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The Adenovirus E1a Gene Induces Differentiation of F9 Teratocarcinoma Cells

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Undifferentiated F9 cells transfected with plasmids encoding adenovirus E1a gene products underwent radical morphological changes. They ceased to express the SSEA-1 stem cell marker antigen and started to express a number of the characteristics of the differentiated state that is induced in F9 cells by treatment with retinoic acid. In particular, they expressed keratin intermediate filaments and acquired the ability to synthesise simian virus 40 tumor antigens after virus infection. The transfected cells expressed the E1a proteins, and this expression was necessary to induce the phenotypic changes, since a coisogenic plasmid encoding only a truncated 70-amino-acid E1a polypeptide and the transfection procedure itself did not detectably alter the morphology or marker expression of the F9 stem cells. The phenotypic change was induced by both 13S and 12S cDNA plasmids. We discuss these results in the context of known E1a functions and with reference to the other oncogenes and external factors that can cause F9 cell differentiation.

The E1a regions of adenoviruses encode at least three related proteins through the production of three alternately spliced mRNAs. The 13S mRNA encodes a protein of 289 amino acids, the 12S mRNA encodes a protein of 243 amino acids, and the 9S mRNA encodes a protein of 55 amino acids (7). The E1a region of the virus is required in most cell types for efficient expression of other early viral gene products, and the E1a proteins appear to be pivotal and powerful regulators of viral gene expression (19). Their activity extends beyond the adenovirus system, however, as the E1a products can both enhance or repress transcription of a range of other genes (8, 13, 20, 21, 33, 35). The target sequences for these regulatory activities have not been unequivocally defined, though there is strong evidence that the negative regulatory activity of E1a is exerted through *cis*-acting enhancer elements (3, 36), whereas the stimulation of transcription is enhancer independent. E1a proteins do not appear to have intrinsic DNA-binding activity (5) but are extensively modified in vivo and associate with a number of specific cellular proteins (15). The E1a region is essential for adenovirus transformation, and although products of the E1b region are also required for efficient transformation, the E1a region alone can in some situations rescue cells from senescence (17, 24). The E1a region also efficiently complements activated *ras* genes to overtly transform primary cells (28) and some established cell lines (6).

An extensive mutational analysis of the E1a region, combined with the use of E1a cDNA clones, has partially assigned these different activities of the E1a region to its different protein products. Although these studies are not completely consistent, the consensus is that the 13S product is polyfunctional and is able to activate transcription of early adenovirus and other genes, to repress transcription by acting on enhancers (for instance, in the simian virus 40 [SV40] system), and, finally, to immortalize. The 12S product is unable (4, 25, 27, 36), except in microinjection systems (5), to activate other genes but can repress and immortalize. Indeed, these last two activities seem to cosegregate in E1a 12S mutants (N. Jones, personal communication).

In the majority of cell types examined, the E1a region is essential for E2 gene expression. Imperiale et al. (18) found that certain E1a mutant viruses are unable to efficiently express an E2 gene product after infection of differentiated F9 cells. In contrast, when the same viruses are used to infect undifferentiated F9 cells, abundant expression of the E2 product is detected. This suggested that the undifferentiated F9 cells contain a cellular factor that has an E1a-like activity and that this activity is lost on differentiation. Further support for this idea has come from an examination of the activity of a range of viral transcription units in F9 cells.

Wild-type polyomavirus is unable to grow efficiently on undifferentiated F9 cells but can grow in the differentiated derivatives (2, 32). This has permitted the isolation of a number of mutant polyomaviruses of extended host range that are now able to grow efficiently within F9 stem cells. Analysis of these mutant viruses has located the enabling mutations in the viral enhancer region, again suggesting that alterations in enhancer activity occur on differentiation and pointing to the existence of a cellular repressor of transcription that is able to act on viral enhancers in F9 stem cells (34).

Strikingly, Hen et al. (16) found that whereas the wild-type polyomavirus enhancer could act as a target for E1a-mediated repression, the F9 selected polyomavirus enhancer mutants were resistant to this repression. Recently, Gorman et al. (12) demonstrated enhancer-dependent repression of transcription of a number of other viral transcription units in undifferentiated F9 cells. This paper also presents evidence that the repression was mediated by a titratable cellular factor. These observations suggest that there is a major shift in transcriptional regulation on F9 cell differentiation that may be mediated by a cellular activity closely analogous to that of the viral E1a region. We hypothesized that this E1a-like activity may control the normal cellular differenti-

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ation process. We set out to introduce the viral E1a region into undifferentiated F9 cells to see whether constitutive expression of this activity would render the cells resistant to the induction of differentiation by external agents, overriding the cellular control factor.

This response was not observed; instead, expression of the E1a proteins in the undifferentiated F9 cells efficiently induced them to undergo a radical phenotypic change, analogous to that induced by retinoic acid.

