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Adenovirus 12S *E1A* gene represses differentiation of F9 teratocarcinoma cells

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ABSTRACT **The F9 teratocarcinoma cell line differentiates *in vitro* after treatment with retinoic acid and cAMP and has been a widely used model system for the study of the molecular events that are responsible for cellular commitment and differentiation during early development. Previous experiments have suggested intriguing parallels between the control of gene expression during F9 cell differentiation and the regulation of gene expression by adenovirus E1A. Transfection of a 12S E1A-expressing plasmid into terminally differentiated, nonproliferating F9 cells generates, at high frequency, colonies of dividing cells, each of which expresses E1A. Cell lines established from these colonies proliferate in the presence of retinoic acid and have lost the fully differentiated phenotype as characterized by the absence of expression of a series of differentiation-specific genes. We conclude that expression of the viral 12S E1A gene product interferes with retinoic acid-induced F9 cell differentiation. Moreover, the results suggest that the differentiation process, as defined by markers of terminal differentiation, may not be a permanent event but can be reversed by E1A expression.**

Teratocarcinoma cells are malignant stem cells that are capable of differentiating *in vivo* into a variety of cell types (1, 2). Upon addition of retinoic acid and cAMP *in vitro*, there is a near complete conversion of the population to a cell type resembling parietal endoderm (3, 4). Concomitant with differentiation is a loss of proliferative capacity. A series of experiments have demonstrated a transcriptional regulatory activity in undifferentiated cells that can complement an E1A-deficient adenovirus for activation of early viral transcription (5). This cellular E1A-like regulatory activity disappears upon differentiation, suggesting a link between this activity, cellular differentiation, and proliferation. Based on this similarity between E1A activity and cellular transcriptional control during F9 cell differentiation, we have examined the possibility that E1A might alter the differentiation process.

METHODS

Cells. The F9 teratocarcinoma cell line, obtained from E. Linney (Duke University), was maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (GIBCO) as described (6). Cultures were induced to differentiate by the addition of retinoic acid (Sigma) to 100 nM and dibutyryl-cAMP (Sigma) to 1 mM.

Plasmids. The plasmid pHβAPr-1-Neo was obtained from L. Kedes (7). The E1A 13S and 12S cDNAs were isolated from simian virus 40 expression vectors as *Hin*dIII/*Pst* I fragments and then inserted into the *Hin*dIII/*Bam*HI sites of pHβAPr-1-Neo vector by using a *Pst* I/*Bam*HI adaptor. The differentiated F9-specific cDNAs have been described elsewhere (8).

RNA Isolation and Analysis. RNA was isolated from F9 cells or differentiated F9 cells as described (6). Northern analysis of RNA was performed as described (6).

Transfection. F9 cells were transfected by a modified calcium phosphate precipitation procedure (9). Twenty micrograms of plasmid DNA was transfected per 100-mm plate of F9 cells. Forty-eight hours after transfection, G418 (GIBCO) was added to 400 μg/ml. Medium was then changed every 2 days until foci were harvested. Transfections of differentiated F9 cells were performed after 2 or 4 days of treatment with retinoic acid and dibutyryl-cAMP.

Immunofluorescent Analysis. Cells in 100-mm dishes were fixed with 4% paraformaldehyde. All staining was carried out in 5% normal goat serum with 0.1% Triton X-100. A rabbit antiserum to laminin (ICN) and a mouse monoclonal antibody to E1A (Oncogene Science, Mineola, NY) were used at a 1:200 and a 1:25 dilution, respectively. Antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG (Calbiochem).

Western Blot Analysis. The procedures for preparing protein extracts, SDS gel electrophoresis, and visualization of specific proteins on blots with antibody and ^{125}I anti-Fab have been described (10).

RESULTS

Stable Expression of E1A Immortalizes Differentiated F9 Cells. We initially attempted to obtain stable expression of the *E1A* gene in F9 cells to examine its effects on the differentiation process. However, despite several attempts, very few G418-resistant foci were formed after transfection with neomycin vectors expressing either the 12S or the 13S E1A cDNA (Table 1). Furthermore, none of these rare foci had detectable levels of the E1A protein, as analyzed by immunofluorescence or Western blotting. Transfection of the neomycin vector alone gave rise to many hundreds of neomycin-resistant foci. We interpret these results to indicate that E1A expression is toxic in undifferentiated F9 cells, preventing long-term cell survival.

