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Antioncogenic effect of adenovirus E1A in human tumor cells

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ABSTRACT **Stable expression of the adenovirus 5 E1A gene reduced anchorage-independent growth and tumorigenic potential, caused cytoskeletal reorganization, induced flat morphology, and restored contact inhibition in three human tumor cell lines. By these criteria, E1A appears to be functionally indistinguishable from a tumor suppressor gene in this context. The apparent paradox accorded by the observations of the ability of E1A to transform rodent cells in cooperation with other oncogenes suggests that E1A may be the prototype of a class of growth-regulatory proteins having context-specific transforming and antioncogenic activities.**

The normal and transformed phenotypes of a cell are thought to be maintained by a balance of activities encoded by oncogenes and antioncogenes (tumor suppressor genes) (1–4). In some cases, viral oncoproteins have been shown to transform by inactivating cellular antioncoproteins (1–6); for example, adenovirus E1A and E1B form complexes with retinoblastoma protein and p53, respectively. Conversely, if a viral antioncoprotein was found, it could in principle inactivate cellular oncoproteins or activate cellular antioncoproteins. Experiments reported here demonstrate that the adenovirus E1A gene unexpectedly influences the phenotype of human tumor cells antioncogenically. These observations suggest that insights into growth control mechanisms may be obtainable by analyzing the interactions of E1A with target cellular proteins in a context where reversal of the transformed phenotype results.

MATERIALS AND METHODS

Cell Lines. The construction of stable E1A-expressing cell lines from A2058 melanoma and HT1080 fibrosarcoma cells, using the plasmid p1Aneo (7), was described (8). The E1A-expressing HeLa cells (9) were obtained from L. Brunet and A. Berk (University of California, Los Angeles). Cells labeled HT1080neo^r and A2058neo^r resulted from transfection with a Bluescript plasmid (unpublished data) containing the simian virus 40 early enhancer-promoted *aph* gene (encoding resistance to G418).

Actin Staining. Cells were stained for 15 min with a 1:100 dilution (in phosphate-buffered saline) of rhodamine-phalloidin stock (Molecular Probes) on glass coverslips after a 15-min fixation in 1% paraformaldehyde and a 10-min permeabilization in 0.1% Triton X-100, prior to photography on a Zeiss Axioplan microscope using the ×62 objective.

Soft Agarose Colony Formation Assays. Cells were plated at 4×10^4 (A2058 and derivatives), 3×10^4 (HeLa and derivatives), or 2×10^5 (HT1080 and derivatives) cells per 60-mm plate (as in refs. 10 and 11) and stained, after a 14- to 17-day incubation, with *p*-iodonitrotetrazolium violet (0.5 mg/ml) for 16 h.

Tumorigenicity Assays. Cells were injected into 4- to 5-week-old athymic nude mice (Harlan–Sprague–Dawley) in 0.25 ml of phosphate-buffered saline at the indicated cell number. Nine mice were injected per cell line, and tumors were dissected at the indicated incubation times.