MATERIALS AND METHODS

Cell culture, Cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum–100 mM glutamine–500 U of penicillin per ml–100 μg of streptomycin per ml. The CO_2 concentration in the incubators was set such that the pH of the medium was equilibrated at 7.3. Care was taken to maintain this pH throughout the experiment.

F9 (1), PCC3, and PCC4 cells were subcultured at a 1:3 dilution every day. Cells were passaged 10 times and then discarded, and new cells were plated from storage.

F9, PCC3, and PCC4 cells were plated as single cell suspensions 16 h before transfection. The calcium phosphate coprecipitation technique described by Gorman et al. (10, 11) was used.

The medium was changed 3 h before addition of the calcium phosphate-DNA coprecipitate, which was left on the cells for 15 h. The cells were then carefully rinsed with DMEM and provided with DMEM supplemented with 10% fetal calf serum.

For the production of stable transformants, cells were treated as above; 48 h after transfection, the medium was changed with fresh DMEM containing 1 mg of G418 (GIBCO Laboratories) per ml and 10% fetal calf serum.

SV40 infection. Cells were infected with the WT776 strain of SV40 at a multiplicity of infection of 50 PFU per cell. The expression of large T was detected by immunoperoxidase staining 48 h after infection with the use of monoclonal anti-T antibodies (14).

Immunoperoxidase staining. The monoclonal antibodies used in this work were as follows: anti-SSEA-1 (31), which recognizes a glycolipid epitope found only in undifferentiated embryonal carcinoma cells; TROMA-1 (22) and LP2K (23), which recognize cytokeratins found only in differentiated cells (E. B. Lane and B. Hogan, unpublished observations); PAb419, which recognizes SV40 large T antigen (14); and M73, which recognizes the 289- and 243-amino-acid products encoded by the adenovirus type 5 (Ad5) E1a region (15).

After transfection (24 or 48 h or 14 days), cells were fixed with freshly made acetone-methanol (1:1, vol/vol) for 3 min. The cells were fixed directly without washing.

All antibodies were diluted in phosphate-buffered saline containing 10% fetal calf serum to give a final concentration of 10 to 20 μg of immunoglobulin per ml.

Fixed cells were incubated with the first antibody overnight at 4°C and then washed five times with phosphate-buffered saline. The second antibody, anti-mouse immunoglobulin conjugated with horseradish peroxidase (DAKO) diluted 1:20 in phosphate-buffered saline containing 10% fetal calf serum, was added and incubated for 3 h at 4°C. Finally, the substrate was added and left in contact with the cells for 30 min. The substrate was always prepared freshly by diluting a stock of *o*-dianisidine (stored as a saturated solution in ethanol at −20°C) to 1 in 100 in phosphate-buffered saline, filtering the solution, and then adding H_2O_2 (30 volumes) to a final concentration of 1 in 5,000.

TABLE 1. Transfection of F9 cells

Construct	DNA (μg)/ plate[a]	No. of G418-resistant colonies	Morphologically altered colonies (% of total)[b]
pRSVneo	3	450	1
pRSVneo + p1A	3 + 10	460	80–90
pRSVneo + pSVE1a	3 + 10	201	95
pRSVneo + pSVN20	3 + 10	180	90
pRSVneo + pSVF12	3 + 10	195	90
pRSVneo + pSVXL105	3 + 10	500	90
pRSVneo + pSVXL3	3 + 10	480	1

[a] Cells (10^4) on a 60-mm petri dish were transfected with the listed amount of DNA and were then grown in G418-containing medium for 14 days.

[b] Morphologically altered colonies were defined as those colonies where more than 30% of the colony area consisted of flattened TROMA-1-positive cells.

Plasmids. Plasmid pRSVneo was derived by the insertion of the long terminal repeat of Rous sarcoma virus (RSV) into the pSV2neo vector (9). It has been shown that transcription of the *neo* gene originates at the correct start site within the RSV long terminal repeat. Plasmid p1A contains residues 1 to 1834 of the Ad5 genome. It has been shown to complement an activated *ras* gene in the DNA transfection-mediated transformation of primary baby rat kidney cells (29).

pSVE1a is a construct containing residues 1 to 1834 from Ad5 inserted between the *Eco*RI and *Pst*I sites of pSVOd, a vector containing the SV40 origin of replication (36).

pSVN20 and pSVF12 were constructed by cloning the *Eco*RI-*Pst*I fragment of the intronless plasmids pJN20 and pJF12 (36), encoding, respectively, the 13S and 12S cDNA sequences of the Ad5 E1a gene into pSVOd. pSVXL105 and pSVXL3 were derived from pSVN20 by small deletions and then by insertion of *Xho*I linkers such that pSVXL105 contains an in-frame insertion, whereas the insertion in pSVXL3 results in a frameshift (36).

