, we transfected P19 cells with genes encoding mutated versions of the adenovirus E1A protein that bind RB and related proteins. When these E1A-expressing P19 cells were induced to differentiate into neuroectoderm, there was a striking rise in the expression of *c-fos* and extensive cell death. The ultrastructural and biochemical characteristics of the dying cells were indicative of apoptosis. The dying cells were those committed to the neural lineages because neurons and astrocytes were lost from differentiating cultures. Cell death was dependent on the ability of the E1A protein to bind RB and related proteins. Our results suggest that proteins of the RB family are essential for the development of the neural lineages and that the absence of functional RB activity triggers apoptosis of differentiating neuroectodermal cells.

THE retinoblastoma (RB)[1] protein is thought to play a key role in the regulation of cell growth and differentiation (reviewed in 27, 30, 48). A role for RB in the control of cell proliferation is indicated by the observations that the loss of functional RB occurs in many human tumors (reviewed in 66), that injection of RB into proliferating cells induces their arrest in G1 phase (25), that RB is phosphorylated in a cell cycle-dependent manner (reviewed in 12), and that oncogenic proteins such as SV40 T antigen and adenovirus E1A proteins bind to RB and this binding is essential for their oncogenicity (8, 50).

A role for RB in differentiation is suggested by a number of observations. Mice carrying homozygous *Rb* mutations die in utero at E15-16 suffering from severe haematopoietic and neurogenic defects (10, 34, 40). The absence of RB did not seem to adversely affect the cell cycle; rather, the embryos appeared to die because erythrocytes and neural tissues failed to mature. In addition, RB and related proteins appear essential for the maturation of skeletal muscle (26, 55).

We set out to examine the role of the *Rb* gene family during neuronal differentiation using cultures of P19 embryonal carcinoma cells. When treated with retinoic acid (RA) these cells differentiate into cultures primarily consisting of neurons and astrocytes (35, 36). During this neuroectodermal differentiation we found that the level of the RB protein rose dramatically from barely detectable levels in the undifferentiated cells (58). To determine if this rise in RB level is required for neural differentiation we set out to use E1A to functionally inactivate RB and related proteins. The adenovirus E1A oncogene encodes a multifunctional nuclear protein (reviewed in 17) that binds to RB and related proteins, p107 and p130. The binding of E1A to this family of related proteins prevents the interaction of these proteins with transcription factors E2F and DP-1 (3, 4). Thus E1A provides a useful perturbant with which to examine the roles of RB family members in regulating cell differentiation.

The expression of wild-type E1A in undifferentiated P19 cells resulted in drastic morphological changes (46, 59, 64, 65); however, these effects of E1A were dependent on the binding of E1A to p300, a cellular protein unrelated to RB (19). We were able to establish clones of P19 cells expressing E1A mutants unable to bind p300 but which retained the capacity to bind pRB, p107 and p130. We report that differentiating P19 cells expressing these E1A proteins undergo apoptosis in those cells that become committed to form neurons and astrocytes.

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1. *Abbreviations used in this paper*: aa, amino acids; Pgk-1, phosphoglycerate kinase; RA, retinoic acid; RB, retinoblastoma.

Materials and Methods

Expression Plasmids

All expression vectors were based on the regulatory sequences of the mouse phosphoglycerate kinase (Pgk-1) gene promoter (1, 44). Plasmids containing E1A coding regions and various deletion mutants were kindly provided by Dr. S. Bayley (McMaster University, Hamilton, Ontario) (Jelmsa et al., 1988) and Dr. P. Branton (McGill University, Montreal, Quebec) (5). Deletion mutants defective in the binding of p300 (see Fig. 1 *A*) are as follows (amino acid deletions shown in parentheses): 1101 (4–25); 1104 (48–60); and 1141 (61–69). The double-deletion mutant, unable to bind p300 or pRB, p107 and p130 was 1101-1108 (4–25 and 124–127). All deletion mutants transcribe only the 12S mRNA. The E1A-coding regions were excised by digestion with AflIII and HincII, and ligated into a vector containing the Pgk-1 promoter and 3′ regulatory sequences (44). The Pgk-1 promoter was also used for expression constructs conferring puromycin resistance (Pgk-puro). A plasmid, B17, containing the Pgk-1 promoter and the first 17 kb of the mouse Pgk-1 genomic clone, was routinely cotransfected to enhance incorporation when stable transformants were generated (45).

Cell Culture and Transfections

P19 cells were maintained in α-MEM containing 5% calf serum and 5% FCS and induced to differentiate as described (52).

DNA was introduced into cells by calcium phosphate precipitation (9). Stable transformants were made by the cotransfection of 10 μg of the E1A carrying plasmid, together with 5 μg of B17 (45), and 2 μg Pgk-puro. Puromycin resistant clones were selected by plating cells in 2 μg/ml puromycin. After 5–7 d of selection, puromycin resistant colonies were screened for E1A expression by immunofluorescence staining with the M73 antibody to E1A (28). Stable cell lines were established expressing the E1A mutants, 1101, 1104, 1141, and the double mutant, 1101/1108. The expression of the transfected DNA in stable clones was verified by western blotting with the M73 antibody (28).

Western Blotting

Western blots and protein extraction were performed as previously described (58). E1A and all deletion mutants were readily detected with a mouse monoclonal antibody (M73) diluted 1:1,000 (28). The rat monoclonal antibody (TROMA-1) detected cytokeratin 55 (38). A rabbit polyclonal anti-NFl60, diluted at 1:2,000, was kindly provided by Dr. W. Mushinski (McGill University, Montreal, Quebec). The mouse monoclonal antibody directed against p53, diluted at 1:10, was purchased from Oncogene Science (Cambridge MA). Anti-GFAP was a mouse monoclonal antibody purchased from Chemicon International Inc. (Temecula, CA). The secondary antibodies were a goat anti-mouse horseradish peroxidase (no. 172-1011; Biorad, Mississauga, Ontario) diluted 1:5,000 and a rabbit anti-rat alkaline phosphatase (DAKOPATTS, Copenhagen, Denmark). The blots were developed by the ECL chemiluminescence system (Amersham, Oakville, Ontario) or the alkaline phosphatase substrate package obtained from GIBCO BRL (Gaithersburg, MD).