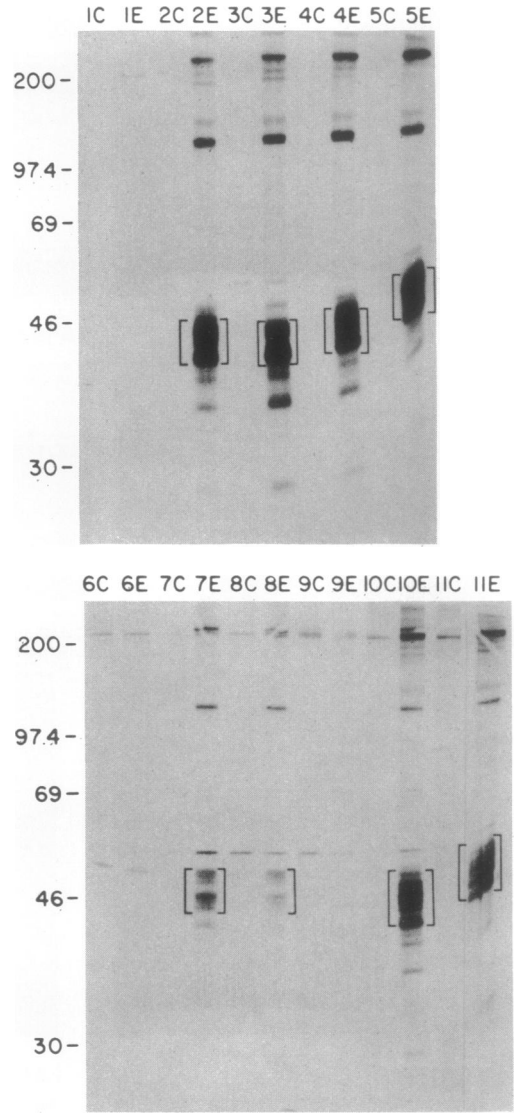

FIG. 1. Immunoprecipitation of E1A proteins from stably transfected and parental cell lines. [^{35}S]Methionine-labeled proteins from E1A-expressing clones derived from HT1080 cells (lanes 1–5), HeLa cells (lanes 6–8), or A2058 cells (lanes 9–11) were immunoprecipitated with anti-E1A antibodies (E) or control antibodies (C). Lanes: 1–5, HT1080, p2AHT2a, p1Aneo3, p1Aneo15, and p1Aneo16, respectively; 6–8, HeLa, Medg18, and Medg28, respectively; 9–11, A2058, 1A58c8-1, and 1A58c11-1, respectively. The bands corresponding to E1A protein species are bracketed.

Immunoprecipitation. Confluent cultures of cell lines (containing 2×10^6 cells) were labeled for 5 h in 35-mm wells with 0.4 mCi (1 Ci = 37 GBq) of [^{35}S]methionine (Tran^{35}S-label, ICN) in methionine-free Dulbecco's modified Eagle's medium

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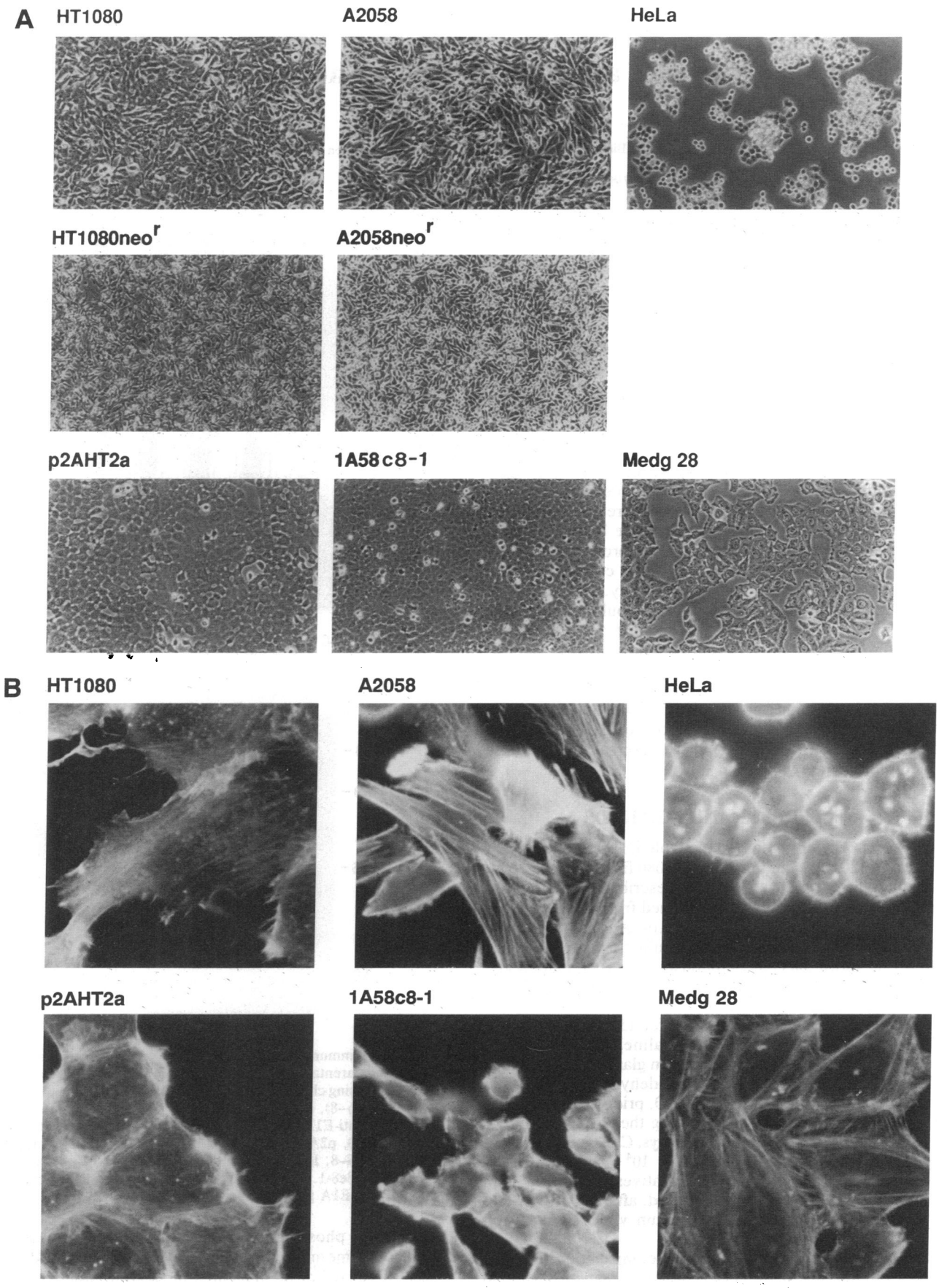