RESULTS

Transfection of F9 cells. Calcium phosphate-mediated transfection of 10^4 F9 cells with 3 μg of pRSVneo DNA reproducibly resulted in the appearance of around 450 colonies able to grown in DMEM containing G418 (Table 1). These colonies grew as a tight cluster of small, almost spherical cells rather loosely bound to the substrate. Their morphology was essentially identical to that of untransfected F9 cell colonies. Their undifferentiated state was confirmed by their appropriate expression of a range of antigenic markers (see below). The failure of transfection and antibiotic selection to induce or select for morphologically altered F9 cells had earlier been observed in transient transfection assays (9) and in stable transfection (26), but in the latter case, only the pSV2neo plasmid was used. The use of the more efficient RSV promoter in these experiments resulted in the appearance of a much higher frequency of antibiotic-resistant colonies but did not otherwise affect the result.

F9 cells transfected with E1a-encoding plasmids. When F9 cells were cotransfected with 3 μg of RSV *neo* DNA and 10 μg of the E1a-encoding plasmid p1A (29) and examined after 14 days of selection with G418, three morphologically distinct colony types were observed. Of the G418-resistant colonies, 10 to 20% (type 1) retained a normal undifferenti-

ated F9 cell appearance typical of those seen after transfection of pRSVneo alone (Fig. 1A). The predominant colony type (type 2, about 60% of the G418-resistant colonies) contained a mixture of two cell morphologies. Typically, the center of the colony contained densely packed cells of stem cell morphology, and these were surrounded by a halo of flattened dispersed cells. The relative proportion of each cell type in the colonies varied greatly (Fig. 1B and C). Quite frequently, the flattened cells appeared as clusters that seemed to have developed from a focus at one point on the periphery of the stem cell core (Fig. 3B), or they developed at the junction between two stem cell-like colonies (Fig. 3C). Colony type 3 (about 20% of the G418-resistant colonies) consisted entirely of flattened extended cells, often with quite large processes (Fig. 1D). These colonies contained far fewer cells than those of the stem cell type, and the cells were typically quite dispersed.

The plasmid p1A contains nucleotides 1 to 1834 of the Ad5 genome and encodes the 13S, 12S, and 9S mRNAs under viral transcriptional control. As transient transfection experiments with p1A suggested that this plasmid induced only a very low level of expression of the E1a gene products in F9 cells, the activity of an alternate E1A-encoding plasmid pSVE1a was determined.

On transient transfection, this plasmid induced higher levels of E1a gene products in F9 cells (see below). Cotransfection of pRSVneo and pSVE1a yielded a higher frequency of morphologically altered colonies. At least 95% showed either a completely flat cell type or flattened cells constituting more than 30% of the colony area. More of the colonies in the transfections were of homogeneously flat morphology, and within those colonies of mixed morphology, the average number of stem-like cells was lower. The plasmid pSVE1a contains nucleotides 1 to 1834 of the Ad5 genome and is thus transcribed to produce the 13S, 12S, and potentially the 9S mRNAs under the control of the normal Ad5 control sequences. The plasmid also contains the SV40 origin of replication.

To assess the role of the different mRNAs and protein products of the E1a region in inducing these morphological alterations, we used two further plasmids. pSVN20 and pSVF12 are similar in construction to pSVE1a but contain Ad5 cDNA sequences such that pSVN20 produces only the 13S mRNA and the 289-amino-acid protein and pSVF12 produces only the 12S mRNA and the 243-amino-acid protein (36). Cotransfection of either plasmid with pRSVneo resulted in the appearance of morphologically altered colonies, and as with the pSVE1a plasmid, these were present at very high frequency (Table 1). Thus, the 13S mRNA and by implication the 289-amino-acid protein or the 12S mRNA and 243-amino-acid protein can independently induce morphological alterations in F9 cells.

The plasmid transfection could have brought about the phenotypic changes in F9 cells, either by a direct effect of the plasmid DNA itself (perhaps titrating out a specific regulatory DNA-binding protein) or through the expression of the RNA transcripts or protein products of the plasmid genes.

To distinguish between these potential mechanisms, two further plasmids were introduced into the F9 cells by transfection. The two plasmids are coisogenic; both are derivatives of the 13S cDNA plasmid pSVN20 which has been mutated by insertion of an *Xho*I linker within the 13S cDNA open reading frame. In plasmid pSVXL105, residues 674 to 693 were deleted and substituted by the linker. This in-frame substitution results in the production of a 286-amino-acid mutant 13S cDNA product which has been shown to be biologically active in both transcriptional activation and repression assays (34). When introduced by transfection with pRSVneo into F9 cells, pSVXL105 induced the same morphological changes that had been seen with the pSVE1a, pSVN20, and pSVF12 plasmids. At least 95% of the G418-resistant colonies examined at 14 days contained a substantial fraction of flattened cells (Table 1).

In the second plasmid, pSVXL3, residues 748 to 753 have been deleted and substituted with the linker. This results in a frameshift such that the plasmid can only encode a truncated 70-amino-acid peptide, which has been found to be biologically inactive in transcriptional regulation (36). When this plasmid and pRSVneo were introduced into F9 cells, the morphology of the resulting G418-resistant cells, examined 14 days after transfection, was indistinguishable from that of colonies resulting from the transfection of the pRSVneo plasmid alone. The alterations in morphology and gene expression seen after transfection of E1a-encoding plasmids into F9 cells seem, therefore, to be a direct consequence of the expression of biologically active E1a protein within these cells.