Retinoic acid treatment of F9 cells leads to a cessation of cell proliferation and expression of the differentiated phenotype (3). The commitment to differentiation appears to be rapid and irreversible, as a brief exposure to retinoic acid followed by its subsequent removal is sufficient to arrest cell growth (4). As expected, transfection of the neo vector into differentiated F9 cells yielded only a few rare colonies, consistent with the loss of growth potential (Table 1). Transfection of pHβAPr-1-neo-13S also failed to generate foci at a significant level. Of those foci that did form, E1A expression was sporadic; we also have not been successful at generating

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Table 1. E1A-mediated immortalization of differentiated F9 cells

Time in RA/cAMP, days	Selection medium	No. of G418-resistant colonies after transfection: With pHβAPr-1-Neo	With pHβAPr-1-neo-12S	With pHβAPr-1-neo-13S
0	G418	>200	2	2
		>1000	4	0
2	G418 + RA	2	220	2
		0	142	3
3	G418 + RA	4	451	15
4	G418 + RA	2	374	2
4	G418	9	619	14

Undifferentiated F9 cells (day 0) or cells grown in retinoic acid (RA) and cAMP for the indicated times were transfected with the indicated plasmid by calcium phosphate precipitation. Twenty micrograms of plasmid DNA was transfected per 100-mm plate of F9 cells. Forty-eight hours after transfection, cells were selected in G418 (400 μg/ml) in Dulbecco's modified Eagle's medium/10% fetal calf serum with or without the addition of 0.1 μM retinoic acid. Medium was replaced every 2–3 days. Colonies on duplicate 100-mm plates were counted after 2 weeks; data are presented as the average number of foci generated on a 100-mm plate. The plasmid pHβAPr-1-Neo was obtained from L. Kedes (7). The E1A 13S and 12S cDNAs were isolated from simian virus 40 expression vectors as *Hin*dIII/*Pst* I fragments and then inserted into the *Hin*dIII/*Bam*HI sites of pHβAPr-1-Neo vector by using a *Pst* I/*Bam*HI adaptor.

stable, long-term cultures. As a result, we have not pursued the analysis of these transfectants.

Expression of 12S E1A in differentiated F9 cells produced a dramatically different result. The E1A 12S expression vector (pHβAPr-1-neo-12S) generated ≈300 foci per plate (Table 1). This result was true whether the cells were transfected 2 or 4 days after addition of retinoic acid and cAMP. At 4 days of growth in differentiation medium, the cells are fully differentiated as defined by a number of criteria including loss of markers characteristic of the undifferentiated cells and acquisition of markers characteristic of the differentiated cell (3, 4). For instance, at this time >98% of the cells expressed laminin, as determined by immunofluorescence (data not shown). Several results indicate that the colonies generated after transfection of 12S E1A were derived from fully differentiated F9 cells and not from the small fraction of cells that may remain undifferentiated. First, the target is clearly not the undifferentiated F9 cell as shown by the sharp reduction in colony formation, using undifferentiated cells, in the presence of E1A. Second, the efficiency of colony formation with 12S E1A in differentiated F9 cells approximates that with vector alone in undifferentiated cells. Third, the number of undifferentiated or only partially differentiated cells clearly decreases between 2 and 4 days of induction, yet there was no decrease in the efficiency of colony formation. Finally, removal of retinoic acid after transfection did not significantly increase the number of colonies generated by vector alone, confirming that the cells are irreversibly committed to differentiation after 4 days of retinoic acid treatment.

That these clones were indeed expressing E1A protein was demonstrated by immunofluorescent staining of a representative plate after transfection of the 12S E1A-expressing plasmid. Greater than 98% of the colonies were positive for E1A, showing nuclear staining (data not shown). A Western blot analysis of E1A protein in two cell lines established from colonies as well as pools of colonies from two plates demonstrated the presence of E1A protein (Fig. 1).