FIG. 2. (*A*) Phase-contrast micrographs of parental (top row), neomycin-resistance transfected (middle row), and E1A-expressing (bottom row) human tumor cells. (×125.) (*B*) Fluorescence micrographs of actin organization in parental (upper row) and E1A-transfected (lower row) cell lines. (×875.)

containing 5% (vol/vol) dialyzed fetal calf serum. Cells were washed twice in phosphate-buffered saline and scraped into 1.0 ml of RIPA-1 [50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/250 mM NaCl/aprotinin (10 μg/ml)/leupeptin (5 μg/ml)/1 mM phenylmethylsulfonyl fluoride/5 mM EDTA/soybean trypsin inhibitor (10 μg/ml)]. After addition of bovine serum albumin to 0.5 mg/ml, lysates were preabsorbed with 100 μl of a 50% (wt/vol) protein A-Sepharose (Pharmacia) slurry (prepared in RIPA-1 containing bovine serum albumin at 0.5 mg/ml) by mixing at 4°C for 30 min and centrifuging for 10 min at 14,000 rpm in an Eppendorf microcentrifuge and 0.5-ml samples were then incubated with 1.5 μg of anti-E1A monoclonal antibody M73 (12) or control antibody [anti-fos Ab-1 (Oncogene Sciences, Mineola, NY)] for 2 h at 0°C. Then, 25 μl of 50% protein A-Sepharose slurry was added and the tubes were mixed for 20 min at 4°C followed by a 2-min centrifugation and five 0.5-ml washes with RIPA-1. Pellets were then resuspended in 60 μl of sample buffer and analyzed by SDS/PAGE.

RESULTS AND DISCUSSION

In a previous report (8), the construction of E1A-expressing HT1080 fibrosarcoma and A2058 melanoma cell lines was described; E1A-expressing HeLa cells (9) were obtained from L. Brunet and A. Berk. In all cases the expression of the E1A gene was documented at the RNA level by Northern blot analysis (8, 9) and, in the present study, protein expression was documented by immunoprecipitation with E1A-specific monoclonal antibodies (Fig. 1). As expected (12), multiple species of E1A protein were detected on the gel.

In each case, the E1A-transfected cells are relatively flat and "epithelioid" compared with the respective parental cell lines or lines transfected with the *aph* gene alone (Fig. 2*A*). These morphologic changes are consistent with the widely reported observation that transformed cells tend to be more refractile and less substrate-attached than normal cells (13). The altered morphology was observed at the time the colonies were first identified on the G418-selection plates, suggesting that secondary stochastic changes in the genome are not required for induction of the altered phenotype.

Transformation is also frequently associated with a reorganization of cytoskeleton (13–16), although the nature of this reorganization varies markedly among cell types (29). To test whether the different morphologies of the parental vs. E1A-transfected cells were accompanied by cytoskeletal changes, the cells were photographed after staining with rhodamine-phalloidin (Fig. 2*B*). The most dramatic effect was in the HeLa cells, where the parental cells showed a narrow band of circumferential staining and amorphous aggregates,