Isolation of Fragmented DNA

To examine DNA fragmentation, 10^6 cells were seeded on a 100-mm dish and treated as described in figure legends. After 48 h cells were subcultured at a density of 3×10^6 in the same medium. The next day, cells were harvested, washed once with PBS, and used for DNA isolation (49). 1.2 ml of lysis buffer was added to 5×10^6 cells suspended in 100 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Lysis was allowed to proceed at room temperature for 15 min and the mixture centrifuged for 15 min at 12,000 rpm. The gelatinous pellet was removed with a pipet and the supernatant digested with 100 μg/ml RNase A at 37°C for 30 min. This was followed by a Proteinase K digestion at 100 μg/ml, 50°C for 30 min. The DNA was then precipitated by adding an equal volume of 100% ethanol and NaCl such that the final concentration was 0.5 M. Following centrifugation the pellet was washed with 70% ethanol and resuspended in 50 μl of TE buffer. DNA ladders could be visualized by running 25 μl on a 1% agarose gel using a 1-kb ladder as a standard.

Northern Blot Analysis

Total RNA was prepared from cells by the lithium/urea method (2). For northern blots showing differentiation time courses, RNA was collected from cells for up to 4 d after RA treatment. Due to the high proportion of dying cells in some cultures there was insufficient material to provide an RNA sample after this time. Time courses with control P19 cells often included RNA samples for up to 10 d after RA treatment to provide a complete expression pattern during normal differentiation. 10-μg aliquots of RNA were separated through a 1.0% agarose gel containing 20 mM 3-(*N*-morpholino)propane-sulfonic acid, 1 mM EDTA, 5 mM sodium acetate, pH 7.0, and 10% formaldehyde. The RNA was transferred to Hybond-N membranes (Amersham) that were then treated with ultraviolet light at 120 mJ with a GS Gene linker UV chamber (Biorad). Hybridizations to random-primed ^{32}P-labeled DNAs, and high stringency washes were performed as described (Maniatis et al., 1982). Signals were visualized by autoradiography. To strip filters before rehybridization, they were washed for 2 h at 65°C in a buffer containing 0.005 M Tris-HCL (pH 8.0), 0.002 M Na_2EDTA and 0.1× Denhardt's solution.

The cDNA probes for *c-jun* (39), *c-myc*, and *c-fos* (63), E2F (37), DP-1 (24), myoD (14), and actin (53) were excised from their plasmid vectors before labeling. The munc-18 probe (56) was a gift from S. Beushausen (NINDS, Bethesda, MD). The probes for cyclins D1, 2, and 3 were excised from a pGEM 7ZF vector by digestion with EcoRI-BamHI.

Electron Microscopy

P19 and P19[1104] cultures were exposed to RA for 0, 3, or 7 d and then fixed in 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h. After initial fixation samples were washed three times in 0.1 M sodium cacodylate buffer, for 15 min each wash. Samples were postfixed on ice in 1% osmium tetroxide for 90 min, dehydrated in ascending concentrations of ethanol and acetone, and embedded in Epon-Araldite. Semithin sections were stained with toluidine blue for light-microscopic examination. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope.

Results

P19 Cells Expressing E1A Undergo Apoptosis when Induced to Differentiate with RA

We have previously established clones of P19 cells expressing high levels of mutant E1A proteins rendered incapable of binding p300 by deletions affecting either the E1A NH_2 terminus or CR1 region (Fig. 1 *A*). P19 cells expressing such E1A mutants were indistinguishable from the parental cells; they proliferated at similar rates and expressed markers of undifferentiated embryonal carcinoma cells (59). When treated with RA, P19 cells normally differentiate into neuroectodermal cells including neurons and astrocytes (35, 36). RA-treated P19 cells expressing any one of the E1A proteins encoded by genes 1101, 1104, or 1141 behaved very differently. For example, P19[1104] cells, which express the E1A protein encoded by the 1104 mutant, continued to proliferate for 48 h in RA after which the cell number in the cultures declined (Fig. 1 *B*). Widespread cell death was evident by 3–4 d. None of the surviving cells had neuritic processes but some cells did survive RA treatment and continued to proliferate.

To determine if the cell death observed was due to apoptosis, DNA was isolated from the cultures of RA-treated P19[1104] cells and was found to be fragmented into multimers of nucleosome size 3–4 d after initial exposure to RA (Fig. 2 *A*). No DNA ladder was evident in untreated P19[1104] cells or from the parental P19 cells either untreated or treated with RA. The cell death in RA-treated cultures was a consequence of the E1A protein since in independently selected clones of transfected P19 cells, RA-induced DNA fragmentation occurred only in those clones that expressed the E1A protein (Fig. 2 *B*).

Clones of P19 cells expressing different E1A mutants were

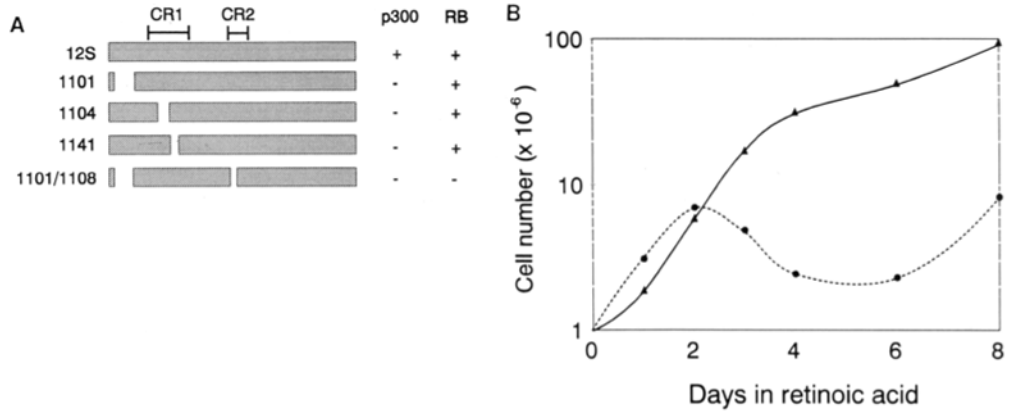

Figure 1. P19[1104] cells die in RA. (*A*) Deletion mutants of the 12S E1A cDNA used in this study. The protein encoded by the 12S cDNA is depicted as a bar of 243 amino acids (aa). The deletion mutants encode proteins with internal deletions: 1101 deletes aa 4–25 from the NH_2 terminus, 1104 deletes aa 48–60 and 1141 deletes aa 61–69, both affecting the CR1 region, and the 1101/1108 double mutant has deletions of aa 4-25 and aa 124–127 altering both the CR1 and CR2 regions of the protein. The ability of each E1A protein to bind p300 and RB is indicated on the right. (*B*) Cell numbers in cultures of RA-treated P19 (▲—▲) and P19[1104] (●···●) cultures. 10^6 cells were seeded into 100-mm culture dishes in 15 ml of medium containing 1 μM RA. Cells were harvested after 1, 2, 3, 4, 6, and 8 d and counted with an electronic cell counter. Each point represents the average from four different experiments.

