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Polyoma Virus Infection of Retinoic Acid-Induced Differentiated Teratocarcinoma Cells

FRANK K. FUJIMURA,[1] PAMELA E. SILBERT,[1] WALTER ECKHART,[2] AND ELWOOD LINNEY[1]*

La Jolla Cancer Research Foundation, La Jolla, California 92037,[1] and Tumor Virology Laboratory, The Salk Institute, San Diego, California 92112[2]

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The mouse teratocarcinoma stem cell line, F9, becomes permissive for productive polyoma infection upon treatment with retinoic acid. Through the use of M13-polyoma recombinant single-stranded DNA probes, spliced and unspliced early viral RNA were detected after polyoma infection of retinoic acid-treated and untreated F9 cultures.

Because of their potential to differentiate into a wide variety of somatic cells, mouse embryonal carcinoma (EC) cells, the malignant stem cells of teratocarcinomas, have elicited considerable interest as a means of investigating mammalian cell differentiation and early embryonic events (11, 20, 25, 33). The first differentiated cell derived from EC cells has the properties of parietal endoderm that arises in very early mouse embryos. These differentiated cells differ morphologically, biochemically, and antigenically from the stem cells. The cellular changes accompanying differentiation of EC cells also is demonstrated by the response of these cells to infection by certain viruses. Undifferentiated EC cells are resistant to infection by simian virus 40 (SV40; 37, 39), polyoma virus (Py; 37), minute virus of mouse (22), and C-type RNA tumor viruses (24, 38). Differentiation of EC cells can lead to the appearance of cell types that are sensitive to these viruses. In the cases of the DNA tumor viruses SV40 and Py, there is no expression of tumor (T) antigens in infected EC cells, although virus adsorption, uncoating, and transport to the cell nucleus apparently are unimpaired (36).

F9 is an EC cell line that undergoes very little differentiation either in vivo or in vitro (4). However, Strickland and Mahdavi (34) found that F9 cells can be induced to differentiate in vitro by low concentrations of retinoic acid. By multiple criteria, some of the differentiated cells initially appearing in cultures of F9 treated with retinoic acid resemble parietal endoderm. In this report, we show that cells sensitive to infection by Py arise in cultures of F9 cells induced to differentiate by retinoic acid.

The kinetics of appearance of Py-sensitive cells in an F9 culture induced with 1 μM retinoic acid are shown in Fig. 1. Sensitivity is measured by immunofluorescence for Py T antigen 24 h after infection and is quantified as the number of colonies containing at least one T antigen-positive cell. F9 cultures required 2 days in retinoic acid before Py-sensitive cells were detectable. After 3 days in retinoic acid, about 90% of the colonies contained Py-sensitive cells. Although the fraction of colonies having Py-sensitive cells remained at 90% between days 3 and 5, the fraction of sensitive cells per colony tended to increase with increasing time in retinoic acid. At any particular time point, the actual number of sensitive cells per colony varied considerably. After 5 days in retinoic acid, over 50% of the cells, by our estimate, were sensitive to Py, but some colonies had very few or no sensitive cells, whereas other colonies had a large proportion of sensitive cells. We did not detect any Py-sensitive cells in uninduced F9 cultures, nor did we see any T antigen immunofluorescence in mock-infected control cultures.

For comparison, the kinetics of appearance of plasminogen activator activity in F9 cell cultures are shown in the insert of Fig. 1. Plasminogen activator is a biochemical marker for parietal endoderm and is induced during early stages of EC cell differentiation (19, 29, 35, 39). Others (23, 34) have shown that plasminogen activator is induced in F9 cells by retinoic acid. The kinetics of plasminogen activator induction in retinoic acid-induced F9 cells closely parallel the appearance of Py sensitivity (Fig. 1). The slight difference in the shapes of the two induction curves may be due to a difference in the sensitivities of the two assays. Immunofluorescence can detect a single T antigen-positive cell within a colony; the plasminogen activator assay, which measures proteolysis of casein by plasmin produced by the action of plasminogen activator on plasminogen, probably is not sensitive enough to detect a single positive cell within a colony. The increase in plasminogen activator-positive colonies between days 3 and 5 in retinoic acid