To look for a residual activity of the 70-amino-acid peptide or for a possible additional weaker activity of the plasmid DNA or its RNA transcripts, the pRSVneo-pSVXL3-transfected cultures were grown for an additional 2 weeks in the selective medium (i.e., until day 28). No increase in colony number manifesting gross morphological change was seen over this period, compared with that of cultures transfected with pRSVneo alone and maintained for the same period.

Expression of E1a protein in transfected F9 cells. The monoclonal antibody M73 was used to detect the expression of E1a proteins in these experiments. This antibody reacts with both the 13S 289-amino-acid protein and the 12S 243-amino-acid protein (15). Cultures of F9 cells were transfected with pSVE1a and stained 48 h later with M73 and a sensitive immunoperoxidase technique. A clear pattern of nuclear staining was seen in 1 to 5% of the recipient cells (Fig. 2A). Untransfected F9 cells completely failed to stain with the antibody. Positive nuclear staining was seen after transient transfection of the plasmids pSVN20, pSVF12, and pSVXL105. No staining of cells transfected with the pSVXL3 plasmid was seen with the M73 antibody (data not shown). When the same antibody was used to stain G418-resistant colonies 14 days after transfection, nuclear staining of the differentiated cells was seen (Fig. 2B). The level of E1a as judged by staining intensity was lower in these established transfectants than that seen in the transient 48-h assay. It was noticeable that the level of E1a expression induced by the pSVE1a, pSVN20, pSVF12, and pSVXL105 plasmids was considerably higher than that induced by p1A as judged by staining with M73 48 h after transfection.

Appearance of differentiation markers. When F9 cells are induced to differentiate with retinoic acid, they undergo changes in morphology closely analogous to those seen in the E1a-transfected cells. They also begin to express a range of keratin polypeptides. Keratin expression in the E1a-transfected cells was therefore tested 2 weeks after transfection with monoclonal antibodies and immunoperoxidase staining. Intense staining of the flattened cells was seen with the antibodies TROMA-1 (22) (Fig. 3C), which is specific for keratin 8, and LP2K, which is specific for keratin 19 (E. B. Lane, personal communication). There was absolutely no staining of the untransfected F9 cells or of the colonies that had a stem cell appearance. These staining patterns coin-