tested in this DNA fragmentation assay. P19 cells expressing E1A mutants 1104, 1101, and 1141 all underwent DNA fragmentation following RA treatment (Fig. 2 *C*). These three E1A mutants do not bind p300 but do bind members of the RB family (18, 32). The double deletion mutant 1101/1108 does not bind p300 or members of the RB family. RA-treated P19 cells expressing the 1101/1108 mutant underwent very little DNA fragmentation (Fig. 2 *D*). These cultures differentiated like the parental P19 cells into neurons and astrocytes.

Light and electron microscopy was used to further characterize the dying cells in P19[1104] cultures (Fig. 3). Untreated populations of P19 and P19[1104] cells contained few or no apoptotic cells (Fig. 3, *A* and *C*) but after 3 d in RA up to half of the P19[1104] cell population consisted of cells with dark pyknotic nuclei (Fig. 3 *D*). Ultrastructural examination of these cells indicated all the expected characteristics of cells undergoing apoptosis (Fig. 3 *F*). These included clumping of dense chromatin near the nuclear periphery, an intact nuclear membrane, extensive cytoplasmic blebbing, and intact cytoplasmic organelles. At later stages apoptotic cells showed marked shrinkage and contained small pyknotic nuclei. These cultures contained large numbers of cytoplasmic fragments.

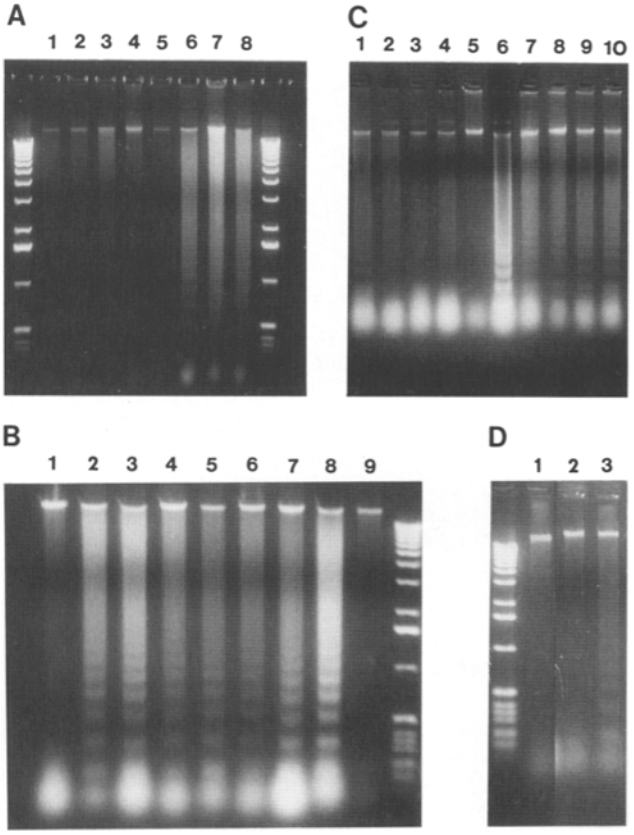

Figure 2. DNA fragmentation in RA-treated P19 cells expressing E1A proteins. (*A*) Kinetics of DNA fragmentation. DNA was isolated from P19 cells after 0 (lane *1*), 3 (lanes *2* and *3*), or 4 d (lane *4*) in RA and subjected to gel electrophoresis and visualization with ethidium bromide. DNA from P19[1104] cells was isolated after 0 (lane *5*), 3 (lanes *6* and *7*), or 4 d (lane *8*) in RA. The cells used in lanes *3* and *7* were exposed to RA as cell aggregates while other cultures were treated while cells were attached to plastic surfaces. (*B*) Only E1A-expressing cells fragment their DNA in RA. P19 cells were cotransfected with plasmids carrying the puromycin resistance gene driven by the Pgk-1 promoter and the E1A mutant 1104 also driven by Pgk-1. Colonies that developed in puromycin were picked and tested for E1A expression using Western blots probed with the M73 antibody. The same clones were treated for 3 d with RA and their DNAs isolated and subjected to gel electrophoresis. Lanes *1* and *9* were from clones of cells expressing no E1A protein while lanes *2–8* were from E1A-expressing cells. (*C*) E1A proteins with intact CR2 regions induce DNA fragmentation. Cells were either untreated (lanes *1–4*) or treated with RA for 3 d (lanes *5–10*) before DNA was isolated and electrophoresed. P19 cells are in lanes *1* and *5*, P19[1101] in lanes *2* and *6*, P19[1104] in lanes *3*, *7*, and *9* and P19[1141] in lanes *4*, *8* and *10*. (*D*) E1A protein with a deletion affecting CR2 did not induce DNA fragmentation. DNA was isolated after 3 d exposure to RA from P19 (lane *1*), P19[1101/1108] (lane *2*), and P19[1104] (lane *3*). The unlabeled lanes in each of *A*, *B*, and *D* contain marker DNA.

