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Differentiation as a requirement for simian virus 40 gene expression in F-9 embryonal carcinoma cells

(gene splicing/tumor antigen)

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ABSTRACT **Infection of differentiated mouse embryo cells by simian virus 40 (SV40) leads to the production of the early mRNAs and the tumor (T) antigens that they encode. In contrast, undifferentiated F-9 murine teratocarcinoma cells do not support these early stages of the SV40 cycle. This block results from the inability to accumulate stable processed early SV40 mRNAs. It has recently been shown that vitamin A and its derivatives can induce *in vitro* differentiation of stem cells. Undifferentiated F-9 cells, upon treatment with a low concentration of retinoic acid, exhibited pronounced morphologic changes as well as the appearance of the H-2 surface antigens. After differentiation, the susceptibility of F9 cells to SV40 infection could be demonstrated by the appearance of large T and small T antigens, as shown by immunofluorescence and immunoprecipitation. Furthermore, S1 nuclease mapping of early SV40 transcripts confirmed the presence of the two spliced early mRNAs. These results indicate that the undifferentiated F-9 stem cells contain the genetic information needed for generating stable processed early SV40 mRNAs but are blocked in the production of functional species.**

Teratocarcinomas consist of embryonal carcinoma stem cells and a variety of differentiated cell types representing all three primary germ layers—endoderm, ectoderm, and mesoderm (1, 2). In the past few years mouse teratocarcinomas have generated considerable interest because of their usefulness as an alternative system to early embryo cells. The analogy between embryonal carcinoma and early mouse embryo cells is based upon the observations that both cell types are pluripotential and can differentiate into a wide range of somatic cells (3, 4). Certain embryonal carcinoma cells can undergo extensive differentiation *in vivo* and *in vitro* (2, 5–7). However, there are several cloned lines of embryonal carcinoma cells that under normal tissue culture conditions do not give rise to differentiated cells. One such line, F-9 (8), has been used in our studies.

It has recently been demonstrated that retinoic acid at concentrations as low as 1 nM induces multiple phenotypic changes in F-9 cells grown *in vitro* (6, 7). In a previous study (9), we showed that simian virus 40 (SV40) infection of undifferentiated F-9 cells did not result in detectable tumor (T) antigen synthesis. Nonetheless, low levels of a full-length, nonspliced early RNA transcripts were detected in the infected cells. Using the method of Strickland and Mahdavi (6), we have been able to induce the F-9 cell line to differentiate. After infection with SV40, the differentiated cells contained spliced early SV40 mRNAs and synthesized SV40 large and small T antigens, as demonstrated by immunofluorescence and immunoprecipitation.

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MATERIALS AND METHODS

Virus Strain and Cell Culture. Small plaque SV40, strain 776, was used in all experiments at a multiplicity of 200 plaque-forming units (PFU) per cell. The F-9 murine tetratocarcinoma cell line (8), obtained originally from A. J. Levine, was cultured on surfaces of T-150 (150 cm^2) flasks that had been coated with gelatin. The cells were maintained in Dulbecco's modified Eagle's medium (4.5 g of glucose per liter) containing 15% fetal bovine serum. The PCC3 murine cell line was obtained from F. Jacob. SV40-transformed monkey kidney cells C_{11} were kindly provided by E. Winocour.

Differentiation of F-9 Cells *In Vitro*. F-9 stem cells were plated at a density of 5×10^5 per T-150 flask; 24 hr later *all-trans*-retinoic acid obtained from M. Sporn was added to the cells at a final concentration of 0.1 μM. Growth in retinoic acid was maintained for different time periods as specified in each experiment. The retinoic acid was dissolved (1 mg/ml) in dimethyl sulfoxide (Spectranalyzed) purchased from Fisher and stored at −70°C. Subsequent dilutions were made in Dulbecco's modified Eagle's medium.