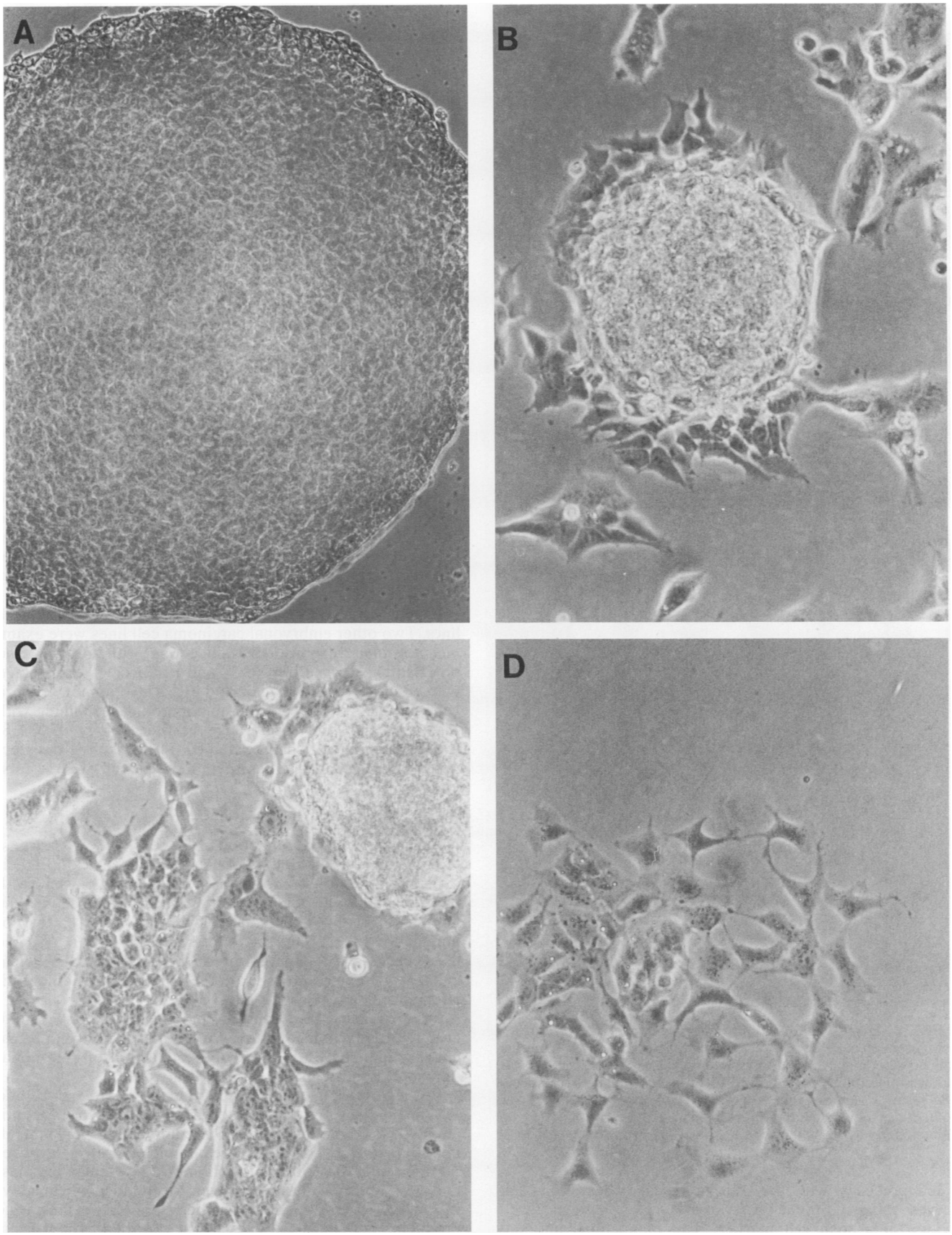

FIG. 1. Colony morphology of E1a-transfected F9 stem cells. Cultures of undifferentiated F9 cells were transfected with p1A and pRSVneo, and colonies were selected for 14 days, using G418-containing medium. (A) Colony with normal F9 stem cell appearance; (B and C) colonies containing a core of F9 stem cells surrounded by flattened differentiated cells: (D) colony of flattened cells with extended processes.

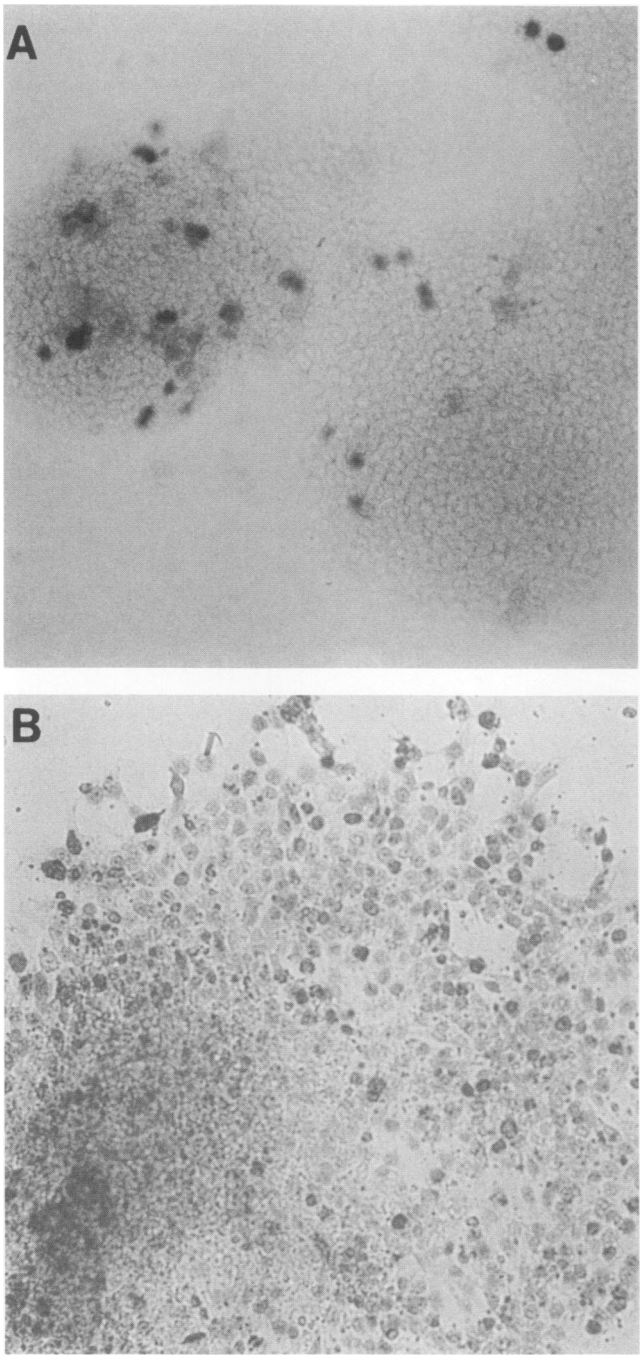

FIG. 2. Expression of E1a proteins in F9 cells. (A) Cultures of undifferentiated F9 cells transfected with pSVE1a, then fixed and stained 48 h later with M73 by the immunoperoxidase method: (B) cultures of undifferentiated F9 cells cotransfected with pSVE1a and pRSVneo. Colonies were selected for 14 days by using G418-containing medium and then were fixed and stained with M73 by the immunoperoxidase method.

cided precisely with the morphological observations and greatly enhanced them.