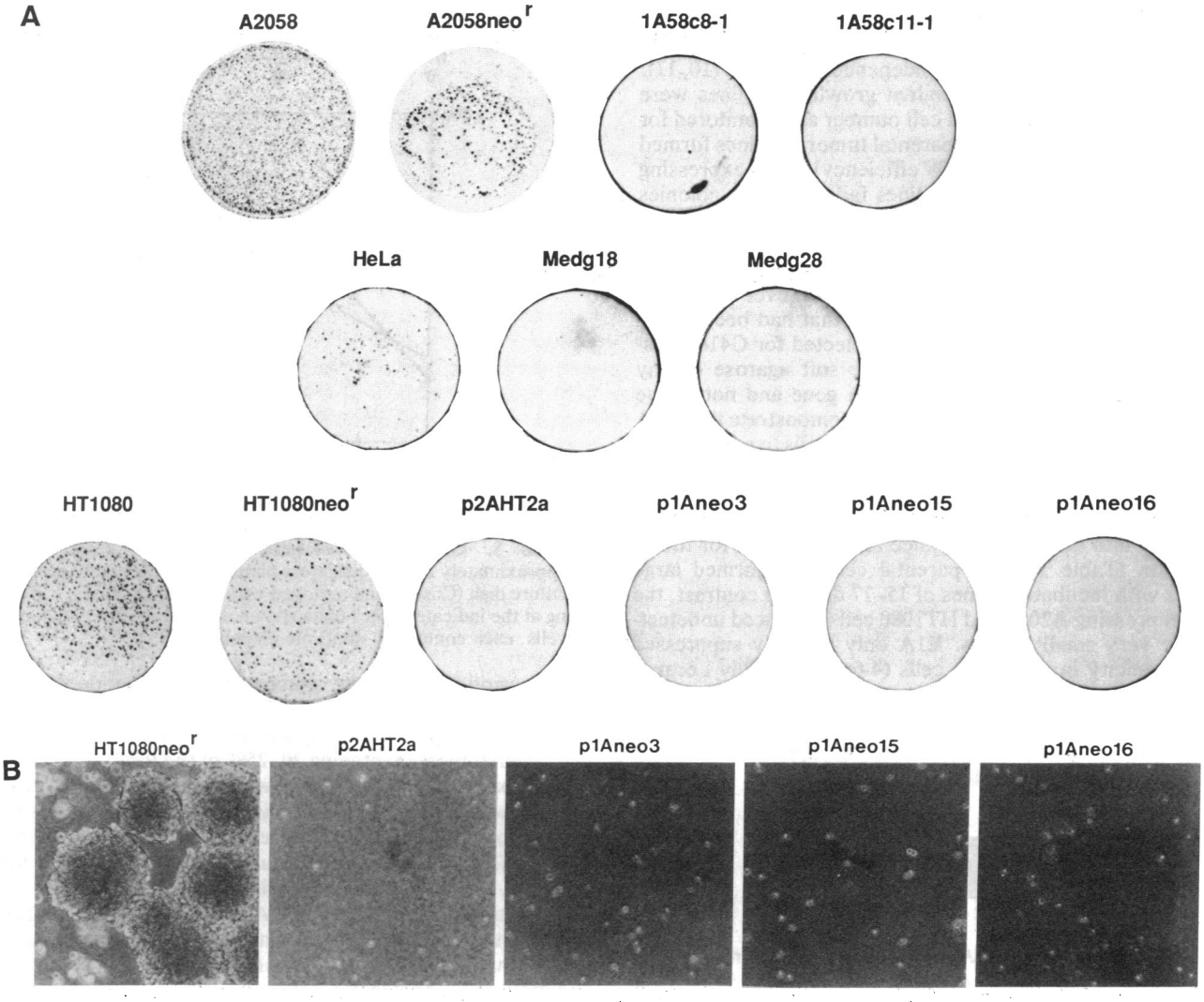

FIG. 3. (*A*) Soft agarose colony formation assays of parental neomycin-resistance transfected, and E1A-expressing human tumor cells. (Bottom row, photomicrographs of HT1080neo^r and E1A-expressing HT1080 soft agarose colonies.) (*B*) Photomicrographs of soft agarose colony formation of representative HT1080neo^r and E1A-expressing HT1080 cells. (×100.)

