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Induction of Differentiation-Associated Changes in Established Human Cells by Infection with Adeno-Associated Virus Type 2

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The nonpathogenic human defective parvovirus adeno-associated virus (AAV) type 2 induced differentiation-associated antigens in cells of the human leukemia cell line HL60 (CD 67), as well as in two different lines of immortalized human keratinocytes, HaCaT and HPK Ia cells (involucrin and cytokeratin 10). Simultaneously, expression of the c-*myc* and c-*myb* oncogenes and the retinoblastoma gene was down regulated whereas c-*fos* expression increased in infected cells. These data point to the potential of AAV to induce functions related to the differentiation pathway in different types of human cells. This phenomenon may be involved in the reported oncosuppressive properties of AAV infections.

Adeno-associated viruses (AAV), as members of the parvovirus family, are small, helper virus-dependent DNA viruses containing a linear single-stranded genome of approximately 5,000 nucleotides (2, 13). Replication of AAV requires the presence of a coinfecting helper virus which can be an adenovirus, a herpesvirus, or vaccinia virus (2, 4, 17, 45). In addition, genotoxic stress of infected cells can substitute for helper functions, allowing AAV replication to some extent (57–59). AAV type 1 (AAV-1) to AAV-5 have not been found to be causally related to any known disease. In contrast, AAV is known to interfere with oncogenesis in vivo and with cell transformation in vitro (9, 14, 17, 30, 34, 38; for a review, see reference 44).

The molecular mechanisms underlying these tumor-suppressive properties of AAV are not understood. We have shown earlier that AAV interferes with mutagenesis (46) and with virus- or carcinogen-induced DNA amplification (24, 45, 47, 48). In addition, AAV infection selectively kills initiated cells (25). Recent studies have shown that induction of tumor necrosis factor or interferon does not account for the tumor-suppressive properties of AAV (44a).

Since an immature state of differentiation is a major characteristic trait of tumor cells, we investigated whether infection with the nonpathogenic parvovirus AAV-2 can induce differentiation in relatively undifferentiated cells. We used well-characterized model systems to study differentiation processes in two different human cell types, the promyelocytic leukemia cell line HL-60 and the human keratinocyte cell lines HaCaT and HPK Ia. We report here that infection of these cell lines with AAV-2 resulted in changes indicating induction of cellular differentiation.

MATERIALS AND METHODS

Cells. Spontaneously immortalized human keratinocytes (HaCaT; provided by N. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany) and human keratinocytes immortalized by human papillomavirus type 16 DNA (HPK Ia; provided by M. Dürst, Deutsches Krebsforschungszentrum) were grown in Dulbecco modified Eagle medium supplemented with 5% heat-inactivated (56°C, 30 min) fetal calf serum. HL-60 cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum. Media and fetal calf serum were from GIBCO (Karlsruhe, Germany). Medium was changed every 2 days. Cells were grown by incubation at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Virus. AAV-2 was propagated in HeLa cells by using adenovirus type 2 as a helper. Cells were lysed by three rounds of freezing and thawing, and purification of AAV-2 virions by CsCl gradient centrifugation was performed as described by Berns et al. (3) and Senapathy and Carter (49).

Infection protocol. Infection with AAV-2 was performed by inoculating phosphate-buffered saline (PBS)-washed cells at a multiplicity of 100 infectious units per cell for 30 min at 37°C. Unabsorbed virus was removed by washing infected cells twice with medium.

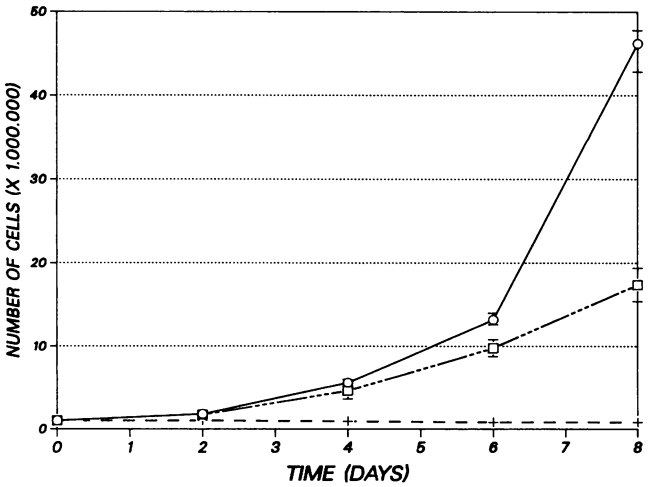

FIG. 1. Growth behavior of HL-60 cells postinfection with AAV-2. Cells (10^6) were seeded in culture flasks and treated with 1.3% DMSO (+), infected with AAV (□), or mock infected (○). After 2, 4, 6, and 8 days, cell numbers were determined by trypan blue exclusion.

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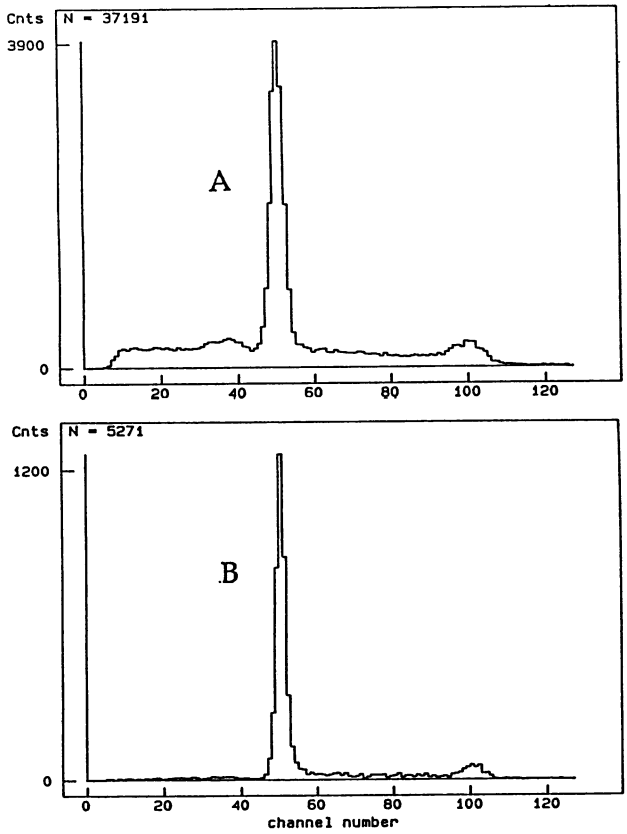