E1A-Immortalized F9 Cells Are Dedifferentiated. The analysis of an E1A-expressing cell line demonstrated clear phenotypic differences from differentiated F9 cells, both in morphology and growth characteristics. The 12S E1A transformants exhibit a more refractile and rounded appearance (Fig. 2*C*) than the flat, fully differentiated F9 cells (Fig. 2*B*). In addition, unlike differentiated F9 cells, the cells continue to divide in culture (now >10 months). The clones also differ, however, from the undifferentiated F9 stem cell with respect to morphology (Fig. 2*A*) and growth rate. The cells expressing the 12S E1A gene have a doubling time of ≈2 days, whereas the undifferentiated F9 cells double every 18 hr. In addition, the 12S E1A transformants are contact inhibited and quiescent when confluent, whereas the undifferentiated F9 cells tend to pile up when they grow.

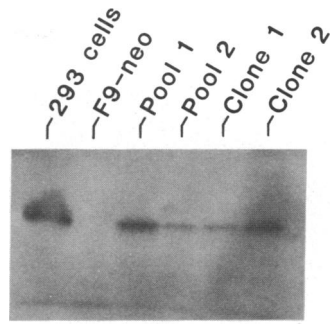

FIG. 1. Western blot analysis of E1A protein expression in E1A 12S transformants. Protein extracts were prepared from 60-mm dishes of two F9 cell lines (clones 1 and 2) derived from transfection with the 12S E1A vector, two pools of colonies of 12S E1A transformants (pools 1 and 2), an F9 cell line selected in G418 after transfection with neo vector alone (F9-neo), and human 293 cells. Aliquots of each extract were subjected to Western blot analysis. An extract of 293 cells was analyzed as a comparison as well as an extract of an F9 cell line selected in G418 after transfection of the neo vector (F9-neo).

With respect to expression of differentiation markers, the 12S transformants have clearly lost expression of genes characteristic of terminal differentiation. Although the fully differentiated cells were strongly positive for laminin staining, the foci that expressed E1A were negative. Fig. 3 shows an example of this immunofluorescence. Differentiated F9 cells are shown in phase contrast in Fig. 3*A* and stained with laminin antibody in Fig. 3*B*. A 12S E1A cell line did not express detectable levels of laminin (Fig. 3*D*). Thus, several observations suggested a loss of some characteristics typical of the differentiated phenotype.

To achieve a more quantitative assessment of the state of differentiation of these cells, we examined the expression of several genes whose expression is regulated by differentiation (11, 12), including laminin, type IV collagen, SPARC (secreted, acidic, cysteine-rich, glycoprotein), protein disulfide isomerase, as well as one previously unidentified gene (*A70-3*) that is specifically expressed in differentiated cells (8). The expression of each of the differentiation-regulated genes was examined in a 12S E1A cell line (referred to as F9-neo-12S). For comparison, an F9 cell line transfected with the neo vector and selected in G418 was analyzed in parallel (F9-neo). RNA was isolated from the F9-neo cell line, the F9-neo cells were treated for 3 days with retinoic acid and cAMP, the F9-neo-12S E1A cells were grown continuously in retinoic acid, the F9-neo-12S E1A cells were grown in the absence of retinoic acid for 1 month, and these retinoic acid-free F9-neo-12S E1A cells were treated with retinoic acid and cAMP for 3 days. As shown in Fig. 4, each of the differentiation-specific RNAs was induced in the F9-neo cell line upon treatment with retinoic acid and cAMP. In contrast, there was no induction in the 12S E1A-expressing cells. This was true whether the cells had been continuously maintained in retinoic acid or removed from retinoic acid and reinduced. Thus, as characterized by the expression of these gene