Table 1. Tumorigenicity of parental tumor cells (HT1080, A2058, and HeLa) and E1A derivatives in nude mice

Cell line	Cells injected, no.	Incubation time, days	Average tumor mass, g	SD
HT1080	6×10^6	17	2.2	0.62
p2AHT2a	6×10^6	17	0.04	0.04
HT1080	2×10^6	17	1.2	0.3
p1Aneo15	2×10^6	17	0.25*	0.3
p1Aneo16	2×10^6	17	0.0	0
A2058	1×10^7	18	1.2	0.3
1A58c8-1	1×10^7	18	0.0	0
1A58c11-1	1×10^7	18	0.0	0
HeLa	1×10^6	15	0.4	0.1
Medg 28	1×10^6	15	0.1	0.03

*See Fig. 4.

but the E1A derivatives displayed long arrays of actin filaments. A different effect of E1A was seen in the other two cell lines: whereas the parental HT1080 and A2058 cells displayed numerous stress fibers, their E1A derivatives carried dispersed arrays of shorter fibers that localized mainly to the peripheries, an effect consistent with their more epithelial morphology (cf. ref. 17). These observations indicate that cytoskeletal reorganizations occurred in the E1A-transfected cells, but the resultant patterns are, not unexpectedly, cell-type-dependent.

The tumorigenicity of cells *in vivo* generally correlates with their ability to form anchorage-independent colonies (10, 11). To test for anchorage-independent growth, cell lines were plated in soft agarose at fixed cell number and monitored for colony formation. While the parental tumor cell lines formed colonies in soft agarose (≈1% efficiency), E1A-expressing clones derived from these cell lines failed to form colonies (Fig. 3). In fact, microscopic examination revealed that the E1A-expressing clones failed even to initiate colony formation on soft agarose (Fig. 3*B*), ruling out a growth-rate effect. The colony-suppressed phenotype was never observed among 15 independent HT1080 clones that had been transfected with the *aph* gene alone and selected for G418 resistance; these results indicate that the soft agarose colony suppression was an effect of the E1A gene and not of the selection process. These observations demonstrate that E1A expression can convert the human tumor cells to a phenotype that is anchorage-dependent for growth.

To test whether E1A expression rendered the human tumor cell lines nontumorigenic *in vivo*, they were injected subcutaneously into nude athymic mice and monitored for tumor formation (Table 1). The parental cell lines formed large tumors with incubation times of 15–17 days. In contrast, the E1A-expressing A2058 and HT1080 cells produced undetectable or very small tumors. E1A only partially suppressed tumorigenicity in the HeLa cells (4-fold), possibly because

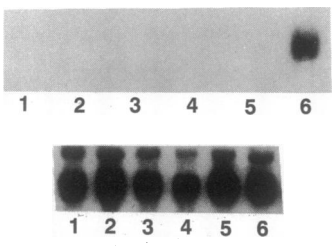

FIG. 4. Expression of E1A in tumors derived from injection with the E1A cell line p1Aneo15. Approximately 2×10^6 p1Aneo15 cells were injected and tumor RNA was isolated after 17 days. RNAs (12 μg) from tumors 1–4 (lanes 1–4), HT1080 RNA (lane 5), or p1Aneo15 cellular RNA (lane 6) were hybridized with an E1A probe (*Upper*) or a γ-actin probe (*Lower*).