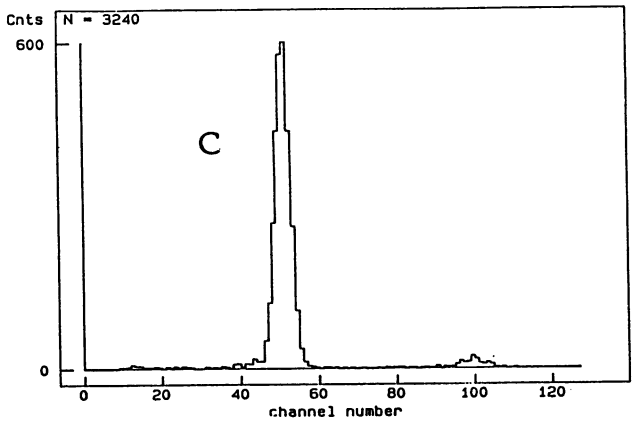

Treatment	Percentage of total cells		
	G1	S	G2/M
Control	54.4	41.1	4.5
AAV-2	70.3	24.6	5.1
DMSO	92.2	2.2	5.6

FIG. 2. Flow cytometry analysis of HL-60 cells infected with AAV-2. Flow cytometry analysis was performed at 6 days postinfection with AAV as described in Materials and Methods. Panels: A, mock-infected HL-60 cells; B, HL-60 cells infected with AAV; C, HL-60 cells treated with 1.3% DMSO. Cnts, counts.

Analysis of differentiation parameters in HL-60 cells. For control purposes, HL-60 cells were induced to differentiate along the granulocyte pathway by adding 1.3% dimethyl sulfoxide (DMSO; Sigma, Deisenhofen, Germany) to the growth medium. Morphological differentiation was visualized on Wright-Giemsa-stained cytospin preparations. Histological parameters of cell differentiation were assessed by nitroblue tetrazolium (NBT; Sigma) reduction capacity. NBT dye reduction was used as a functional marker of the terminal maturation of HL-60 cells. Cells (10^6) were washed twice with PBS and suspended in 1 ml of PBS containing 0.1% NBT and 1 μg of 12-*O*-tetradecanoylphorbol-13-acetate (all reagents were from Sigma). After incubation of cells at 37°C for 30 min, the percentage of cells containing black-blue formazan deposits was assessed by microscopic examination.

Flow cytometry analysis. Flow cytometry was performed by using a System 30-L fluorescence-activated cell sorter (Ortho-Instruments, Hannover, Germany). Cells were fixed in 70% ethanol and stored at 4°C for up to 1 week prior to flow cytometric analysis. The percentages of cells in the G_1, S, and G_2-M phases of the cell cycle were determined by the method described by Dean and Jett (16).

Indirect immunofluorescence and antibodies. Indirect immunofluorescence microscopy was performed for keratinocytes grown on glass coverslips. After being washed twice with PBS, cells were fixed with methanol (−20°C), briefly rinsed in acetone (−20°C), and stained with monoclonal antibodies (MAbs) to cytokeratin 10 (Bio Science, Emmenbrücke, Switzerland) and involucrin (Dianova, Hamburg, Germany) by using the single-label immunofluorescence staining technique described by Moll et al. (37). HL-60 cells were washed twice in PBS, and cytospin preparations were treated as described for keratinocytes for staining with MAbs against granulocyte-specific antigens (CD67; Dianova). As the second antibody, fluorescein-conjugated goat anti-mouse immunoglobulin (Dianova) was used for 30 min. Cells on coverslips were washed three times with PBS, mounted with 90% glycerol–10% PBS, and analyzed by using a Leitz (Wetzlar, Germany) fluorescence microscope.

Southern blot analysis. Genomic DNA was prepared from AAV-2-infected cell lines. Cells were washed twice in PBS, suspended in lysis buffer (3% sarcosyl, 0.07 M Tris-HCl, 0.025 M EDTA) containing proteinase K (100 μg/ml; Sigma), and incubated for 14 h at 37°C. The solution was extracted with an equal volume of redistilled phenol and subsequently extracted with an equal volume of phenol and chloroform-isoamyl alcohol (24 vol%/1 vol%). Isopropanol (0.3 vol%), sodium acetate (0.1 vol%), and isopropanol (0.7 vol%) were added to the DNA solution. The DNA precipitate formed at room temperature was washed in ethanol and suspended in 10 mM Tris-HCl–1 mM EDTA. Aliquots of restriction enzyme-digested high-molecular-weight DNA were electrophoresed on a 0.8% agarose gel, blotted onto GeneScreen Plus filters (DuPont, NEN, Boston, Mass.) as described by Southern (51), and hybridized with ^{32}P-labeled (α-[^{32}P]dCTP; Amersham, Braunschweig, Germany), randomly primed (20), cloned AAV-2 DNA (pAV-2 or pTAV) for 16 h