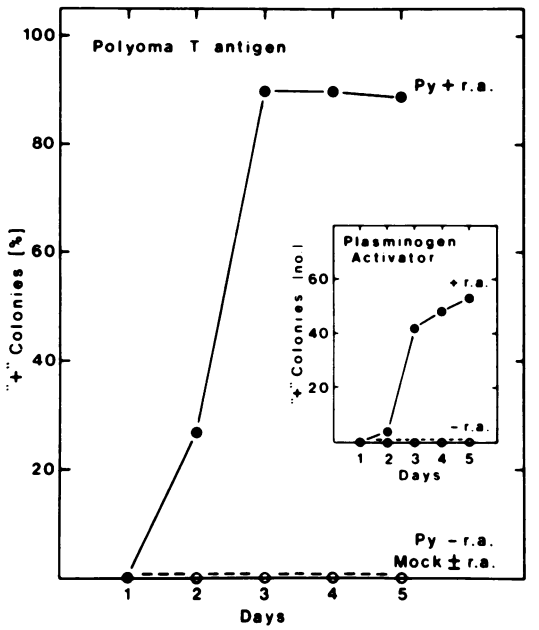

FIG. 1. *T antigen immunofluorescence of Py-infected F9 cells. F9 cells were plated onto gelatinized glass cover slips in 60-mm dishes at clonal densities in Dulbecco-modified Eagle medium (4.5 mg of glucose per ml; Flow Laboratories, Inglewood, Calif.) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and antibiotics (50 U of penicillin and 50 μg of streptomycin per ml). Four hours after plating, retinoic acid (Sigma Chemical Co., St. Louis, Mo.) was added to appropriate cultures at a final concentration of 1 μM. Stock solutions of retinoic acid were prepared at 1 mM in 95% ethanol and stored over a period of 6 weeks at −20°C. Dilutions of the stock solution were made in culture medium before use. Control cultures lacking retinoic acid contained 0.95% ethanol. At daily intervals for 5 days after addition of retinoic acid, separate cultures were infected (or mock infected) with Py at multiplicities between 50 and 200. At 24 h after infection, cells were fixed and stained for Py T antigen. Cells on the cover slips were washed in phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ (0.01% each) and then fixed in acetone-methanol (3:1). Fixed cells were stained for Py T antigen by the indirect method, using rat anti-Py tumor serum and fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.). Cover slips were washed between and after staining in steps in phosphate-buffered saline containing 1 mg of bovine serum albumin per ml. Washed cover slips were mounted and examined on a Leitz fluorescent microscope. Individual colonies were scored for the presence (+) or absence of T antigen-fluorescent cells. At least 100 colonies were scored for each time point. The insert shows the kinetics of appearance of plasminogen activator activity in F9 cultures. One hundred F9 cells were plated per 60-mm dish and either treated or untreated with retinoic acid as described above. At daily intervals, plasminogen activator was measured by the casein-agarose method (10). The number of plasminogen activator-positive (+) colonies per plate was scored for duplicate plates at each time point. The efficiency of plating of F9 cells was about 60%.*

may reflect an increase in the number of cells producing plasminogen activator up to a threshold number needed for detection.

Although the appearance of Py-sensitive cells seems to parallel the kinetics of appearance of plasminogen activator, we do not know if these two phenomena are related to a single event induced in F9 cells by retinoic acid. We have performed double assays for plasminogen activator and for T antigen immunofluorescence on single cells of Py-infected, retinoic acid-induced F9 cells. Our results indicate that some of the cells are positive for both assays, but we have not been able to determine whether or not the induction by retinoic acid of plasminogen activator and of T antigen expression occur simultaneously in a single cell. Retinoic acid-treated F9 cultures contain cells of differing morphologies, so it is possible that several different cell types arise in retinoic acid-induced cultures.

Synthesis of T antigen occurs during early Py gene expression. In permissive cells, early gene expression precedes and is a prerequisite for Py DNA replication. Late gene expression, with synthesis of viral structural proteins, follows viral DNA replication. Differentiated cells derived from F9 by retinoic acid are permissive for Py infection. This is shown by the fact that complete virus particles, measured by immunofluorescence with antivirion serum, by hemagglutination, and by plaque assay, were produced in retinoic acid-treated F9 cells after infection with Py (data not shown). The yield of virus from retinoic acid-treated F9 cells was comparable to that from permissive 3T6 cells.