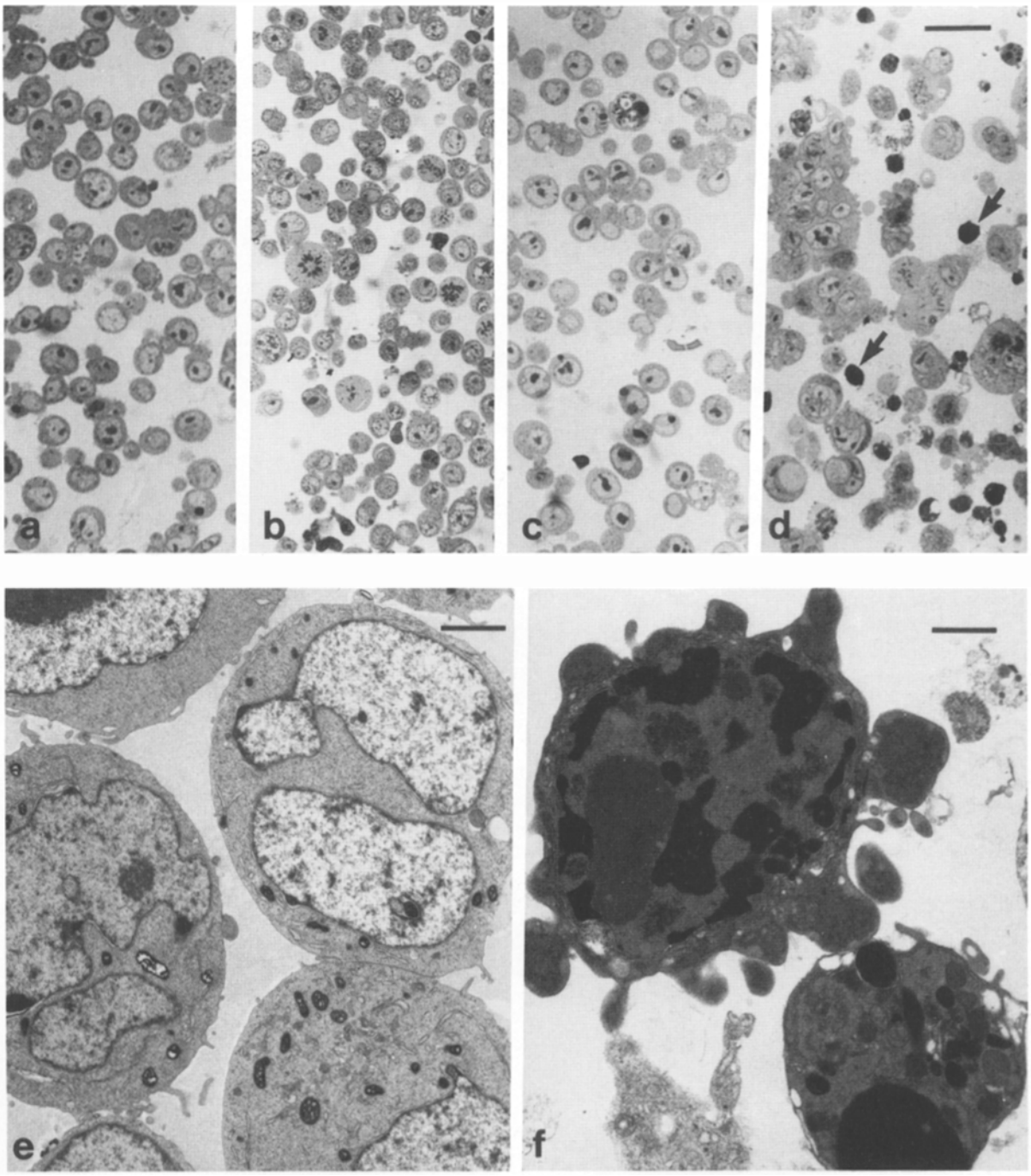

Figure 3. RA-treated P19[1104] cells undergo extensive apoptosis. P19 cells (*A* and *B*) and P19[1104] cells (*C* and *D*) were either untreated (*A* and *C*) or treated with RA for 3 d (*B* and *D*). In toluidene blue stained sections we saw apoptotic nuclei and apoptotic bodies only in RA-treated P19[1104] cells (*D, arrows*). *E* shows the typical ultrastructural appearance of P19 cells with multilobulated nuclei and relatively light chromatin. The cytoplasm contains numerous free ribosomes, few mitochondria and few stacks of rough endoplasmic reticulum. *F* shows an apoptotic cell from an RA-treated P19[1104] culture. This cell has multiple cytoplasmic blebs, dense chromatin clumps along the nuclear envelope, and intact cytoplasmic organelles. Bars: (*A–D*) 10 μm; (*E*) 2 μm; (*F*) 1.2 μm.

Cells Committed to the Neural Lineage Undergo Apoptosis

RA-treated P19 cells differentiate primarily into neurons and astrocytes (35, 36) along with some fibroblast-like cells (54) and a few epithelial cells. RA-treated P19 cultures developed high levels of neurofilament 160 (NF160) and GFAP, markers of neurons and astrocytes respectively (Fig. 4 *A*). In RA-treated P19 cultures, neurons mature within 3 d when NF160 is detected while astrocytes mature more slowly so GFAP is detected only after 8 d. Surviving cells in RA-treated cultures of M3, a clone of P19[1104] cells, contained barely detectable levels of these two proteins. Similar results were obtained with P19[1101] and P19[1141] cells (data not shown).