Detection of SV40 T Antigen. *Immunofluorescence.* Undifferentiated and differentiated F-9 cells grown on microscope slides (coated with gelatin) were inoculated with SV40. Twenty-four hours after infection the cells were fixed in ice-cold acetone (5 min). Anti-SV40 T serum was incubated with the fixed cells for 30 min at 37°C. Excess serum was removed by serial washes in phosphate-buffered saline. Fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster IgG was added to the cells for 30 min at 37°C. FITC-conjugated goat anti-hamster IgG was mixed with rhodamine contrast stain. Slides were then washed in phosphate-buffered saline, air dried, and analyzed by using a Leitz fluorescence microscope. Anti-SV40 T serum from tumor-bearing hamster, and FITC-conjugated goat anti-hamster IgG was obtained from Huntington Research Center, Brooklandville, MD. For detection of H-2^b surface antigens, indirect immunofluorescent staining was used (7). H-2^b-specific alloantiserum was kindly provided by D. Sachs.

Immunoprecipitation and polyacrylamide gel electrophoresis. All the steps from infection of cells through analysis of the proteins on 20% polyacrylamide slab gel were described before (9), except for the selection of antigen–antibody complexes, which was done by the procedure of Witte *et al.* (10), using a 10% formalin-fixed solution of *Staphylococcus aureus* Cowan I strain, kindly provided by S. Ruscetti.

Analysis and Quantitation of Viral RNA. *S1 nuclease*

Abbreviations: SV40, simian virus 40; T antigen, tumor antigen; PFU, plaque-forming units; VTC, viral transcriptional complex.

mapping. The method of Berk and Sharp (11) was used to map the SV40 early RNA from differentiated SV40-infected F-9 cells. Total cellular RNA was extracted 10 hr after infection of differentiated F-9 cells (9). The RNA was hybridized to ^{32}P-labeled single-stranded probe of SV40 DNA cleaved with *Bam*HI/*Hpa* II restriction endonucleases, and hybrids were analyzed on an alkaline agarose gel as described (9).

Analysis of SV40 primary transcripts. RNA obtained *in vitro* from viral transcriptional complexes (VTCs) was analyzed by hybridization with single strands of SV40 DNA that had been cleaved with *Bam*HI/*Hpa* II restriction endonucleases and immobilized on nitrocellulose filters (12).

Exhaustive filter hybridization. RNA was extracted from VTCs (9) and hybridized to denatured SV40 DNA immobilized on small nitrocellulose filters (6-mm-diameter filter containing 1 μg of SV40 DNA). Hybridization was carried out in 0.1 ml of 0.6 M NaCl/0.06 M sodium citrate/0.5% sodium dodecyl sulfate/10 μg of purified yeast RNA at 68°C for 3 days, using three consecutive DNA filters (13). Each filter was incubated with RNA for 18–20 hr, then replaced with a new filter. The filters were washed four times with 0.3 M NaCl/0.03 M sodium citrate and treated for 1 hr at 37°C with pancreatic RNase at 15 μg/ml in the same buffer.

RESULTS

***In Vitro* Differentiation of F-9 Cells by Retinoic Acid.** F-9 cells grow in culture as an extremely dense monolayer or as tightly packed colonies of embryonal carcinoma cells (Fig. 1 *a* and *b*). Addition of 0.1 μM retinoic acid to undifferentiated F-9 cells grown *in vitro* induces several morphologic changes (6). Within 48 hr, the cells move apart, flatten, and acquire numerous cellular projections (Fig. 1*c*). If the cells are maintained in medium containing retinoic acid for a long period of time (e.g., 12 days) the flattened cells become more numerous and their cellular projections longer (Fig. 1*d*). The population of cells obtained by retinoic acid treatment does not appear homogeneous, but it is nonetheless morphologically distinct from F-9 stem cells. To confirm that a significant fraction of the cells were differentiated, we examined the cultures that had been maintained for 12 days in retinoic acid for the presence of the H-2 histocompatability antigens. It has been demonstrated previously by others that H-2 cell surface antigens are not found on undifferentiated F-9 cells and that these antigens are acquired during cell differentiation (D. Solter, personal communication). Using an indirect immunofluorescence technique (7), we found that approximately 80% of the differentiated F-9 cells were H-2^b-positive after incubation with H-2^b-specific alloantiserum; in contrast, only 3–4% of the undifferentiated cells were positive. Thus, on the basis of morphologic and antigenic criteria, retinoic acid-treated cells differentiate in tissue culture.