Loss of a stem cell marker. The monoclonal antibody SSEA-1 (31) reacts with a glycolipid antigen found on undifferentiated F9 cells but not on those treated with retinoic acid. Colonies transfected with RSV *neo* alone or untransfected F9 cells stained intensely and homogeneously with this antibody (Fig. 4A). The morphologically altered cells present in the E1a-transfected F9 colonies failed completely to react with this antibody (Fig. 4B), and the staining pattern obtained was the reciprocal of that seen with the TROMA-1 and LP2K antibodies.

T antigen expression. Undifferentiated F9 cells abortively infected with SV40 virus failed to express SV40 T antigen. The nature of the block to expression is unclear, since the same cells transfected with SV40 virus DNA express both large and small T (9). Retinoic acid-induced differentiation of F9 cells renders them permissive for T antigen expression after virus infection. When E1a-transfected F9 cell colonies were abortively infected with SV40 virus and stained with anti-T monoclonal antibodies 48 h later, nuclear T antigen was readily detected only in the cells of flattened morphology (Fig. 5). These cells were quite susceptible to abortive infection, as 30 to 40% of them expressed T when infected at a moderate multiplicity (50 PFU cell). In keeping with previous studies, absolutely no T antigen was detected in cells of stem cell morphology.

Continuous culture of E1a transfectants. Cultures of cells resulting from the transfection of pRSVneo with either pSVE1a, pSVN20, or pSVF12 have been maintained for periods of up to 4 months in the presence of the selective medium. Keratin-expressing E1a-positive cells persist and can come to dominate these mass cultures, implying that these cells retain some proliferative potential. The cells are, however, very slow growing. It has not yet been possible to grow isolated colonies for prolonged periods.

Effect of E1a expression on other embryonal carcinoma cell lines. Two other embryonal carcinoma cell lines were examined for their response to transfection with the p1A and pSVE1a plasmids. Both PCC4 and PCC3 cells, transfected with either of these plasmids and pRSV neo, expressed E1a gene products detected by immunoperoxidase staining with M73 (15) and showed a range of morphology of G418-resistant colonies, when examined 14 days after transfection, that was similar to that seen in the F9 cell experiments.

DISCUSSION

Morphological differentiation of F9 cells after DNA transfection was first reported by Muller and Wagner (26). In their experiment, cotransfection of a c-*fos*-encoding plasmid with pSV2neo led to the development of differentiated cells in the population of G418-resistant colonies. Using a battery of immunological markers of differentiation similar to those used here, they demonstrated that the morphological changes were accompanied by the expression of differentiation-specific proteins.

Our results with E1a-encoding plasmids mirror quite closely those obtained with c-*fos* in that the morphological changes induced by E1a and the acquisition of differentiation-specific proteins are clearly analogous.

It is legitimate to ask what functional properties c-*fos* and E1a might share, in that they both trigger F9 cell differentiation. This is not apparently a common property of all immortalizing oncogenes, as we completely failed to trigger F9 differentiation with transfection of c-*myc*-encoding plasmids (unpublished data). However, as no biochemical activity has yet been assigned to either the c-*fos* or E1a gene products, any suggested convergence in their properties is purely speculative. Both proteins are located in the cell nucleus, and both E1a and v-*fos* at least can (30) transactivate other viral and cellular genes. E1a can also down regulate