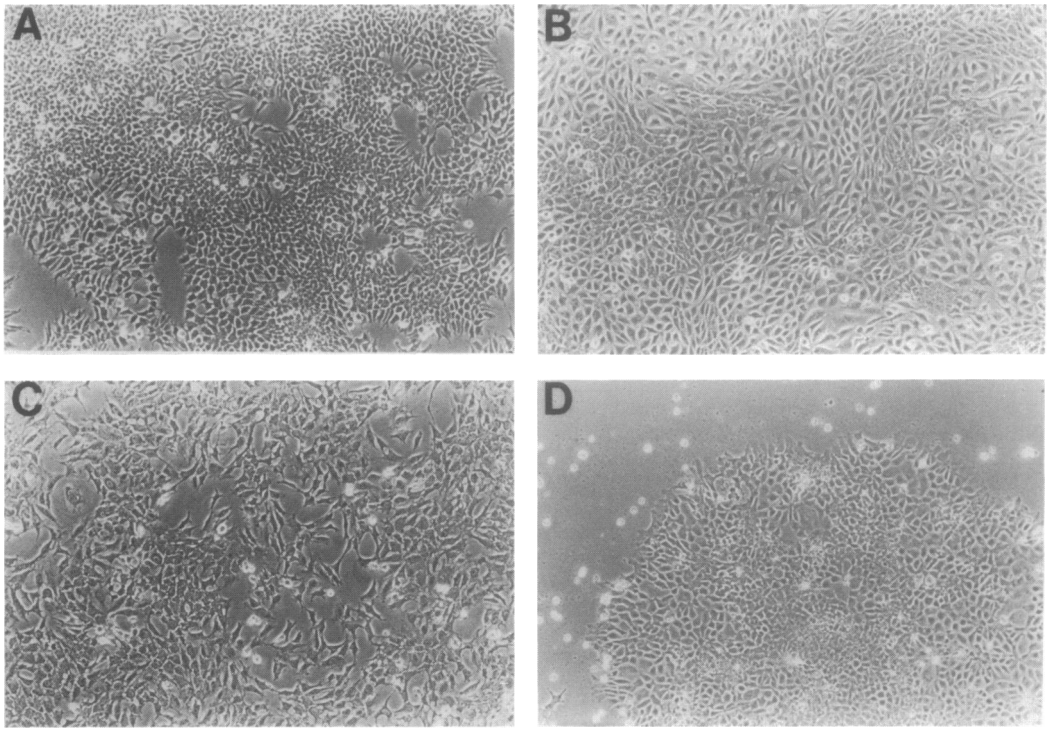

FIG. 2. Morphology of F9 cell lines immortalized by E1A expression. Phase-contrast micrographs of undifferentiated F9 cells (*A*), F9 cells after 3 days in retinoic acid and cAMP (*B*), F9-neo-12S cells (*C*), and a portion of the colony (*D*) that gave rise to the cell line shown in *C*.

products, the 12S E1A cell line has lost the differentiated phenotype.

DISCUSSION

A series of previous experiments have documented the immortalizing capacity of the *E1A* oncogene. That is, introduction of *E1A* into primary cell cultures, cells that exhibit little or no capacity for continued proliferation, results in the establishment of permanent cell lines (13–15). Utilizing differentiated F9 cells as the target, cells that also have no proliferative capacity, we demonstrate that E1A can induce cell proliferation, resulting in the establishment of permanent cell lines. What distinguishes our experiments from most previous assays of E1A immortalization is the link with cell differentiation, since in the F9 cell system we show that there is a loss of the fully differentiated phenotype together with immortalization. One earlier study did demonstrate adenovirus transformation of differentiated rat hepatocyte cultures, although this was dependent on E1B as well as E1A