Analysis of SV40 RNA Transcribed in Differentiated F-9 Cells. Spliced early SV40 mRNAs are efficiently transcribed in differentiated mouse embryo cells. In contrast, we showed earlier (9) that undifferentiated F-9 cells infected with SV40 at high multiplicities (50–250 PFU per cell) contain small amounts of SV40-specific RNA representing a colinear (primary) but unspliced early transcript. In order to determine if retinoic acid-treated (120 hr) F-9 cells can accumulate stable spliced transcripts of SV40 RNA, we examined total RNA isolated from cells infected with the virus for 10 hr. This time corresponds to the peak of SV40 RNA synthesis in mouse cells

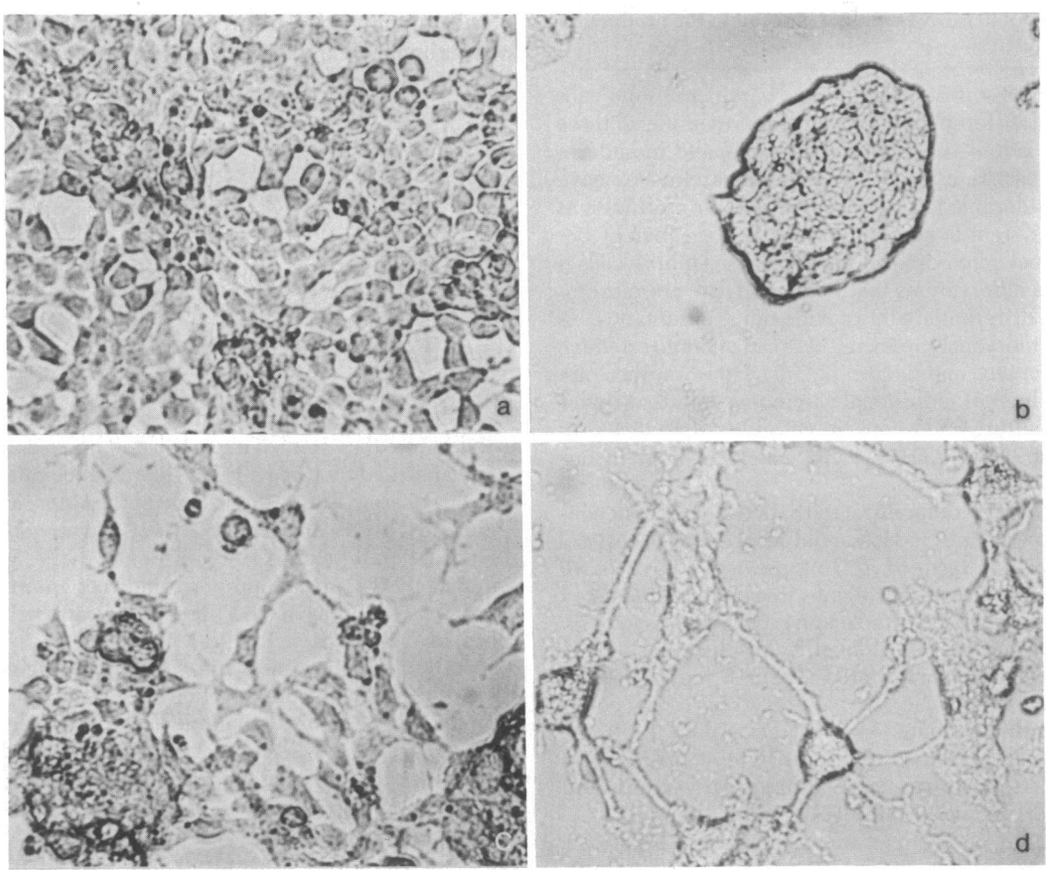

FIG. 1. Differentiation of F-9 teratocarcinoma cells in the presence of retinoic acid. (×200.) (*a*) Dense monolayer of F-9 cells under normal culture conditions; (*b*) tightly packed colonies under normal culture conditions. (*c*) F-9 cells maintained in 0.1 μM retinoic acid for 48 hr; (*d*) cells maintained in 0.1 μM retinoic acid for 12 days.