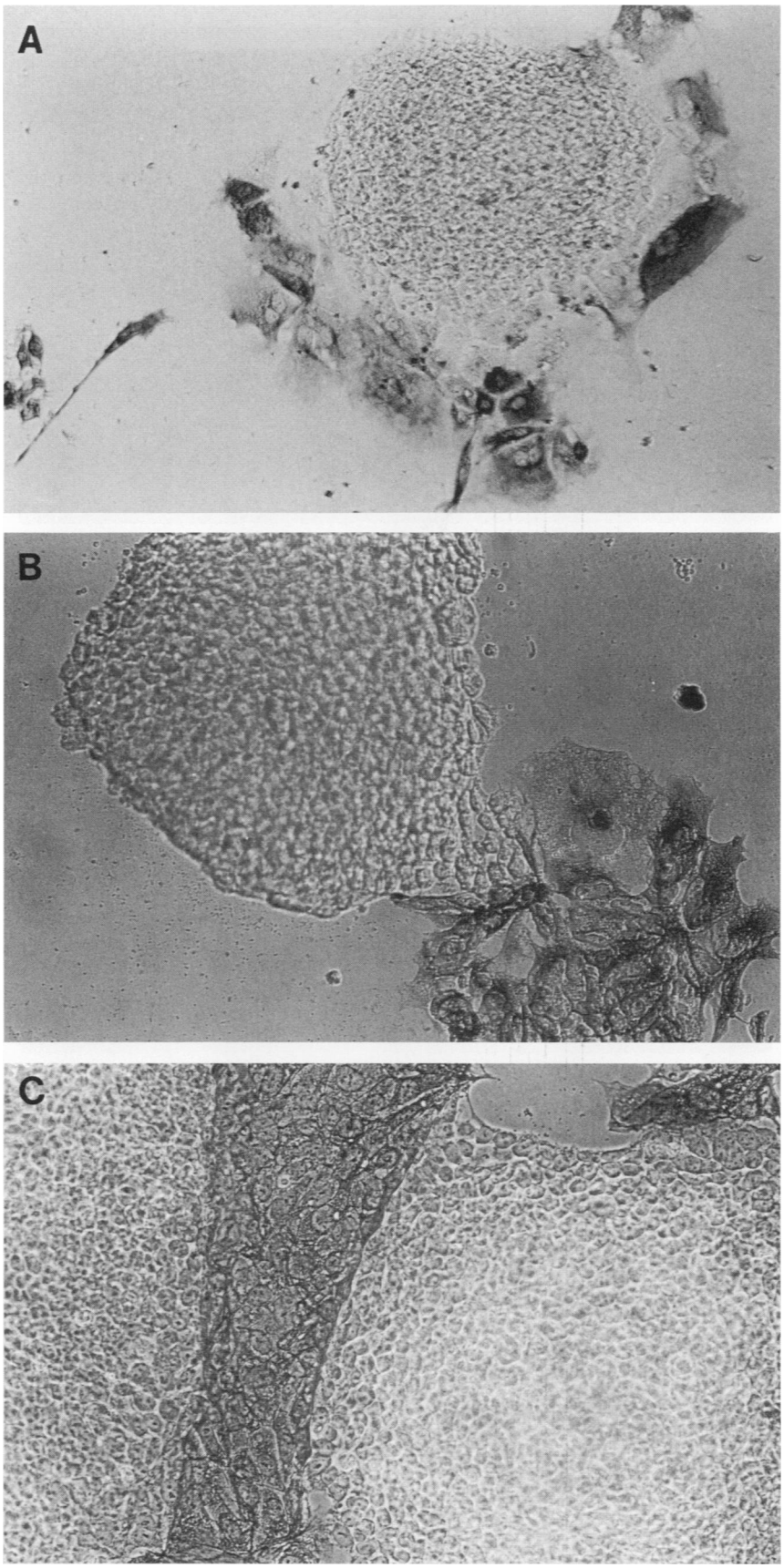

FIG. 3. Expression of differentiation markers. Undifferentiated cells were cotransfected with p1A and pRSVneo. Colonies were selected with G418-containing medium and then were fixed and stained by the immunoperoxidase method. (A and B) Staining with LP2K, a monoclonal antibody specific for keratin 19; (C) staining with TROMA-1, a monoclonal antibody specific for keratin 8.