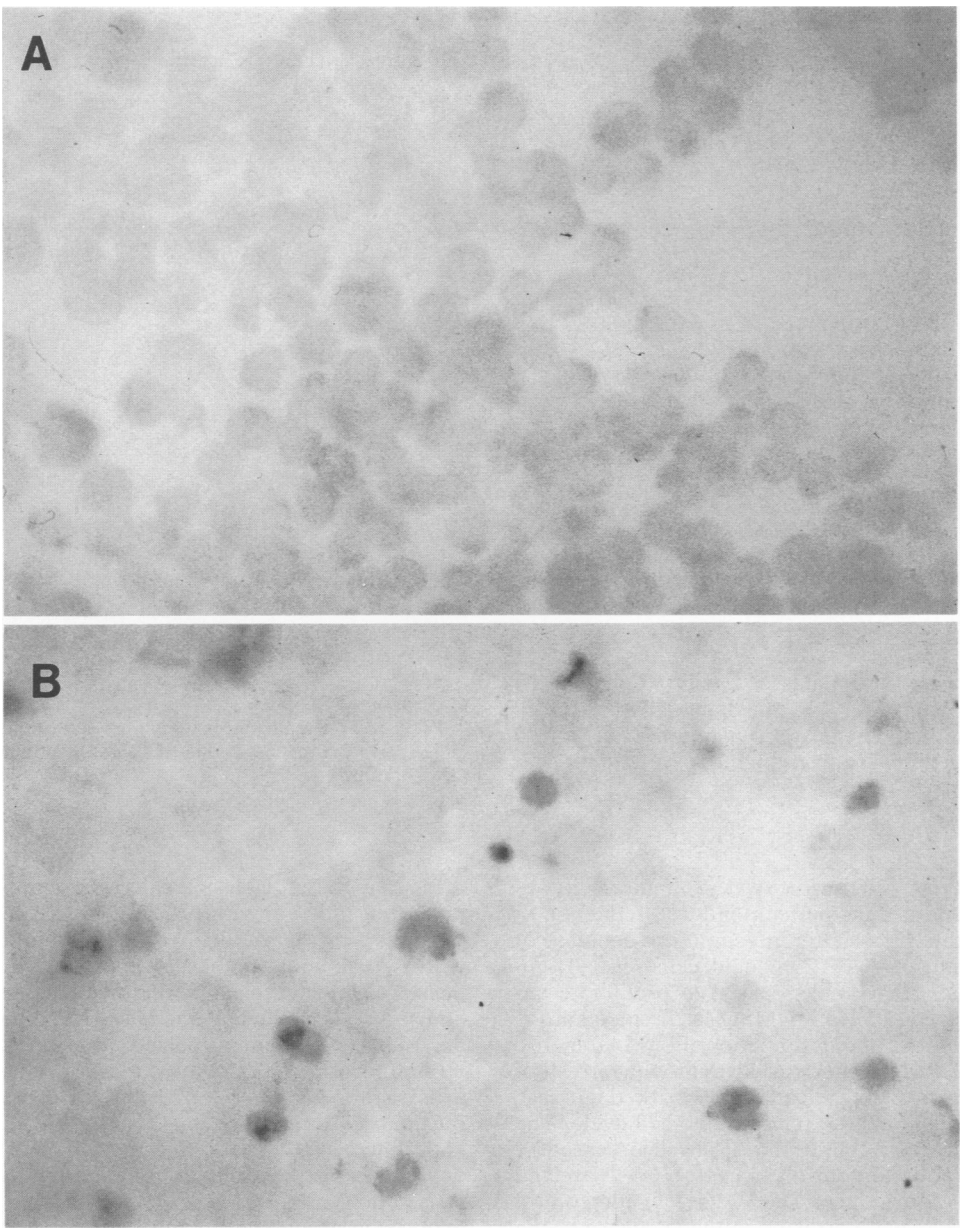

FIG. 3. Immunofluorescence analysis of fixed HL-60 cells with granulocytic-specific MAb CD67. Panels: A, mock-infected cells; B, AAV-2-infected cells.

at 65°C in 0.5 M Na_2HPO_4–7% sodium dodecyl sulfate (SDS)–1 mM EDTA. Hybridized filters were washed extensively at 65°C three times with 1% SDS–50 mM Na_2HPO_4 and autoradiographed.

Northern (RNA) blot analysis. Total RNA was extracted by the acid guanidinium-phenol-chloroform method (10). Aliquots of denatured total RNA were separated by 1% agarose gel electrophoresis and transferred onto GeneScreen membranes. Hybridization was performed with randomly primed (20) DNA probes, as indicated in the figure legends, for 16 h at 65°C in 0.5 M Na_2HPO_4–7% SDS–1 mM EDTA. Hybridized filters were washed extensively at 65°C three times with 1% SDS–50 mM Na_2HPO_4 and autoradiographed.

Western immunoblot analysis. Proteins were prepared from AAV-2-infected or mock-infected cells by suspending PBS-washed cells in a Tris-HCl-buffered 2% SDS solution containing 10% glycerol, 5% mercaptoethanol, and 0.1% bromphenol blue. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (33) performed with a 5% acrylamide stacking gel and a 10% acrylamide separating gel. Prior to electrophoresis, samples were heated to 100°C for 3 min. A fifty-microgram protein sample was loaded per lane. Molecular mass estimates were obtained by simultaneous electrophoresis of molecular mass markers from 15,000 to 240,000 kDa (Sigma). After electrophoresis, the gels were transferred onto nitrocellulose membranes and stained with Ponceau (Sigma). Each lane was cut into three pieces and blocked for at least 1 h with 5% TBST (5% milk powder, Tris-HCl, NaCl), incubated with MAbs (Dianova) against the proteins specified by c-*fos*, c-*myb*, *p53*, CK10, and *Rb*