To confirm the presence of T antigen in Py-infected, retinoic acid-treated F9 cells, we analyzed polyacrylamide gels of proteins immunoprecipitated by anti-Py T serum. Infection of permissive cells by Py leads to the synthesis of at least three tumor antigens, having apparent molecular weights of 90,000 to 100,000 (90 to 100K), 50 to 60K, and 22K, referred to as large, medium, and small T antigens, respectively (14, 15, 26). Immunoprecipitates of Py-infected, retinoic acid-induced F9 cells contained proteins having electrophoretic mobilities identical to the large, medium, and small T antigens from Py-infected 3T6 mouse fibroblast cells (Fig. 2a and c). The large, medium, and small T antigens are not present in immunoprecipitates of mock-infected, retinoic acid-induced F9 cells (Fig. 2b).

No T antigen proteins could be detected in

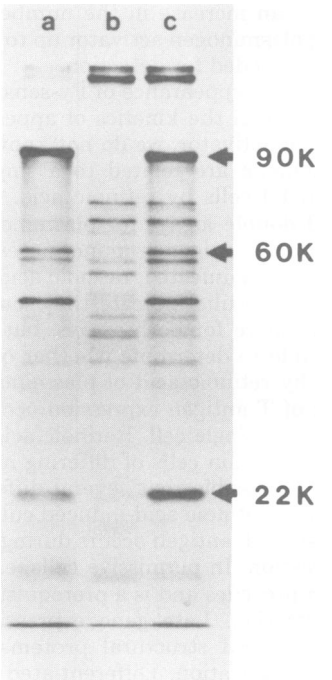

FIG. 2. *Electrophoretic analysis of immunoprecipitated Py T antigen proteins. F9 cells were plated at a density of 2 × 10⁴ cells per 60-mm dish and treated with 1 μM retinoic acid as described for Fig. 1. At 3 days after the addition of retinoic acid, cells were infected with Py at a multiplicity of 200 and labeled with [³⁵S]methionine between 43.5 and 45.5 h postinfection. Details of infection, radiolabeling, immunoprecipitation, and gel electrophoresis are described by Hutchinson et al. (14). Electropherograms are of ³⁵S-labeled proteins immunoprecipitated by anti-Py T serum from: Py-infected 3T6 cells (control) (a); mock-infected, retinoic acid-treated F9 cells (b); and Py-infected, retinoic acid-treated F9 cells (c). The arrows indicate the positions of the 90K-, 60K-, and 22K-dalton species of Py T antigen.*

immunoprecipitates of uninduced F9 cells (Fig. 3). Immunoprecipitates of uninduced F9 cells do contain material having an electrophoretic mobility very similar to that of large T antigen (Fig. 3a and h). To show that this background band does not obscure a low level of T antigen present in undifferentiated cells, F9 cultures were infected with a deletion mutant of polyoma, d18 (12), that synthesizes modified medium and large T antigens. The large T antigen specified by this mutant has an apparent molecular weight 5,000 lower than that of the wild-type (16) and has an electrophoretic mobility different from those of the background bands. As shown in Fig. 3b, large T antigen, although present in immunoprecipitates of retinoic acid-induced F9 cells infected with d18 (Fig. 3g), could not be detected in uninduced cells infected with d18.

Recently, evidence suggesting a reason for the lack of viral T antigen expression in EC cells has been published. Like some other eucaryotic genes, expression of Py and SV40 T antigens requires posttranscriptional splicing of RNA to remove noncoding intervening sequences (1, 3, 6, 9, 17, 18, 30). In EC cells infected with SV40, early viral RNA is present but is not spliced (28). Differentiated mouse cells (28) or retinoic acid-induced F9 cells (27), when infected with SV40, contain spliced early RNA.

To examine this possibility for Py, the *Pst*I-1 and *Pst*I-2 fragments of Py DNA were cloned, using the single-stranded DNA bacteriophage vectors M13 mp 73 (21) and fd 101 (13), respectively. The *Pst*I-1 fragment lies in the early region of the Py genome including part of the intervening sequences for all three T antigen mRNA's. The sequence of Py DNA (7, 9, 30, 31) in this area suggests that the three spliced early RNAs would hybridize with a predicted 1,554- to 1,568-base region of the *Pst*I-1 fragment, whereas unspliced early RNA would hybridize with a predicted 1,876-base region of the *Pst*I-1 fragment. Figure 4 illustrates four of these recombinants and shows how recombinants with complementary-strand inserts are identified.