(ref. 14; unpublished data). ^{32}P-Labeled SV40 DNA was cleaved with *Bam*HI and *Hpa* II restriction endonucleases. A single strand of the DNA fragment from 0.145 to 0.725 SV40 map units (*Bam*HI/*Hpa* II-A_E), which represents the coding region for the early viral functions, was purified (9) and hybridized with the RNA described above. After annealing, DNA·RNA hybrid molecules were digested with nuclease S1 and the products were analyzed by alkaline agarose gel electrophoresis. Three principal bands measuring approximately 1900, 660, and 330 nucleotides were observed (Fig. 2*b*; although the 330-nucleotide band appears light in the figure, it was clearly present in the autoradiogram). These DNA bands have been shown to represent the spliced SV40 early RNA species found in SV40-transformed (Fig. 2*a*) and lytically infected cells (330 + 1900 nucleotides = spliced large T mRNA; 660 + 1900 nucleotides = spliced small T mRNA) (15). To confirm the finding of spliced early SV40 mRNAs and to determine if they are expressed, we examined SV40-infected differentiated F-9 cells for virus-specific early proteins.

Synthesis of SV40 Large T and Small T Antigens in Differentiated F-9 cells. Undifferentiated embryonal carcinoma cells infected with SV40 fail to accumulate detectable amounts of T antigen (9, 16, 17). F-9 cells that had been carried for 120 hr in the presence of retinoic acid were inoculated with high multiplicities of plaque-purified wild-type SV40 (200 PFU per cell). Twenty hours after infection, cells were examined by indirect immunofluorescence staining for the presence of SV40 T antigen. In contrast to undifferentiated F-9 cells, which do not express early SV40 gene products (Fig. 3*a*), 75% of the infected differentiated cells became T antigen-positive (Fig. 3*b*). To confirm this observation and to analyze differentiated F-9 cells for the synthesis of SV40-specific large T and small T antigens, we examined [^{35}S]methionine-labeled proteins by immunoprecipitation and polyacrylamide gel electrophoresis. Protein extracts from undifferentiated and differentiated F-9 cells 20 hr after infection were immunoprecipitated either with an anti-T serum (obtained from hamsters bearing SV40 tumors) or with a control hamster serum (10). The immunoprecipitates were analyzed on 20% polyacrylamide/sodium dodecyl sulfate gels (18). As shown in Fig. 4 *b* and *c*, undifferentiated SV40-infected F-9 cells do not contain detectable quantities of either of the known early SV40 gene products (the 90,000 M_r large T antigen and the 17,000 M_r small T antigen) (19, 20). In contrast, both SV40-specific tumor antigens (Fig.4 *d* and *e*) were present in an immunoprecipitate of the infected differentiated cells. These results clearly indicate that retinoic acid added to F-9 stem cells induces cellular differentiation to a stage compatible with early SV40 gene expression. In analogous studies, differentiated F-9 cells were infected with polyoma virus and labeled for 48 hr with [^{32}P]orthophosphate. Restriction enzyme cleavage of Hirt extracts (21) indicated that the retinoic acid-differentiated cells support efficient replication of polyoma virus DNA (data not shown).