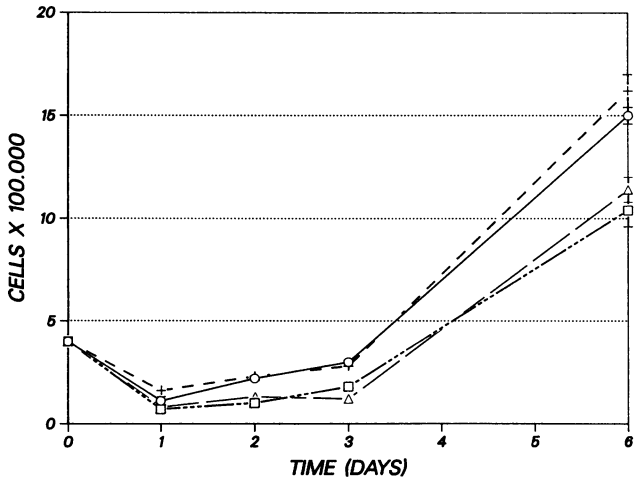

FIG. 4. Growth behavior of immortalized keratinocytes postinfection with AAV-2. HaCaT or HPK Ia cells (4×10^5) were infected with AAV-2 (□ and △, respectively) or mock infected (○) and +, respectively). After 1, 2, 3, and 6 days, the numbers of viable cells were determined by trypan blue exclusion.

for at least 3 h, washed five times in 1% TBST, incubated with alkaline phosphatase for 1 h, and stained with NBT–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) for 30 min (detection reagents were from Sigma).

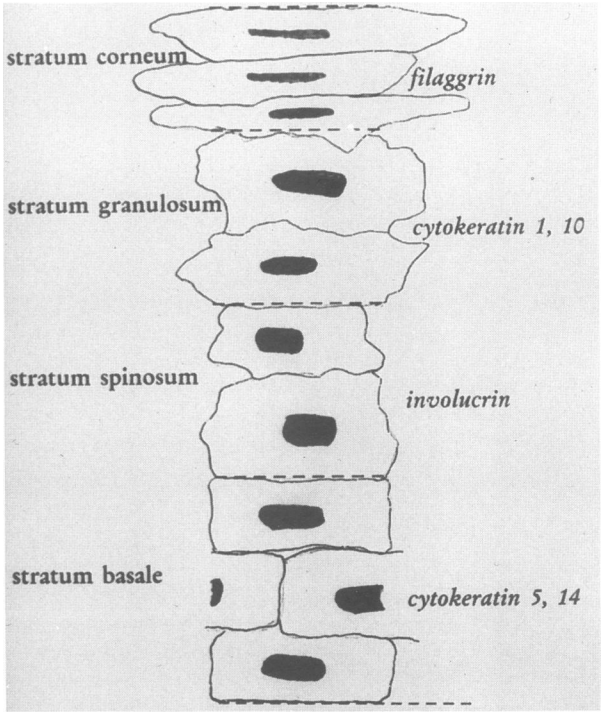

FIG. 5. Schematic representation of the maturation of HaCaT cells in cell culture indicating the sequential appearance of specific cytokeratins.

RESULTS

Infection of HL-60 cells with AAV-2 (multiplicity of infection, 100 infectious units per cell) induced reduction of cell growth detectable 4 days after infection by a decrease of the proliferation rate of AAV-2-infected cells (Fig. 1). In control experiments, HL-60 cells ceased to proliferate immediately after addition of 1.3% DMSO in the medium, as expected for this inducer of differentiation. Flow cytometric analysis 8 days after infection revealed growth arrest of AAV-2-infected HL-60 cells indicated by a marked increase of the number of cells in the G_1 phase of the cell cycle. The number of AAV-2 infected cells in the G_1 phase increased by about 25% compared with controls. In HL-60 cells treated with the differentiation inducer DMSO (1.3%) for control purposes, 96% of cells were arrested in the G_1 phase of the cell cycle, indicating induction of terminal differentiation (Fig. 2).

The NBT test is used to distinguish between differentiated and undifferentiated myelocytic cells, since differentiated cells (e.g., granulocytes) are able to reduce soluble tetrazolium to insoluble formazan deposits which are visible microscopically. After 4 days, 64% of HL-60 cells treated with 1.3% DMSO were NBT positive (positive control), and in 12% of AAV-2 infected cells black-blue formazan deposits could be detected. After 8 days, nearly all (96%) of the DMSO-treated and 26% of the AAV-2-infected HL-60 cells were NBT positive.

The flow cytometry data, as well as the NBT reduction assay data, suggest that infection with AAV-2 induced terminal differentiation in about 25 to 30% of HL-60 cells.

To evaluate these findings, we investigated expression of differentiation-associated antigens after AAV-2 infection in this leukemia cell line. Immunofluorescence analysis of fixed cells with granulocyte-specific MAb CD67 demonstrated the appearance of antigens specific for granulocytic differentiation after infection with AAV-2 (Fig. 3).

To test whether induction of differentiation after infection with AAV-2 could be observed in other than leukemic cell lines, we infected cells of the spontaneously immortalized human keratinocyte cell line HaCaT and the human papillomavirus type 16-immortalized foreskin keratinocyte line HPK Ia with AAV-2. Infection of HaCaT and HPK Ia cells with AAV-2 induced a decrease in the proliferation rate at 2 days after infection (Fig. 4). When transplanted onto nude mice, HaCaT cells reform a regular epithelium with expression of the specific differentiation markers schematically represented in Fig. 5 (6). Therefore, we analyzed the expression of involucrin (detectable early in the differentiation process) and cytokeratin 10 (detectable late in the differentiation process) with MAbs at various days after infection. Immunofluorescence analysis with anticytokeratin 10 antibodies revealed the appearance of this marker of differentiation of HaCaT cells at 3 days postinfection with AAV-2 (Fig. 6). These findings were confirmed by Western blot analysis, which also showed an increase in the expression of cytokeratin 10 (Fig. 7; 4 days after AAV-2 infection).

We also analyzed expression of cytokeratin 10 in cells of the HPK Ia cell line by using the immunofluorescence technique. The HPK Ia line was established by immortalizing primary human keratinocytes with DNA of human papillomavirus type 16 (18). Similar to the findings obtained with HaCaT cells, infection of HPK Ia cells with AAV-2 resulted in an increase of cytokeratin 10 expression. Similarly, induction of involucrin was found in HaCaT cells after infection with AAV-2 (data not shown).