These recombinants were used to examine the RNA from Py-infected F9 cells by using the Berk and Sharp procedure (2). Polyadenylic acid (+) total RNA was hybridized to the EL-M2-8 recombinant DNA and then treated with S1 nuclease. The protected DNA was sized on an alkaline agarose gel, blotted onto nitrocellulose, and identified by hybridizing the blot against ^{125}I-labeled EL-M2-14 DNA. Figure 5 shows the results of this analysis compared with cytoplasmic and nuclear polyadenylic acid (+) RNAs from Py-infected 3T6 cells. Both the F9 cells and the retinoic acid-induced F9 cells have a major spliced component and a minor unspliced component (note that the RNA concentrations and autoradiographic exposure times are greater for the uninduced RNA, indicating a lower concentration of Py RNA in the uninduced F9 cells). In the control RNAs from 3T6 cells, only the nuclear component has a minor unspliced band.

To summarize, Py infection of F9 cells induced to differentiate by the addition of retinoic acid led to synthesis of all three Py T antigens, and the kinetics of appearance of T antigen-positive cells appeared to follow the kinetics of appearance of differentiated cells secreting plasminogen activator. Both uninduced and retinoic acid-induced F9 cultures when infected with Py had more spliced than unspliced early viral RNA.

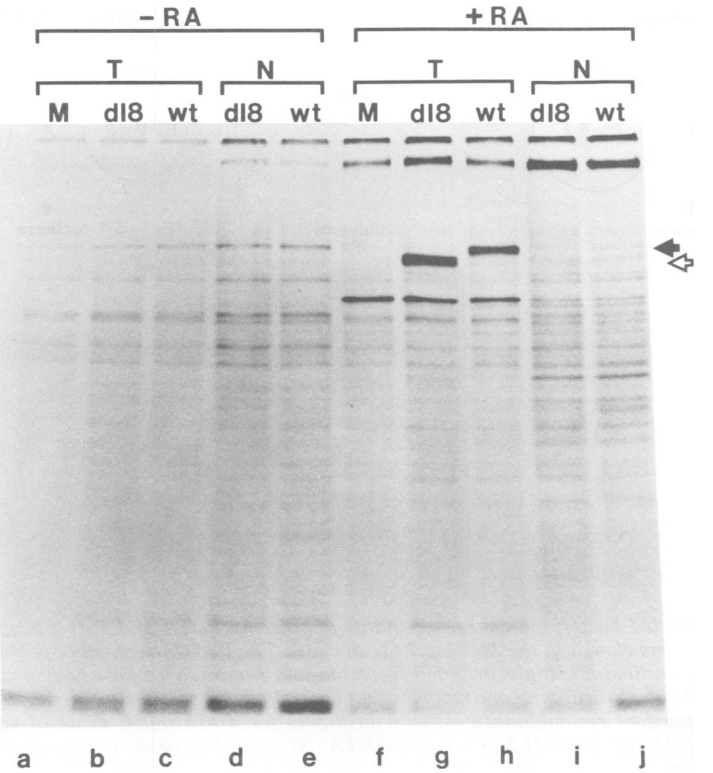

FIG. 3. *Electrophoretic analysis of immunoprecipitates. Experimental details were as described in the legend for Fig. 2. F9 cells either untreated (−RA) or treated for 3 days with 1 μM retinoic acid (+RA) were mock infected (M) or infected with wild-type Py (wt) or with the Py deletion mutant, d18 (12). Electropherograms are of [^{35}S]methionine-labeled proteins precipitated with anti-Py T serum (T) or with normal rat serum (N). The solid arrow indicates the position of wild-type large T antigen; the open arrow indicates the position of d18 large T antigen.*