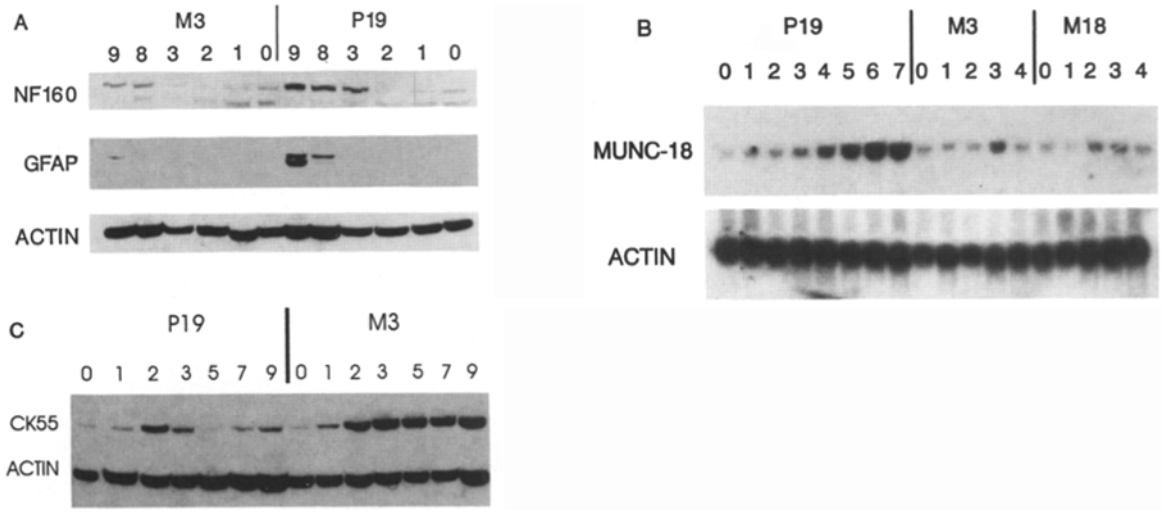

Figure 4. E1A-expressing P19 cells express few neuronal markers following RA treatment. Cellular protein (*A* and *B*) or RNA (*C*) were isolated from cultures at intervals following exposure to RA. These cells were P19, M3, a clone of P19[1104], and M18, a clone of P19[1141]. The numbers above each lane represent the number of days in RA. (*A*) An immunoblot of protein probed with antibodies to the 160-kD component of neurofilament (*NF160*), with antibodies to glial fibrillar acidic protein (*GFAP*), and with antibodies to actin. Each lane was loaded with 50 μg of protein and separated by SDS–polyacrylamide gel electrophoresis before being transferred to nitrocellulose. Immunoreactive material was detected by chemiluminescence. (*B*) A Northern blot showing the expression of the early neuronal marker munc-18. RNA was isolated from cultures at the times indicated and 10 μg loaded on each lane before being electrophoresed, blotted, and probed with cDNAs for munc-18 and actin. (*C*) An immunoblot probed with antibodies to cytokeratin 55 (CK55) and actin. Protein preparation was similar to that described in *A*.

These results suggest that most of the cell death in these cultures was of cells differentiating into neurons and astrocytes.

Both NF160 and GFAP are relatively late markers of differentiation while munc-18 (29) is a gene expressed early during neuroectodermal differentiation (56). The expression of munc-18 was markedly enhanced as P19 cells differentiated in RA (Fig. 4 *B*). In M3 and M18 cells, clones of P19[1104] and P19[1141] cells, respectively, munc-18 mRNA levels increased following RA treatment but declined after day 3. The loss of munc-18 mRNA from these cultures coincided with abundant cell death consistent with the idea that neural cells were induced and then eliminated from this cell population.

RA-treated P19 cultures normally develop very few epithelial cells (36, 62). Cytokeratin 55 is a protein marker for early epithelium and it is expressed at low levels in RA-treated P19 cultures (Fig. 4 *C*). In contrast, surviving cells in RA-treated P19[1104] cultures contained very high levels of cytokeratin 55. Immunofluorescence with this antibody indicated that the majority of P19[1104] and P19[1141] cells surviving RA treatment contained cytokeratin filaments (data not shown).

An ultrastructural comparison of P19 and P19[1104] cells 7 d after RA treatment was performed. RA-treated P19 cultures consisted of large numbers of neurons with characteristic processes (Fig. 5 *A*). No such neurons were evident in cultures of P19[1104] cells. Many of the surviving P19[1104] cells were epithelioid in nature with characteristic intercellular junctions (Fig. 5 *B*). The other cell type evident in these P19[1104] cultures were large multinucleate cells containing cytoplasmic aggregates of intermediate filaments (Fig. 5 *C*). The nature of these aberrant large cells is unknown.

Gene Expression in RA-treated Cells

The level of RB expression in P19 cells increases to 10 times the level in untreated cells 3-4 d after RA treatment (58). It is at this time that extensive cell death occurs in RA-treated P19[1104], P19[1141], and P19[1101] cells. In addition, the RB-binding domain of E1A is essential for the RA-induced apoptosis. These results imply that apoptosis in E1A-expressing P19 cells undergoing differentiation occurs as a result of the binding of the mutant E1A protein to proteins of the RB family. We therefore examined the expression of genes believed to be targets for regulation by the RB-like proteins. In addition, we investigated the expression of genes whose products mediate and modulate the effects of RB-like proteins.

The transcription factors E2F and DP-1 are thought to heterodimerize (4, 31) and be negatively regulated by binding to RB (3, 7, 67). The transcripts of both are present in P19, P19[1104], and P19[1141] cells before and during RA-induced differentiation (Fig. 6 *A*).

The phosphorylation of RB proteins is at least in part mediated by cyclin-dependent kinases and recent evidence suggests a role for cyclin D-1 in apoptosis (22). The transcripts encoding the D cyclins are low in untreated P19 cells and increase following RA treatment (Fig. 6 *C*). The level of D-1 cyclin appears to be elevated in untreated P19[1104] and P19[1141] cells and this level of expression was maintained following exposure to RA. The level of D-2 cyclin mRNA also appeared to be higher in RA-treated cells than in the undifferentiated parental cultures while little change was seen in the level of D-3 cyclin mRNA following RA treatment (Fig. 6 *C*).