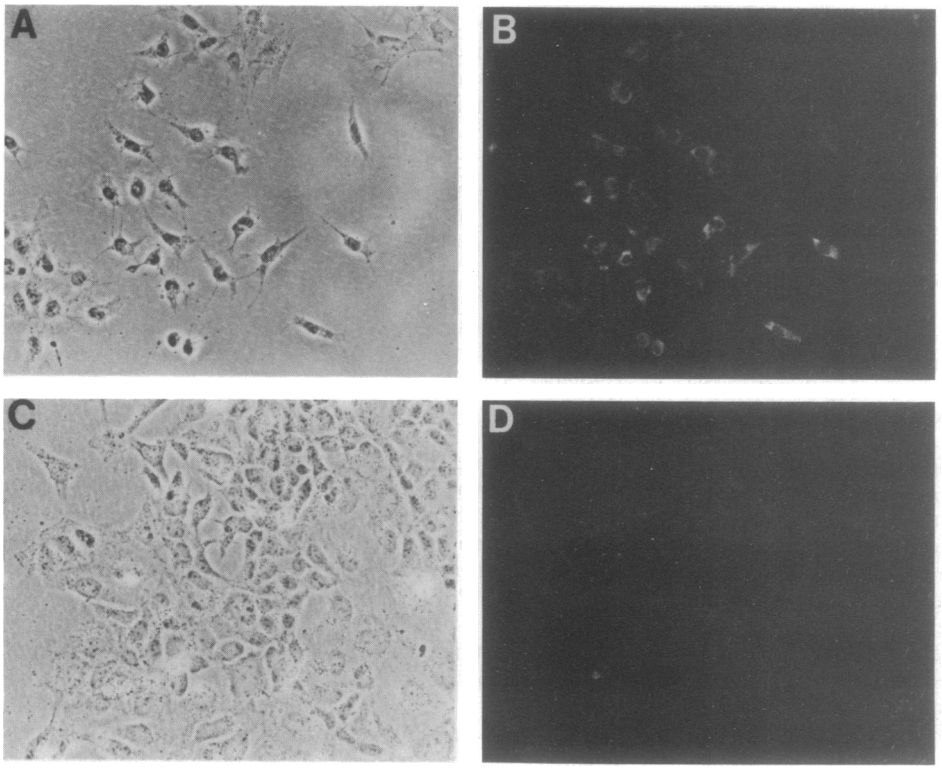

FIG. 3. Laminin expression in differentiated F9 cells and E1A 12S transformants. Differentiated F9 cells (4 days in retinoic acid and cAMP) were examined by phase contrast (*A*) as well as by immunofluorescence using a laminin monoclonal antibody (*B*). (*C*) Phase-contrast micrograph of a colony derived from transfection of differentiated F9 cells with the E1A 12S expression vector. (*D*) Immunofluorescent staining of the field of cells shown in *C* using the laminin monoclonal antibody.

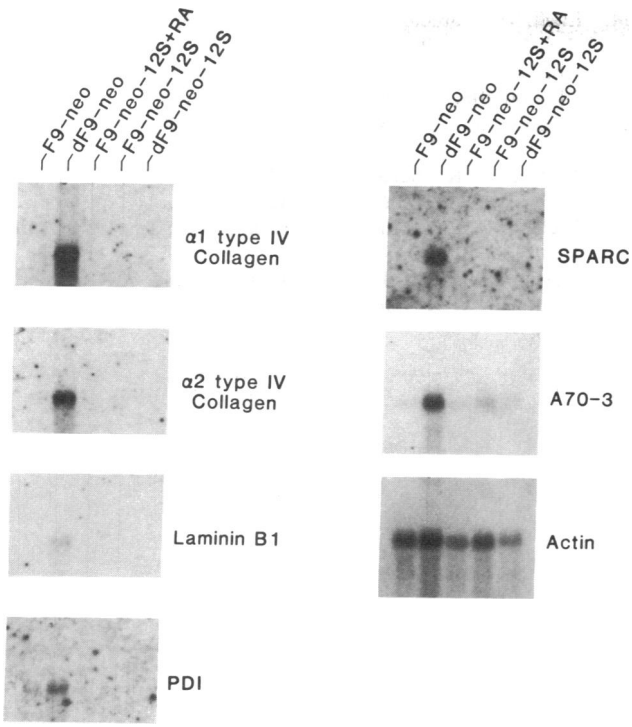

FIG. 4. Expression of differentiation-specific RNAs in E1A immortalized F9 cells. Total cytoplasmic RNA was isolated from untreated F9-neo cells, F9-neo cells grown in differentiation medium for 3 days (dF9-neo), F9-neo-12S cells that had been continuously maintained in retinoic acid (F9-neo-12S+RA), F9-neo-12S cells maintained in normal medium (F9-neo-12S), and F9-neo-12S cells that had been grown in normal medium for 1 month and then grown in differentiation medium for 3 days (dF9-neo-12S). Identical Northern blots were probed with each of the differentiation-specific cDNAs as well as actin as a control. PDI, protein disulfide isomerase; SPARC, secreted, acidic, cysteine-rich, glycoprotein.