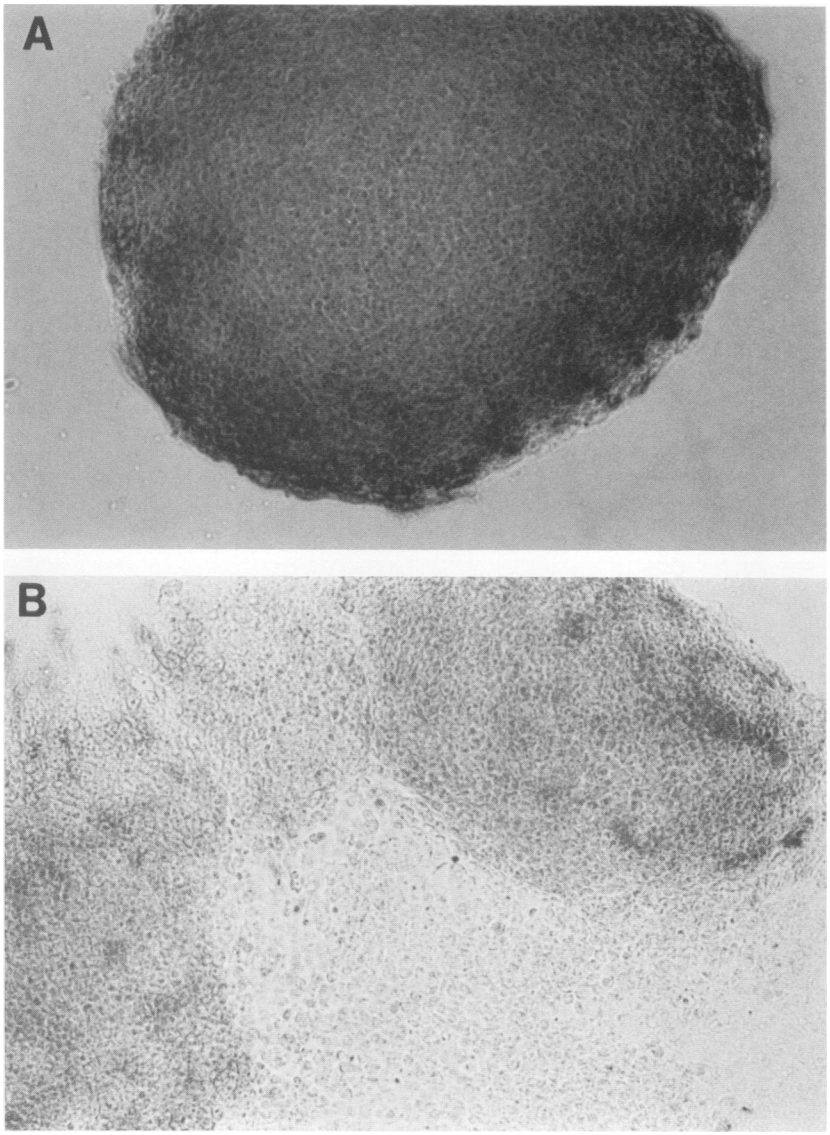

FIG. 4. Expression of SSEA-1 marker. (A) Undifferentiated F9 cells were transfected with pRSVneo, and G418-selected colonies were stained with anti SSEA-1, a monoclonal antibody that recognizes an epitope on a glycolipid found only on undifferentiated cells; (B) undifferentiated F9 cells were cotransfected with p1A and pRSVneo, and G418-resistant colonies were stained with anti-SSEA-1.

certain enhancer-dependent transcription, but such activity has not been described for c-*fos* or v-*fos*. The positive induction of differentiation and continuous growth by the 12S cDNA plasmid imply that the direct transcriptional activation of the E1a products is not involved in the differentiation-inducing event, since a large body of work (with the exception of direct microinjection of protein into Vero cells [5]) implies that the 12S product lacks transactivating activity.

Instead, the hypothesis can be put forward that it is the repression of certain genes that brings about the phenotypic response. If this is correct, then it has important implications for the control of gene expression in development, implying that the stem cell state is actively maintained by certain gene products and that differentiation occurs in the functional absence of these products. The mortal state of primary cells in culture may similarly be actively maintained.

It is important to realize that the cellular E1a-like factors in F9 cells apparently possess both the negative and positive transcriptional regulatory potential of the viral proteins. This is implicit in the finding of both transcriptional activation of later viral genes in E1a mutant viruses and in the repression of specific enhancer-dependent genes within F9 cells. It is likely that in both the viral and cellular systems, these two activities compete and interact with fine specificity for a given control region, allowing delicate differential control of gene expression. In this context, the apparent paradox of our findings is more readily understood.

It is important to further define the precise alterations of gene expression induced in F9 cells by the E1a proteins, compared with those induced by external stimuli and by other oncogenes. We also plan to extend our initial observations on other embryonal carcinoma cell types and to attempt further long-term culture of the E1a-expressing F9 cells to obtain well-characterized cell lines.

We share the view of Muller and Wagner (26) that the

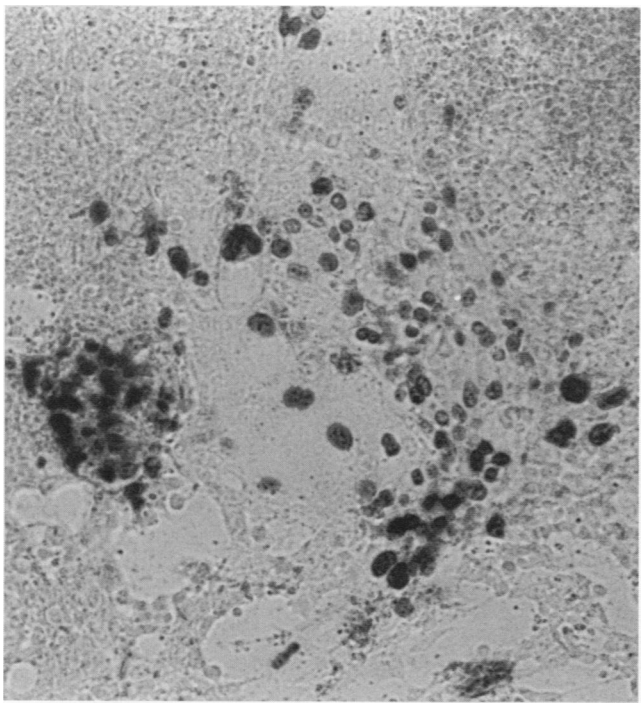

FIG. 5. Expression of SV40 large T antigen in E1a-differentiated F9 cells. Undifferentiated F9 cells were cotransfected with p1A and pRSVneo. Colonies were selected by using G418-containing medium and were then infected with SV40 virus at 50 PFU per cell for 48 h. The cells were fixed and stained with PAb419, a monoclonal antibody that recognizes the N terminus of large T.

specificity of induction of differentiation is implicit within the recipient F9 cell and can be activated by a range of signals. Nevertheless, the recruitment of the E1a gene products into the group of intracellular inducers is significant and can be analyzed in great detail in the future with the wide range of available E1a mutants.

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