Since induction of differentiation in HL-60 cells (e.g., by

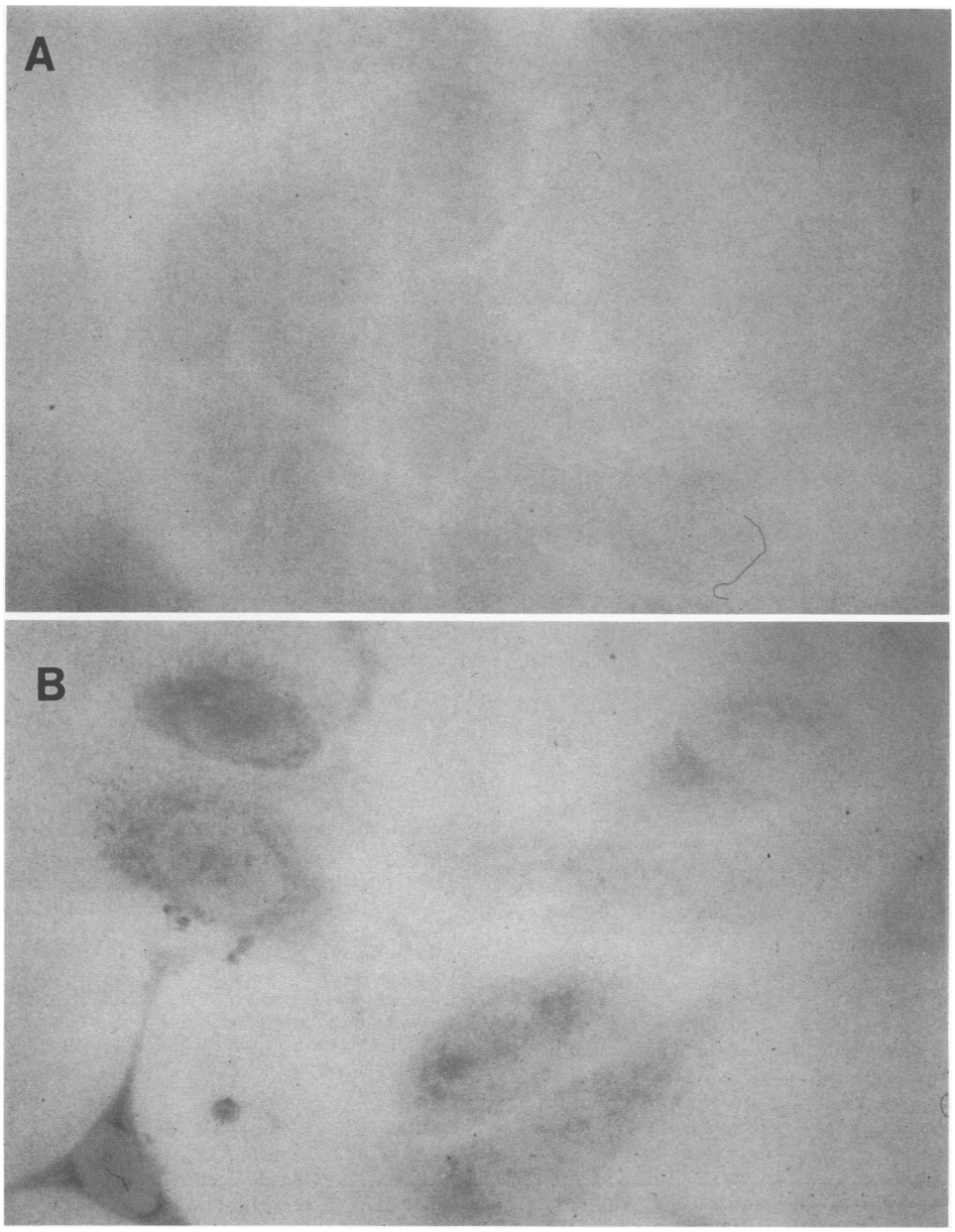

FIG. 6. Immunofluorescence analysis of fixed HaCaT cells with a cytokeratin 10-specific MAb. Panels: A, mock-infected cells; B, AAV-2-infected cells.

DMSO or retinoic acid) is known to be associated with down regulation of transcription of the c-*myc* and c-*myb* oncogenes from the highly amplified c-*myc* sequences present in these cells (11, 15, 39, 42), we determined whether synthesis of the proteins encoded by c-*myc*, c-*myb*, and c-*fos* would also be changed by AAV-2 infection. Analysis of total c-*myc* RNA in AAV-2-infected HL-60 cells showed a decrease of transcription from the c-*myc* gene starting at 1 day postinfection with AAV-2 (Fig. 8), paralleling the effects of DMSO. Similarly, Western blot analysis with anti-c-myb antibodies showed reduced expression of the c-*myb* oncogene product in AAV-2-infected HL-60 cells (Fig. 9). In contrast, expression of the c-*fos*-encoded protein in HL-60 was increased for up to 3 days postinfection with AAV-2. Similarly, expression of the c-*fos* protein was increased in HaCaT cells (Fig. 10).

Expression of oncogenes c-*myc*, c-*myb*, and c-*fos* in control of cell proliferation is interrelated with expression of the retinoblastoma susceptibility gene *Rb* (54). Therefore, we analyzed the expression of this gene, as well as that of *p53*, postinfection with AAV-2. Yen et al. (60) reported that in HL-60 cells, induction of terminal differentiation is associated with down regulation of expression of the Rb protein. We analyzed Rb expression after AAV-2 infection in HL-60 and HaCaT cells by using immunofluorescence and Western blotting techniques and also found down regulation of expression of the Rb protein in HL-60 cells starting at 3 days