Some immediate early genes are thought to play roles in

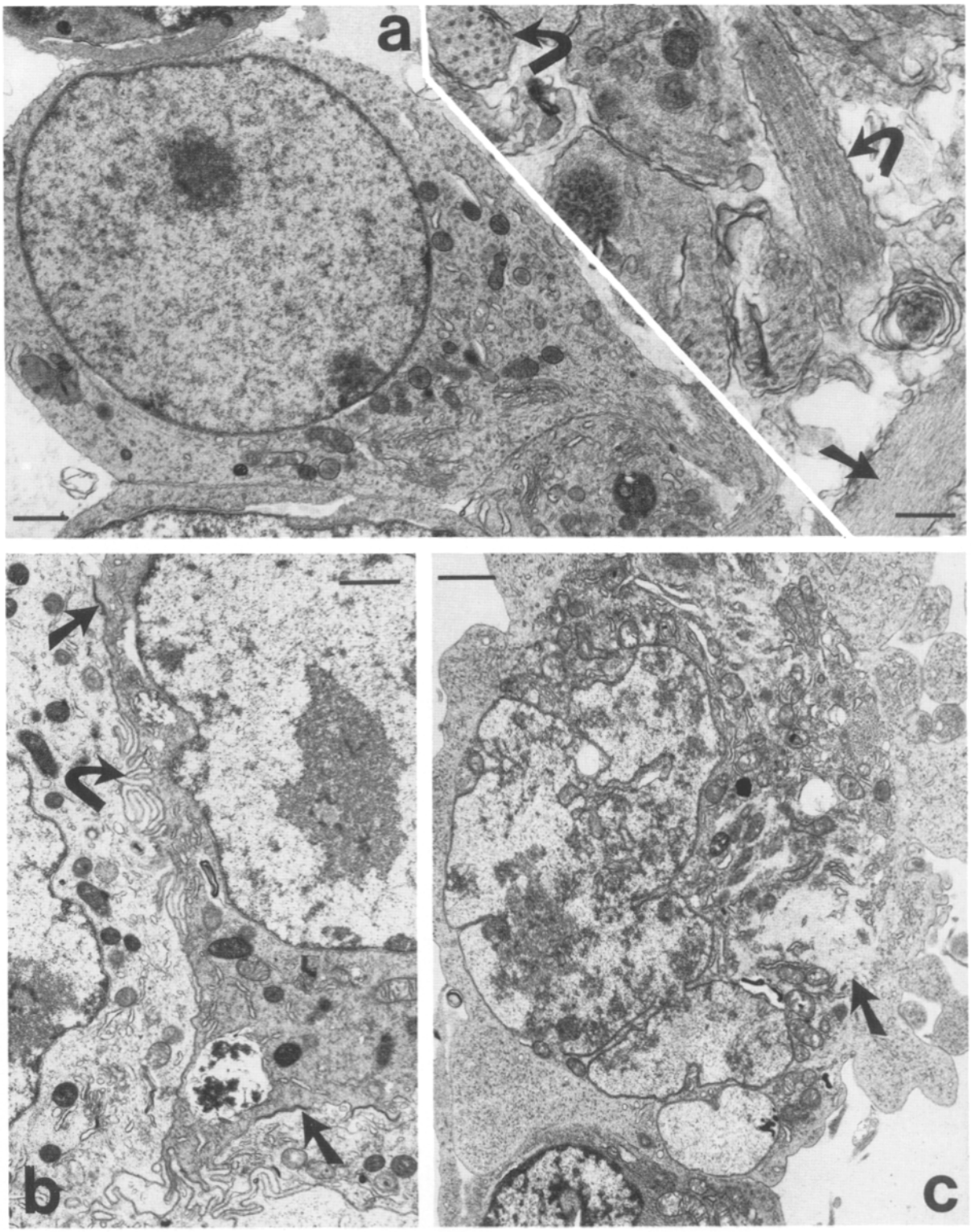

Figure 5. Ultrastructure of P19 and P19[1104] cells 7 d after RA treatment. (*A*) Electron micrograph of RA-treated P19 cells with large vesicular nuclei, multiple Golgi complexes, a few neurosecretory-like granules,and neuritic processes with microtubules. The insert shows a number of neuritic processes containing microtubules (*curved arrows*) along with a single astrocytic process (*straight arrow*) filled with abundant intermediate filaments. (*B*) RA-treated P19[1104] cultures contained epithelial cells with intercellular junctions (*arrows*) and lateral short interdigitating processes of two opposing cellular membranes (*curved arrow*). (*C*) RA-treated P19[1104] cultures also contained large abnormal cells of undetermined nature. These cells contained multilobulated nuclei and focal accumulation of intermediate filaments in the cytoplasm (*arrow*). Bars: (*A*) 1 μm; (*A*, *inset*) 0.3 μm; (*B*) 1 μm; (*C*) 1.4 μm.