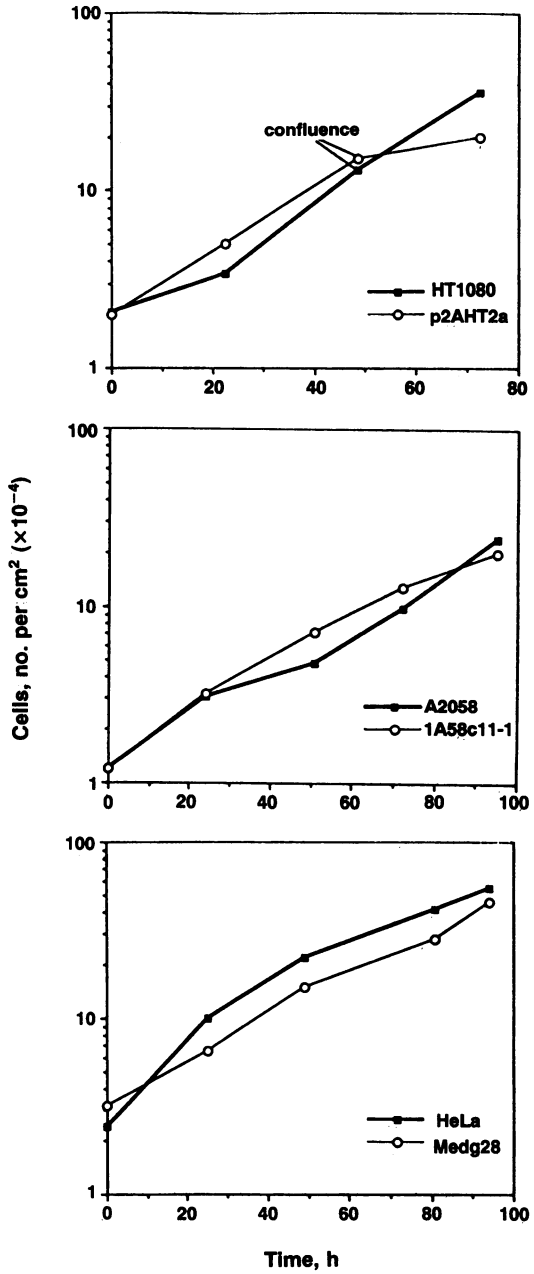

FIG. 5. Growth rates of parental and E1A-expressing cells. Approximately 2×10^4 cells were plated into wells of a 12-well tissue culture dish (Costar) and removed with trypsin treatment for counting at the indicated times (data shown are averages of two duplicate wells, each counted in duplicate chambers).

transcription from the glucocorticoid-dependent murine mammary tumor virus promoter, which was driving the E1A gene, was compromised in the subcutaneous environment.

Small tumors (averaging 20–25% of the tumor mass arising from parental HT1080 cells) were obtained upon injection with the E1A-expressing cell line p1Aneo15. RNAs isolated from four of these tumors were analyzed on a Northern blot (Fig. 4). No E1A mRNA could be detected in the tumor RNAs. Normal levels of γ-actin mRNA were present, demonstrating that the tumor RNAs were intact. These results suggest that the small tumors arose from a subpopulation of cells that had reacquired tumorigenicity by losing E1A gene expression. Analogous selection against expression of a tumor suppressor gene was reported in experiments where p53 expression was shown to partially prevent focus formation in oncogene-transfected rat cells (6): the small number of

foci that formed in the presence of transfected p53 were shown to lack detectable wild-type p53 gene expression.

To further rule out the possibility that E1A simply slowed growth rates so as to render soft agar colonies and tumors undetectable at short time points, growth curves were constructed (Fig. 5). E1A did not substantially affect the growth rate of subconfluent tumor cells.

The possibility that the tumor suppression could have been augmented by the ability of E1A to sensitize certain cells to lysis by tumor necrosis factor α (18–20) was also considered. This was, however, unlikely, in that ^{51}Cr-labeled p2AHT2a cells gave only 18% more lysis than parental HT1080 cells in tumor necrosis factor α at 500 units/ml—a dose high enough to kill nearly 100% of sensitive rodent cells (data not shown).

The phenotypic effects of E1A in human cells have not previously been described, although it has been noted that human cells are relatively refractory to transformation by E1A when assayed in collaboration with other oncogenes (21). The experiments reported here suggest that E1A converts three diverse types of human tumor cells into an apparently nontransformed state. Paradoxically, a salient phenotypic effect of adenovirus E1A is collaboration with the *ras* oncogene to transform primary rodent cells (7). Since specific rodent cell types may turn out to be affected antioncogenically by E1A or specific human cells may be transformable by E1A, it would be premature to ascribe these results to species differences only, especially since the effects of E1A expression in spontaneously transformed rodent cell lines have not been reported. Nevertheless, these disparate effects suggest the concept that E1A can transduce signals into both positive and negative growth control pathways, depending upon the specific cellular proteins with which it interacts and their cell contexts.

Some of the cellular proteins that interact with E1A protein to mediate its transforming effects have been identified (22–25). Future experiments will indicate whether these same or different proteins mediate the antioncogenic effect reported here. The antioncogenic effect of E1A is reminiscent of "reverse-transformation" effects of cAMP on certain transformed cell lines such as CHO-K1 (26), suggesting that the functional interaction of E1A with the cAMP-responsive activating functional transcription factor (ATF) (27) could play a role. A second possibility is that the interaction of E1A with retinoblastoma protein (5) could have opposite phenotypic consequences in human tumor cells vs. rodent cells. Inhibition of transcription factor AP-1 by E1A (8) or of other transcription factors could also be hypothesized to play a role, as could the repression of oncogene transcription (28).

Human cells are the natural host for adenovirus infection, suggesting, in an evolutionary sense, that some aspect of the adenoviral life cycle may benefit from the antioncogenic effect. Speculatively, cellular transcription or replication factors that are utilized by the virus could be expressed preferentially in cells converted to another state of differentiation by E1A.

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