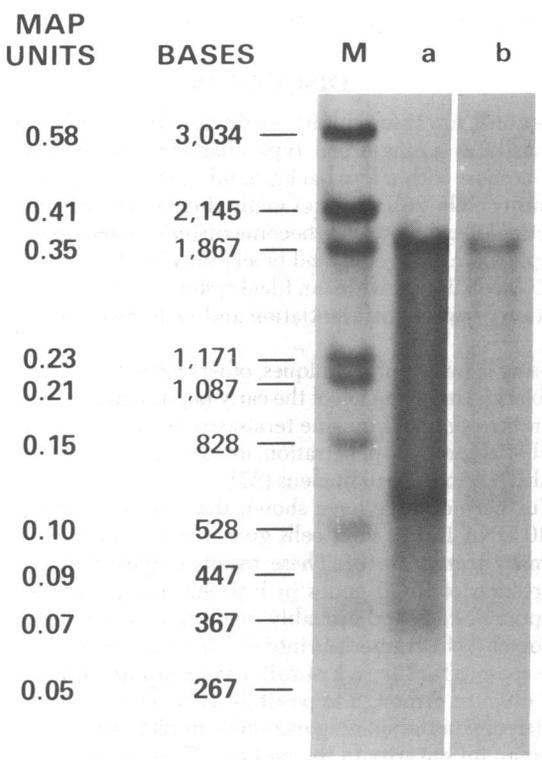

FIG. 2. Nuclease S1 analysis of RNA from differentiated F-9 cells infected with SV40. RNA extracted from retinoic acid-treated F-9 cells 10 hr after infection with SV40 was annealed with a ^{32}P-labeled single-strand DNA probe (A^-) representing the early coding region of SV40 (9). DNA·RNA hybrid molecules were analyzed by the nuclease S1 technique of Berk and Sharp (11). Lane M, marker ^{32}P-labeled SV40 DNA restriction fragments of sizes indicated in fractional length of the SV40 genome and in numbers of nucleotides. Lane a, cytoplasmic RNA from SV40-transformed monkey kidney cells annealed with the (A^-) ^{32}P-labeled probe; lane b, cytoplasmic RNA from differentiated SV40-infected F-9 cells hybridized to the same probe. The three bands observed with these RNA preparations have the same size as the common RNA "body" (1900 bases) and the "leaders" for small T (660 bases) and large T (330 bases) SV40 lytic mRNAs (15).

Comparative Analysis of SV40 RNA from Undifferentiated and Differentiated F-9 Cells. In a previous study (9) we established that a major factor in the failure of undifferentiated F-9 cells to express SV40 early gene products is related to the inability to accumulate stable spliced early transcripts. In order to evaluate the possibility that initiation of SV40 transcription is also depressed in the undifferentiated F-9 cells, we attempted

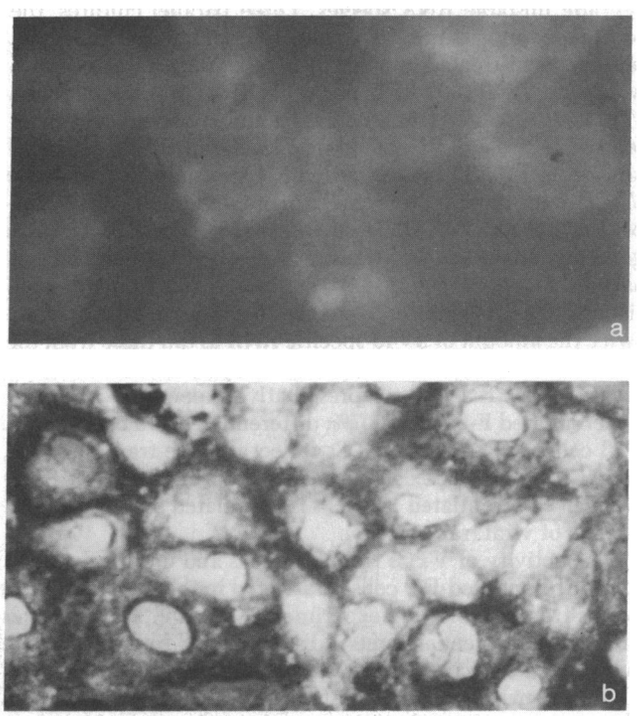

FIG. 3. T antigen immunofluorescence in SV40-infected F-9 cells. (*a*) Undifferentiated F-9 cells. (×460.) (*b*) Differentiated F-9 cells. (×330.) The same photographic exposure time was used for *a* and *b*.