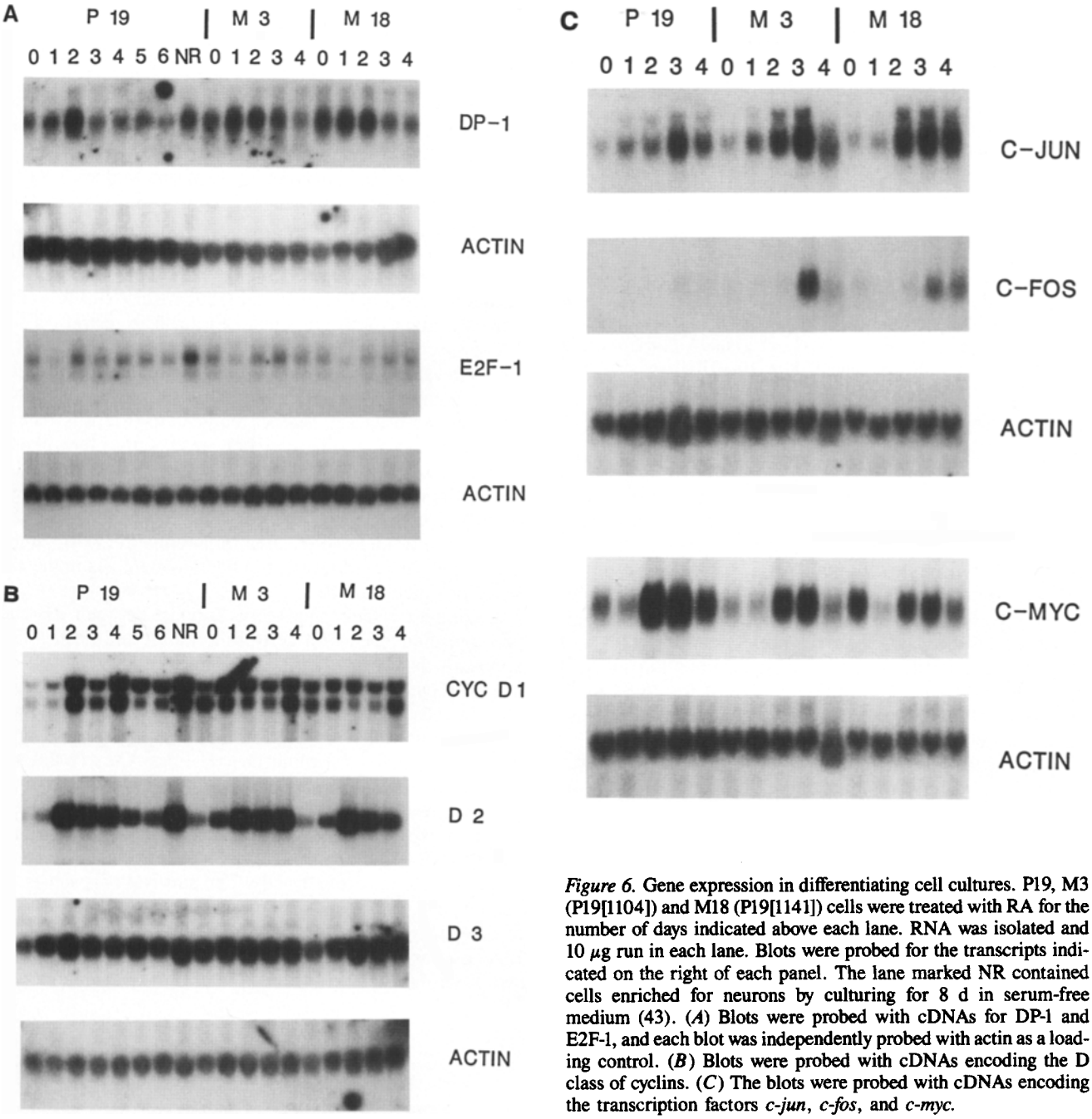

Figure 6. Gene expression in differentiating cell cultures. P19, M3 (P19[1104]) and M18 (P19[1141]) cells were treated with RA for the number of days indicated above each lane. RNA was isolated and 10 μg run in each lane. Blots were probed for the transcripts indicated on the right of each panel. The lane marked NR contained cells enriched for neurons by culturing for 8 d in serum-free medium (43). (*A*) Blots were probed with cDNAs for DP-1 and E2F-1, and each blot was independently probed with actin as a loading control. (*B*) Blots were probed with cDNAs encoding the D class of cyclins. (*C*) The blots were probed with cDNAs encoding the transcription factors *c-jun*, *c-fos*, and *c-myc*.

apoptosis (21, 61) and are known to be modulated during RA-induced differentiation of P19 cells (15, 63). *c-jun* and *c-myc* are induced following RA treatment of P19 cells and expression of both was similarly increased in P19[1104] and P19[1141] cultures (Fig. 6 *B*). *c-fos* mRNA levels are very low in P19 cells (13) and remain low for many days following RA-induced differentiation. In P19[1104] and P19[1141] cells *c-fos* expression was also low; however, the abundance of the *c-fos* transcript markedly increased after 3–4 d of RA treatment, the time during which apoptosis became evident in the cultures (Fig. 6 *B*).

The p53 protein is believed to play a critical role in apoptosis (11, 16, 42, 68). Very high levels of p53 are present in untreated P19 and in E1A-expressing P19 cells and these levels declined markedly after exposure to RA (Fig. 7). High p53 levels are a characteristic of embryonal carcinoma cells such as P19 and F9 (41) and the reduced levels seen in differentiated cells are similar to those found in fibroblast cell cultures.

E1A-expressing P19 Cells Fail to Differentiate into Mesoderm

P19 cells are multipotent and aggregates of these cells can be induced to differentiate into mesodermal and endodermal

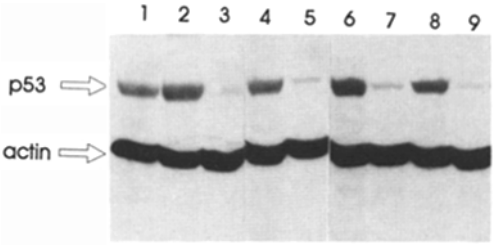

Figure 7. Differentiating P19 cells down-regulate p53. Protein from cell cultures was subject to SDS-polyacrylamide gel electrophoresis, electroblotted, and probed with antibodies to p53 and to actin. Visualization was by chemiluminescence. Lane *1*, F9 embryonal carcinoma cells; lane *2*, P19 cells; lane *3*, P19 cells treated for 72 h with RA; lane *4*, P19[1101] cells; lane *5*, P19[1101] cells treated for 72 h with RA; lane *6*, P19[1141] cells; lane *7*, P19[1141] cells treated for 72 h with RA; lane *8*, P19[1101+1108] cells; lane *9*, P19[1101+1108] cells treated for 72 h with RA.

cell types. When exposed to 1% DMSO, cardiac muscle is formed abundantly from P19 cells. When cultured under the same conditions, none of the E1A-expressing P19 cells differentiated. For example, no muscle was formed in P19[1104] cultures treated with 1% DMSO and a marker of undifferentiated cells, oct-3, was not reduced in abundance (Fig. 8 *A*).

RB expression is elevated in skeletal muscle (57) and skeletal muscle is formed from aggregated cultures of P19 cells exposed to 1.5% DMSO and 10^{-9} M RA (20). However, none of the E1A-expressing P19 cells exposed to these conditions differentiated (Fig. 8 *B*).

When aggregated and treated with 1% DMSO, there was undetectable cell death in cultures of P19[1101], P19[1104], and P19[1141] cells (Fig. 8 *C*). The failure of these E1A-expressing cells to differentiate means that it is impossible to determine if E1A induces apoptosis of cells differentiating into mesodermal lineages. Rather, it appears that the E1A protein interferes with the signal responsible for the initiation of differentiation into the mesodermal lineages suggesting that a member of the RB family plays a role in the commitment of P19 cells to initiate mesodermal development.

Discussion

Our results indicate that RB has a crucial role in the differentiation of cells into neuroectodermal lineages. The levels of RB expression in embryos (6) and in differentiating P19 cells (58) rise by 10-fold as cells develop into neuroectoderm. Inhibiting the function of RB with the E1A protein in differentiating P19 cells resulted in extensive cell death and a significant decrease in the proportion of surviving cells forming neurons and astrocytes. P19 cells expressing mutant E1A proteins unable to bind RB did not die but differentiated normally. The simplest interpretation of this data is that RB or other members of the RB family are not only essential for the development of neuroectoderm but are also linked to the signals leading to programmed cell death in neural cells.