(16). Furthermore, although some foci that grew out were reduced for albumin expression, the effect on the state of differentiation was not clearly determined. Two previous studies have also linked E1A expression with an arrest of differentiation—in one case nerve growth factor induction of PC12 cell differentiation (17) and in another the differentiation of myoblast cultures to myotubes (18). The approach in these two studies was different than ours in that the cells were already proliferating and E1A expression was found to block the subsequent induction of differentiation. In contrast, our experimental approach provided evidence that E1A expression might actually reverse the process by providing a proliferative capacity to the cells. Nevertheless, the sum of each of these experiments supports the conclusion that E1A expression can interfere with cellular differentiation.

Our experiments clearly demonstrate that 12S E1A expression blocks differentiation and results in cell lines that do not express markers of terminal differentiation even in the presence of retinoic acid and cAMP. In contrast, two previous reports have suggested that E1A might induce the differentiation of F9 cells. Obviously, the major difference between our work and these other studies lies in the target for E1A transfection. In both previous cases, the effects of E1A introduction into undifferentiated F9 cells was measured. In one case (19), the frequency of colony formation upon E1A transfer to undifferentiated cells was quite low, similar to our findings. The extent of differentiation in the few cell lines that did arise from this selection was clearly limited; only one marker of differentiation was increased in expression and the expression of this gene (*endo A*) could be further increased, along with other markers of differentiation, by treatment with retinoic acid and cAMP. The experiments reported by Montano and Lane (20) revealed a high frequency of colony formation using F9 cells and E1A. The discrepancy between this result and our observation that E1A prevented colony formation in F9 cells could be explained by a difference in the E1A vector used, or by a subtle difference in the cell lines used. That is, the response of a cell to E1A may depend critically on the amount of E1A expressed and on the exact physiological state of the cell. It is also possible that the cell lines generated by introduction of E1A into undifferentiated or differentiated F9 cells may not be that drastically different. Although the E1A-expressing cells derived in our experimental system have clearly lost the fully differentiated phenotype, it is also true that they are not identical to the undifferentiated F9 stem cells. The cells generated by transfection of undifferentiated F9 cells (19, 20), although they clearly have acquired some aspects of differentiated cells, also do not appear to be fully differentiated. Thus, E1A expression may drive F9 cells into an intermediate stage of differentiation.

Oncogenesis is a process whereby a cell escapes from the normal cellular proliferative controls. Often it can be demonstrated that such cells are also phenotypically less well differentiated than the tissue from which they are derived. The results presented here clearly demonstrate that expression of the *E1A* oncogene alters the retinoic acid-induced differentiation of F9 cells leading to a proliferating cell that has lost expression of terminal differentiation markers. Indeed, we wish to suggest that E1A expression brought about a reversal of the differentiated phenotype; that is, a cell that had already differentiated and that was expressing the differentiation genes now reversed the process as a result of E1A expression and dedifferentiated. The critical issue in this argument is the nature of the cell that acquired the E1A expression vector and then gave rise to stably growing colonies. As already described, several lines of evidence suggest that such a cell was in fact a differentiated cell, expressing genes characteristic of terminal differentiation. Most importantly, the transfection efficiencies argue that the target was not a small percentage of cells that had not yet differentiated since one might have anticipated a significant reduction in colony formation, as a function of days in retinoic acid, if only a subset of the transfectable cells could give rise to these colonies. Of course, it is possible that the cell target for E1A is at an intermediate stage of differentiation, expressing differentiation markers but not yet fully committed to the terminal step. Regardless, these data suggest that the differentiation-dependent activation of cellular gene expression, and thus the differentiated cell phenotype, is not permanent. These findings, as they relate to tumor biology, have important implications. Most tumors are generally believed to arise from an undifferentiated, proliferating stem cell population. Our data suggest that in certain cases a tumor may derive from a fully differentiated cell due to the expression of an activity such as E1A that stimulates cell proliferation, leading to dedifferentiation and ultimately oncogenic conversion.

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