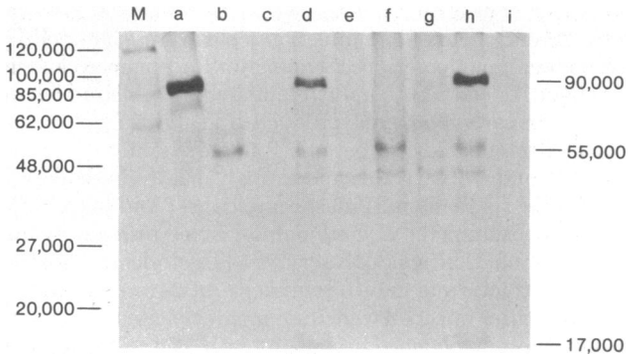

FIG. 4. Analysis of viral proteins by immunoprecipitation. Proteins were labeled and harvested from undifferentiated and differentiated F-9 cultures infected with SV40 (9). The extracts were immunoprecipitated either with 25 μl of hamster anti-SV40 T serum or with normal hamster serum as described by Witte *et al.* (10). Immunoprecipitates were electrophoresed on a 20% polyacrylamide slab gel containing 0.2% sodium dodecyl sulfate (18). Lane M, marker adenovirus 2 proteins. Lane a, control proteins from SV40-infected monkey kidney cells immunoprecipitated with anti-SV40 T serum; lane b, undifferentiated F-9 cell proteins immunoprecipitated with anti-SV40 T serum; lane c, same proteins as in b, precipitated with normal hamster serum; lane d, differentiated F-9 cell proteins with anti-SV40 T serum; lane e, proteins as in d with normal serum; lane f, uninfected differentiated F-9 cells with anti-SV40 T serum; lane g, proteins as in f with normal hamster serum. Proteins extracted from a spontaneously differentiated embryonal carcinoma line (PCC3) infected with SV40 and immunoprecipitated with anti-SV40 T serum (lane h) and normal hamster serum (lane i).

to compare viral transcriptional initiation in undifferentiated and differentiated F-9 cells by assaying the incorporation of radiolabeled precursor into nascent RNA chains of VTCs by using the Sarkosyl extraction method of Gariglio and Mousset (22). This technique has the advantages of isolating viral (as opposed to cellular) transcription complexes in a supernatant fraction and eliminating the potential enzymes that might degrade unstable RNA species. Three parallel cultures, the continuous mouse line 3T3, undifferentiated F-9 cells, and retinoic acid-treated (96 hr) F-9 cells were infected with wild-type SV40 (200 PFU per cell). At 10 hr after infection VTCs were isolated from the same number of cells in each infected culture and incubated in a reaction mixture (23) containing [α-^{32}P]ATP. Radiolabeled RNA was purified from the VTCs of the three cultures and quantitated by exhaustive hybridization to SV40 DNA on filters (13). The 3T3 cultures generally contained 4 to 6 times as much virus-specific RNA as did the undifferentiated F-9 cultures. VTCs from differentiated F-9 cells, however, generated the same or no more than twice the amount of SV40-specific RNA as did those from the undifferentiated F-9 cells. For example, the fractions of virus-specific RNAs in a representative experiment were: undifferentiated F-9 cells, 0.07%; differentiated F-9 cells, 0.14%; 3T3 cells, 0.40%. If the VTCs reflect relative transcriptional activity (23, 24), these data suggest that expression of early SV40 RNA in differentiated F-9 cells is not related solely to a stimulation of viral transcription.

The activity of VTCs from 3T3 cells and undifferentiated and differentiated F-9 cells is reflected in the intensity of the bands from the blotting experiments shown in Fig. 5. In this analysis, equivalent amounts of RNA purified from VTCs were hybridized with the separated strands of SV40 *Bam*HI/*Hpa* II fragments on blots. This hybridization experiment, furthermore, confirmed earlier results indicating that SV40 transcriptional activity in nonpermissive mouse cells can occur on both early and late template strands (25; unpublished data).