The susceptibility of neural tissues to the absence of RB during development is consistent with other recent investigations. Embryonic mice nullizygous for the *Rb* gene (10, 34, 40) show extensive cell death throughout the nervous system at the time terminal differentiation was about to take place. In transgenic mice expressing HPV-16 E7 (which, like E1A binds RB) driven by the interstitial retinol-binding protein (IRBP) promoter, photoreceptor cells die at the time when they normally undergo terminal differentiation (33). Thus, neural cells are predisposed to undergo apoptosis in the absence of functional RB.

A complex relationship seems to exist between RB and p53 and their effects on cell proliferation and death. The apoptosis that occurs in RB deficient mice is dependent on p53 (47). On the other hand, undifferentiated embryonal carcinoma cells such as P19 express almost undetectable levels of RB along with very high levels of p53, yet these cells proliferate rapidly as immortal cell lines. Clearly the absence of RB in a p53 containing cell is not sufficient to trigger apoptosis in all cell types.

In E1A-expressing P19 cells we found no normal neurons while in RB deficient mice certain subsets of neurons were found to differentiate normally. The survival of a subset of neurons in RB deficient mice could be due to the expression of other members of the RB family in these neurons. The E1A proteins expressed in P19 cells are able to bind RB, p107 and p130, thus affecting the function of the entire family of

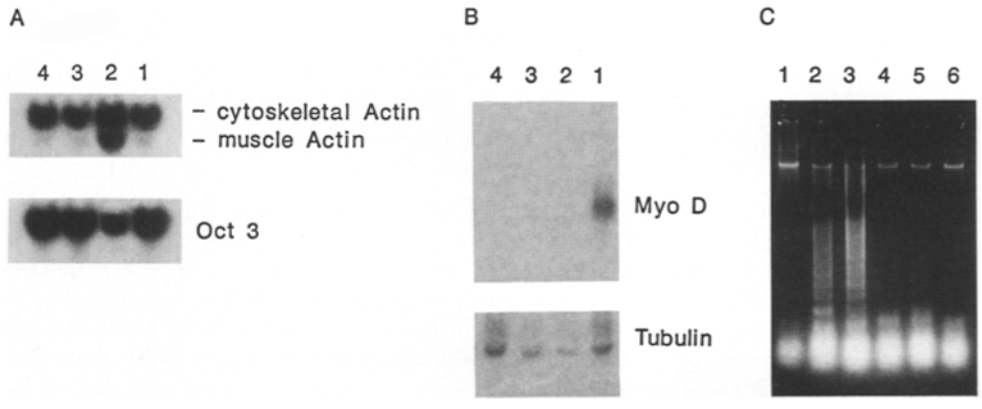

Figure 8. E1A-expressing P19 cells fail to differentiate into muscle. (*A*) P19 (lanes *1* and *2*) and P19[1104] (lanes *3* and *4*) cells were aggregated and treated with 1% DMSO. On day 0 (lanes *1* and *3*) and day 6 (lanes *2* and *4*) RNA was isolated and probed for actin and Oct-3. The muscle specific α-actin transcripts were expressed in DMSO treated P19 cultures (lane *2*) but not in P19[1104] cells (lane *4*). All cultures expressed the larger β-actin mRNA. The Oct-3 transcript is abundant in embryonal carcinoma cells but is reduced in differentiating cultures (lane *2*). (*B*) P19 (lanes *1* and *2*) and P19[1104] (lanes *3* and *4*) were aggregated and treated with 1.5% DMSO and 10^{-9} M RA. On day 0 (lanes *2* and *4*) and day 10 (lanes *1* and *3*) RNA was isolated and probed for the skeletal muscle specific marker, myoD, and for tubulin. (*C*) P19 (lanes *1* and *4*), P19[1104] (lanes *2* and *5*) and P19[1141] (lanes *3* and *6*) cells were aggregated and treated for 3 d in RA (lanes *1–3*) or 1% DMSO (lanes *4–6*). DNA from these cultures was isolated and examined for fragmentation as in Fig. 2.

proteins. No defect in glial differentiation has been reported in RB deficient mice perhaps because RB deficient embryos die before maturation of this cell type. Our studies suggest that the *Rb* gene family is not only essential for the differentiation of neurons, but also their supporting glial cells.

Among the surviving cells in RA-treated cultures of E1A-expressing P19 cells, the reduced development of neurons and glia was accompanied by an increased abundance of cells expressing the epithelial cell marker, cytokeratin 55. Because extensive cell death occurs in these cultures, the abundance of epithelium may be a consequence of the resistance of this cell type to E1A-induced cell death. Alternatively, the E1A protein in differentiating P19 cells may alter the developmental program of these cells to favor the formation of epithelium. We suspect that both selection and reprogramming account for the abundance of epithelium.

A number of proto-oncogene products are known to play essential roles in the induction of apoptosis and many of these proteins appear to be present in the RA-treated P19 cells. It seems particularly notable that *c-myc* and cyclin D1 are upregulated during neuroectodermal differentiation and both are expressed at high levels in E1A-expressing P19 cells when apoptosis is initiated. On the other hand, p53 is expressed at very high levels in undifferentiated P19 cells and its level declines in RA-treated cultures. Although the level of p53 is low when E1A-expressing P19 cells undergo apoptosis the concentration of p53 protein is nevertheless detectable and similar to that seen in normal cells and tissues.

The expression of *c-fos* is thought to be a harbinger of apoptosis because *c-fos* expression precedes programmed cell death in vivo (61). In parental P19 cells, *c-fos* mRNA levels are very low both before and during differentiation. However, in E1A-expressing P19 cells there was a dramatic increase in *c-fos* expression at the time cells undergo apoptosis. This increase in *c-fos* expression may be mediated directly or indirecly by the E1A protein. Studies with human *c-fos* promoter have identified a sequence motif that can down regulate promoter activity in the presence of RB (51) although this sequence is not well conserved in the murine promoter.

RB function appears essential for cells to arrest properly in G1 phase when challenged with ionizing radiation (60). The cell death seen in E1A-expressing P19 cells after RA treatment occurs when many of these cells are differentiating into neurons and would normally be permanently withdrawing from the cell cycle. The loss of glia from these cultures may be due to the failure of these cells to arrest in G1 before maturing. Alternatively, the differentiation of cells into the astrocyte lineage may depend on inductive signals from the neurons.

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