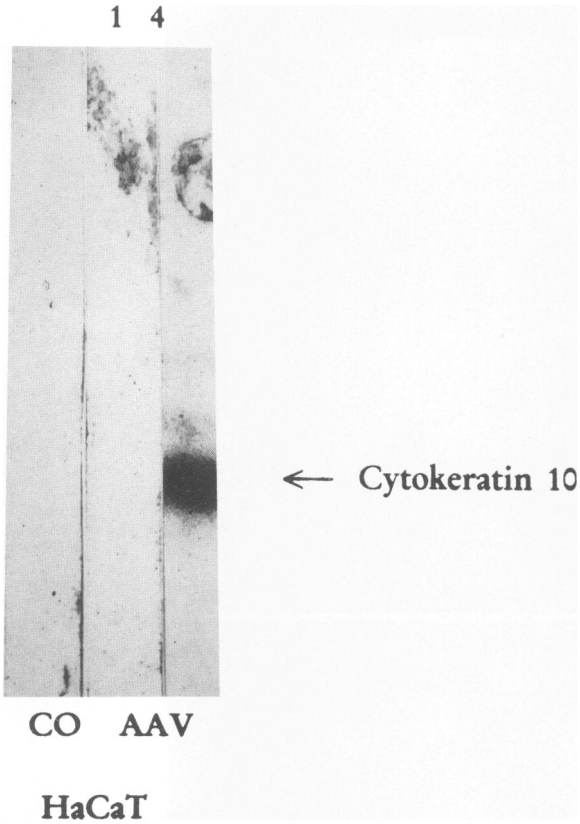

FIG. 7. Western blot analysis with a MAb against cytokeratin 10 showing the levels of cytokeratin 10 protein at 1 and 4 days postinfection of HaCaT cells with AAV-2 and an untreated (mock-infected) control (CO). A 50-μg protein sample was loaded per lane.

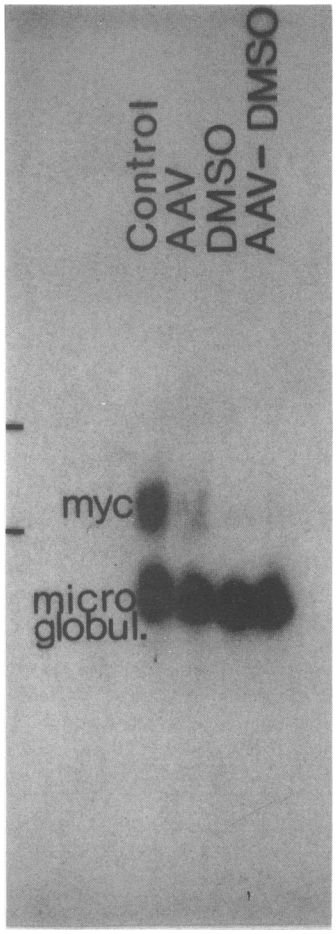

FIG. 8. Transcription of c-*myc* in AAV-2 infected HL-60 cells. One day after infection with AAV, treatment with 1.3% DMSO, treatment and infection (AAV-DMSO), or mock infection (Control), c-*myc* expression was analyzed by Northern blot analysis of HL-60 cells. Hybridization with a β-2 microglobulin probe was used to compare amounts of RNA loaded per lane.

postinfection (Fig. 11). However, with the same MAb used for analysis of Rb expression in HL-60 cells we could not find a change in its expression in HaCaT cells.

In addition, we determined the amount of p53 protein present after infection of HaCaT cells with AAV-2 (Fig. 12). This cell line is known to express large amounts of p53 (5). We could not find an effect of AAV-2 infection on p53 expression in HaCaT cells as detected by the Western blot technique. Since Wolf and Rotter (56) reported that shutoff of p53 gene expression in HL-60 cells is induced by major deletions and rearrangements in the p53 genomic structure, we did not analyze p53 expression in this cell line.

After infection with AAV-2 (with no helper virus) we extracted genomic DNA and total RNA from HL-60 and HaCaT cultures. By using Southern blot analyses, we detected AAV-2-specific DNA at 1 h postinfection (Fig. 13). At times when differentiation-related antigens appeared, neither AAV-2-specific DNA nor RNA was detectable.

DISCUSSION

Lack of terminal differentiation is a characteristic trait of tumor cells. In recent years, efforts have been made to induce differentiation in leukemic cells for cancer therapy (21). Agents such as retinoic acid and hexamethylene bisacetamide have been found to induce differentiation in leukemic cells (22, 52). In view of the apparent nonspecificity of the antitumor effects of parvovirus infections (see references cited in the Introduction), we analyzed whether these viruses are possibly inducers of differentiation. We focussed on effects caused by infection with the nonpathogenic human parvovirus AAV-2 in two representative cell lines which are well-established model systems for analysis of differentiation. The human promyelocytic leukemia cell line HL-60 can be induced to differentiate into mature functional granulocytes by agents such as DMSO and retinoic acid (12) or into monocytic or macrophagelike cells after exposure to phorbol myristate acetate (27). HaCaT cells are spontaneously immortalized human keratinocytes which reform orderly structured and differentiated epidermal tissue when transplanted onto nude mice (6).

We induced differentiation-associated markers in both cell lines in up to 30% of the cells by infection with AAV-2. Induction of differentiation by AAV-2 was detected later than that by DMSO, the classical inducer of the granulocyte pathway in HL-60 cells. In addition, AAV-2 infection of HL-60 cells resulted in decreased expression from the c-*myc* and c-*myb* oncogenes, as has been reported to

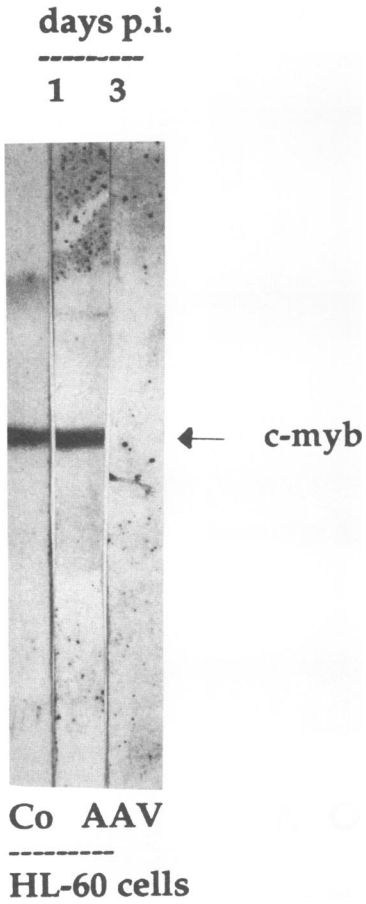

FIG. 9. Western blot analysis of c-myb protein levels in HL-60 cells at 1 and 3 days postinfection (p.i.) with AAV-2 and in an untreated control (Co). A 50-μg protein sample was loaded per lane.