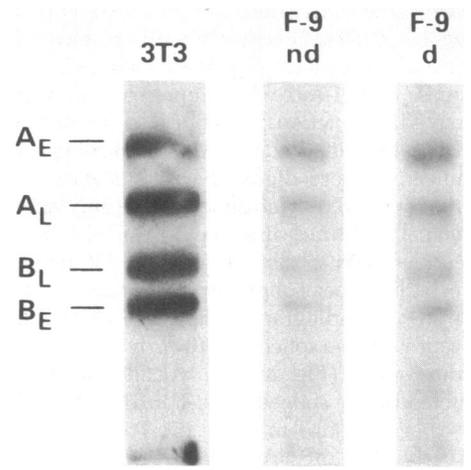

FIG. 5. Comparative analysis of *in vitro* extended viral RNA from SV40-infected 3T3 cells and undifferentiated or differentiated F-9 cells. Viral transcriptional complexes were obtained from the same number of SV40-infected 3T3 cells and undifferentiated or differentiated F-9 cells. Ten hours after infection RNA chains were elongated *in vitro* with [α-^{32}P]ATP (23). RNA was isolated as described (9) and equivalent portions from the three preparations were hybridized to the separated strands of *Bam*HI/*Hpa* II-cleaved SV40 DNA immobilized on nitrocellulose blots. A_E, SV40 early coding strand; B_E, early noncoding strand; B_L, late coding strand; A_L, late noncoding strand; nd, nondifferentiated; d, differentiated.

DISCUSSION

Under ordinary tissue culture conditions, the F-9 cell line grows primarily as a single cell type characteristic of embryonal carcinomas, with a low background of spontaneously differentiating cells (26). Cultures maintained in low concentration of retinoic acid, however, become differentiated as judged by morphologic, antigenic, and biochemical criteria (6, 7). Thus, the F-9 cell line provides an ideal system for studying the correlation between differentiation and early SV40 gene expression.

Using a variety of techniques, others have demonstrated that the block(s) in expression of the early papovavirus gene products in undifferentiated murine teratocarcinoma cells is not at the level of absorption, penetration, uncoating, or migration of the viral DNA to the cell nucleus (27).

Furthermore, we have shown that active transcription of SV40 DNA in F-9 stem cells gives rise to an unspliced early primary transcript (9). These results suggest that a block in expression of SV40 genes in F-9 cells occurs at a posttranscriptional stage and probably involves an inhibition of maturation of RNA transcripts into stable early mRNA molecules. The reasons for the lack of mRNA maturation in F-9 cells are still obscure. Among the possibilities we have considered is a deficiency in the splicing enzyme complex, which may result from an immaturity in the host cell. This, in turn, would presume that unspliced nuclear transcripts are unstable. A precedent exists for the instability of such RNA molecules, because SV40 deletion mutants that are unable to generate spliced transcripts do not produce stable RNAs (28–31). Thus, splicing may be a requirement for the stabilization of certain mRNAs. There are, of course, other possibilities for the absence of mature SV40 transcripts in infected undifferentiated F-9 cells, and these are not mutually exclusive with the above hypothesis. For example, (*i*) there may be a defect in an additional step of SV40 RNA maturation; (*ii*) there may exist high concentrations of RNase in undifferentiated cells; (*iii*) untranslated RNAs may be unstable, and differentiation may be required for factors involved in translation of certain messages; (*iv*) the primary

defect in undifferentiated F-9 cells could result from an inefficient rate of initiation of viral transcription that is, in turn, reflected in deficient processing. Because the results with VTCs suggested that SV40 transcription was almost as active in undifferentiated and differentiated F-9 cells, we consider this last explanation less likely. Nevertheless, a comparative analysis of the amount of pulse-labeled SV40 RNA in undifferentiated and differentiated F-9 cells may provide further information on this point.

This study demonstrates that undifferentiated F-9 cells contain the genetic information needed for transcription, processing, and stabilization of SV40 RNAs and subsequent synthesis of the early viral proteins. However, stable SV40 mRNA can be generated in these cells after a treatment with retinoic acid, which has been shown to induce cellular differentiation (6, 7). In future experiments it will be important to determine if the inability of undifferentiated F-9 cells to express SV40 early genes is specific to this virus or if it applies to other eukaryotic genes that require splicing for the generation of a mature mRNA.

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