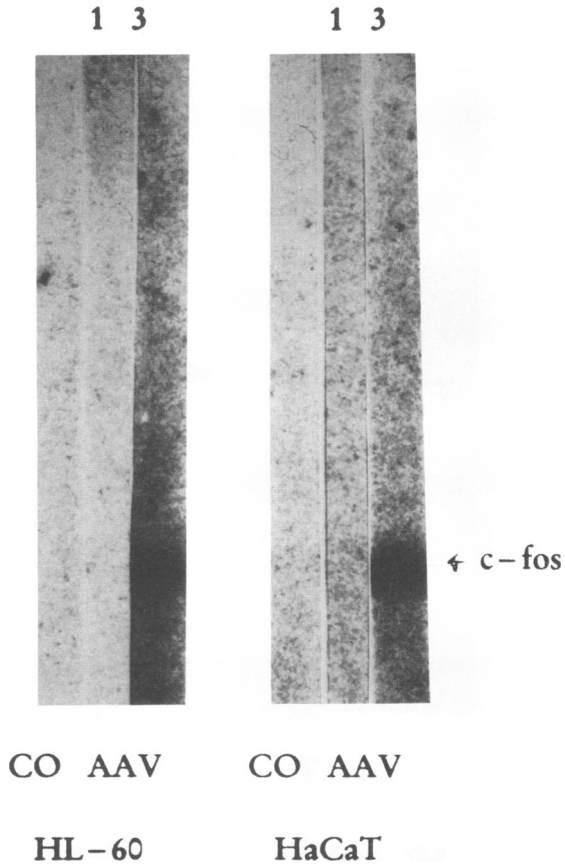

FIG. 10. Western blot analysis of c-fos protein levels in HL-60 and HaCaT cells at 1 and 4 days postinfection with AAV-2 and in untreated (mock-infected) controls (CO). A 50-μg protein sample was loaded per lane.

occur after treatment of HL-60 with DMSO or retinoic acid (39).

The protein products of c-*myc* and c-*myb* are expressed in a variety of tissues in a highly conserved manner (19, 40). Several studies have demonstrated that expression of both c-*myc* and c-*myb* is induced in cells stimulated to proliferate, i.e., c-*myc* in the transition from G_0 to G_1 (23) and c-*myb* in the transition from the G_1 to the S phase of the cell cycle (53). Decreased expression of c-*myc* and c-*myb* when hemopoietic cell lines are induced to differentiate terminally (26, 28, 31, 42) has shown that differentiation accelerates when expression of both oncogenes is inhibited, implying a causative role in hemopoietic differentiation. A decline in the expression of c-*myc* and of c-*myb* in HL-60 cells after AAV-2 infection might therefore reflect inhibition of proliferation and onset of differentiation. Expression of c-*fos*, c-*myb*, and c-*myc* in control of cell proliferation is associated with regulation of retinoblastoma susceptibility gene *Rb* (54) and probably with the p53 protein. Involvement of this gene in cell cycle regulation was implied by changes in Rb phosphorylation during progression of the cell cycle (7, 36). Rb regulates the activity of c-*fos* (43) by repressing c-*fos* transcription (54). Down regulation of Rb expression could therefore result in up regulation of c-*fos* transcription, as observed in this study. This is in line with our observation of the levels of expression of these genes after infection of HL-60 cells with AAV-2 as reported herein.

Wild-type p53 protein has many properties that hint at its role as a tumor suppressor protein (35). p53 mRNA and protein levels were found to decrease in a number of cell types when they were induced to differentiate (29, 41). HaCaT cells are known to express high levels of p53 protein. Infection of HaCaT cells with AAV-2 had no effect on the expression of p53. However, we used a MAb which recognizes wild-type and mutant p53 proteins. Therefore, we could not assess differential regulation of mutated versus wild-type p53-encoding genes in HaCaT cells.

The mechanism of AAV-2-induced differentiation is not clear, and we do not know whether cessation of cell proliferation (marked by a decrease of c-*myc* expression), arrest of cells in the G_1 phase, or down regulation of expression of the retinoblastoma gene is induced directly by AAV-2 infection. It is possible that AAV-2 interferes with targets other than those investigated here and that effects described in this study are due to indirect influences of AAV-2 infection on the cell cycle. Relatively late induction of differentiation and late up regulation of c-*fos* and down regulation of *Rb* may support this hypothesis.

In comparison with the DMSO results, only a fraction of HL-60 cells responded to AAV-2 by showing the capacity of NBT reduction and CD67 expression as markers of differ-

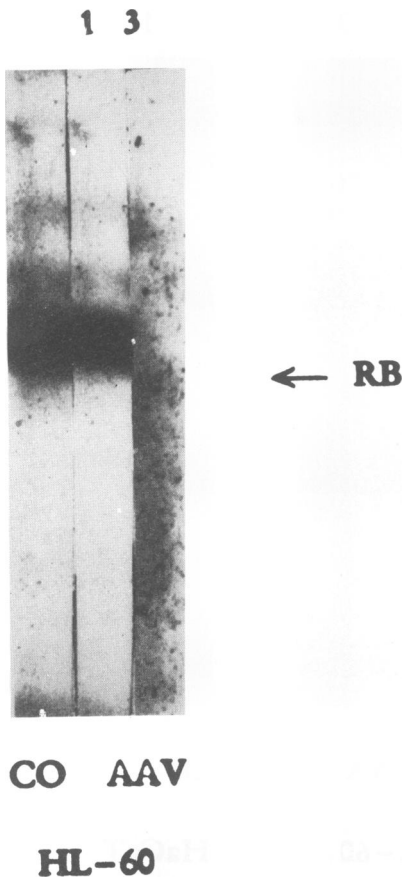

FIG. 11. Western blot analysis of retinoblastoma susceptibility gene (*Rb*)-encoded protein levels in HL-60 cells at 1 and 3 days postinfection with AAV-2 and in an untreated control (CO). A 50-μg protein sample was loaded per lane.

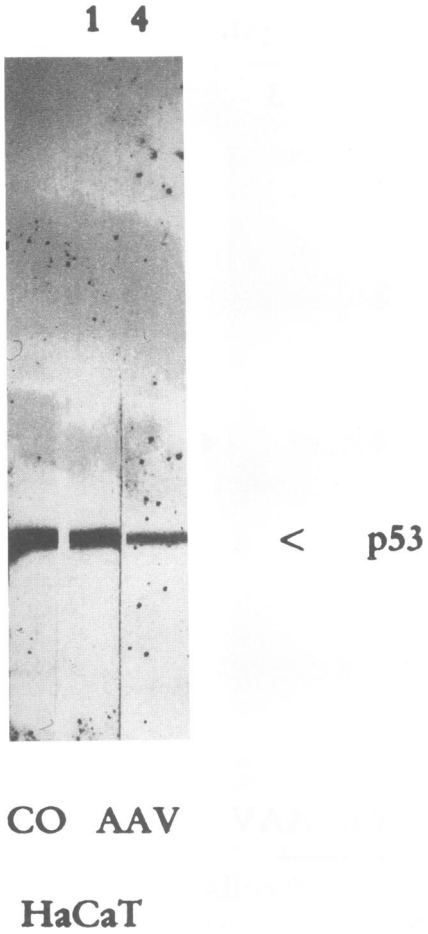

FIG. 12. Western blot analysis of p53 protein levels in HaCaT cells at 1 and 4 days postinfection with AAV and in an untreated (mock-infected) control (CO). A 50-μg protein sample was loaded per lane.

entiation. It is noteworthy that HL-60 and HaCaT cells infected with AAV revealed substantial abrogation of c-*myc* expression and absence of c-myb and Rb production. The dramatic down regulation of c-*myc* transcription and of the c-myb and Rb protein expression suggests that these changes are induced more efficiently and earlier after AAV infection. With no helper virus, AAV does not replicate when infecting cells. At the times postinfection when expression of differentiation-associated antigens was most pronounced, no AAV-2 DNA or viral transcripts were detectable by the Southern or Northern blotting technique. This is possibly due to a relatively low percentage of cells containing AAV-2-specified macromolecules at these stages postinfection or to early loss of AAV-2 molecules from cells committed to differentiation.

This could indicate that very early postinfection transient expression of AAV-2 proteins is sufficient to trigger pathways that eventually lead to the phenomena observed. Furthermore, we cannot exclude the possibility that the infecting AAV-2 single-stranded DNA molecules interact with cellular DNA-binding factors involved in the differentiation pathway. It may also be that interaction of infecting AAV-2 particles with the cellular membrane can mediate the effects observed. Preliminary data of our group suggest that differentiation can be (at least partially) induced by membrane interactions. Winocour et al. (55) described inhibition of cell division postinfection with AAV in several cell lines. They concluded that this inhibition can result from a reaction between parental AAV virions and target cells. In this study, detectable parental viral gene expression was not required for inhibition of cell growth. The high input multiplicities of AAV which Winocour et al. required for inhibition of cell growth and cell cycle progression are consistent with the conclusion that a component of the incoming parental virion (viral capsid protein or parental viral genome) is involved. Generally, reactions between virus particles and the outer membrane have been reported to induce physiologically significant modifications in membrane permeability (8, 32). For example, binding of UV-inactivated human adenovirus type 2 to its cell surface receptor increases the permeability of the host cell to a variety of low-molecular-weight compounds and alters the activity of the membrane-associated pump (50). We cannot exclude the possibility that a reaction between input AAV particles and the membranes of particular target cells alters the uptake of ions and nutrients known to influence the progression of the cell cycle (1). Further analysis of cellular and viral functions involved in AAV-2-induced differentiation is necessary to assess the role of differentiation in the tumor-suppressive properties of AAV.

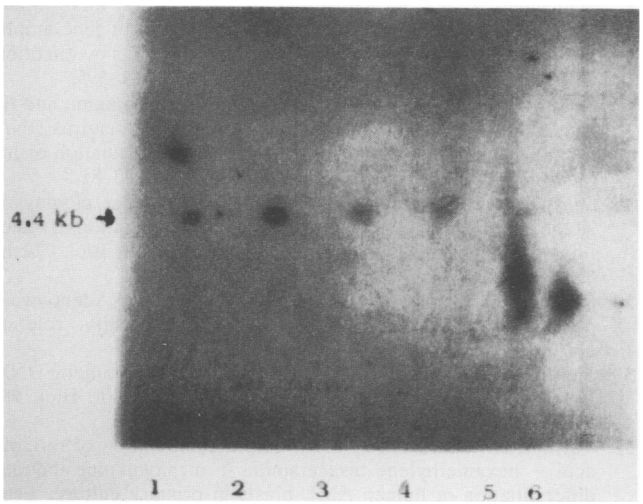

FIG. 13. Southern blot analysis of AAV-2-infected HaCaT and HL-60 cells. Southern blot analysis of cellular DNA of cells at 1 h postinfection with AAV. Lanes: 1, HaCaT cells; 2, HeLa cells (positive control); 3, HL-60 cells; 5 and 6, sensitivity controls consisting of 10 and 1 pg, respectively, of nonlinear, supercoiled plasmid pTAV2.

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