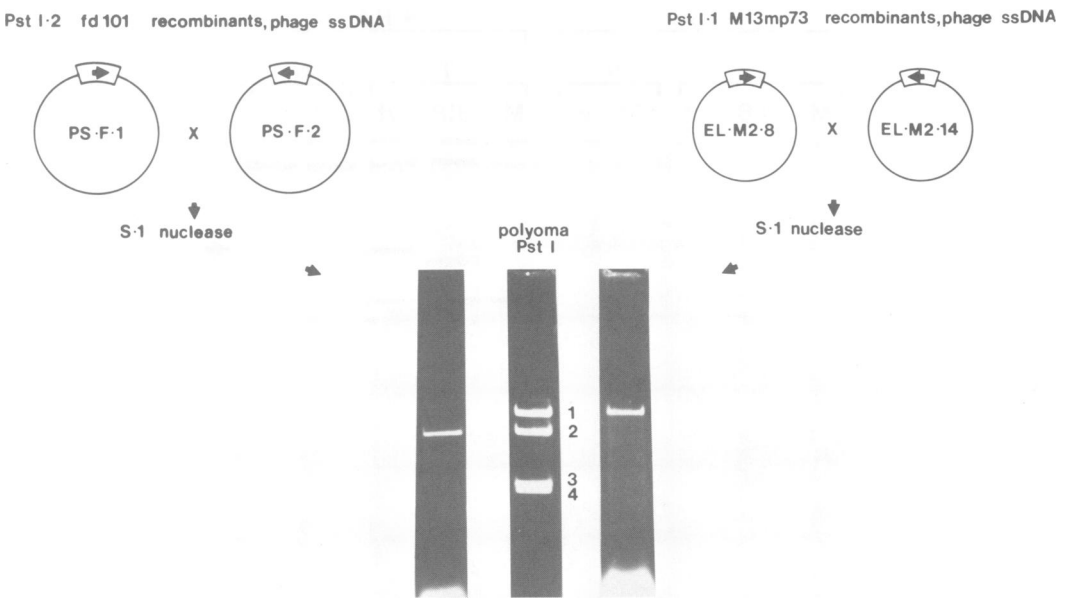

FIG. 4. *Single-stranded bacteriophage recombinants of polyoma PstI-1 and PstI-2 fragments. The PstI-1 fragment of polyoma was cloned in the M13 mp 73 vector (21). The PstI-2 fragment of polyoma was cloned in the fd 101 vector (13). Individual single-stranded recombinants were assayed for which strand of the Py DNA fragments was inserted by hybridizing one recombinant bacteriophage single-stranded DNA with DNAs from other twice-plaque-purified recombinants. After hybridizing for 4 h at 60°C (5 μg of each DNA, 50 mM Tris-hydrochloride [pH 8.0]–200 mM NaCl–1 mM EDTA; 20 μl total volume); the samples were treated with S1 nuclease for 30 min at 45°C (S1 buffer [8]; 500 U in 0.3 ml added to sample). The samples were then run on a 1.4% agarose horizontal slab gel along with PstI-digested Py supercoiled DNA as the marker. The S1-resistant product of the fd 101 recombinants (PS F-1 × PS F-2; left lane) runs with the Py PstI-2 fragment. The S1-resistant product of the M13 mp 73 recombinants (EL-M2-8 × EL-M2-14; right lane) runs with the Py PstI-1 fragment. Restriction enzyme analysis of the replicative form DNAs of these recombinants and hybridization with electrophoretically separated strands of Py DNA indicate that EL-M2-14 and PS-F-1 carry the strand of Py DNA having the same sense as early polyoma RNA.*

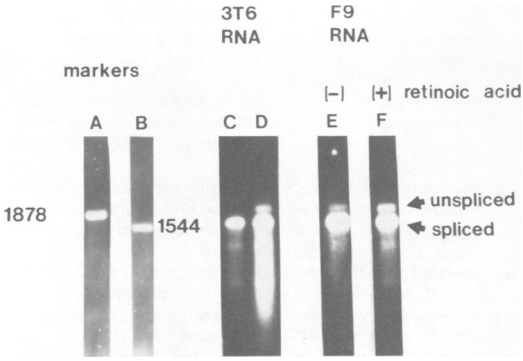

FIG. 5. *Analysis of RNA from polyoma-infected F9 embryonal carcinoma cells. Polyadenylic acid (+) RNAs from Py-infected 3T6 and F9 cells were analyzed by hybridizing the RNAs to EL-M2-8 single-stranded DNA (ssDNA) for 16 h (100 ng of EL-M2-8 DNA–40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] [pH 6.4]–400 mM NaCl–1 mM EDTA–80% recrystallized formamide; 20 μl final volume; samples heated at 85°C for 15 min and then hybridized at 52°C for 16 h). After hybridization, the samples were S1 nuclease digested as described in the legend to Fig. 3. The samples were electrophoresed on a horizontal alkaline agarose gel (1.5% agarose–50 mM NaOH–1 mM EDTA). The DNA was blotted onto nitrocellulose by a modification (8) of the Southern procedure (32). The nitrocellulose was processed and hybridized against ^{125}I-labeled EL-M2-14 DNA ($\sim 3 \times 10^7$ cpm/μg) and washed according to Favaloro et al. (32). Lanes A and B, ethidium bromide fluorescence of M13 and fd recombinants processed as in Fig. 3 and used as markers of 1,878 and 1,544 bases in the alkaline gel. Lanes C and D, autoradiographs of hybridization blots (2-day exposures) of RNAs processed by the Berk and Sharp procedure (2): cytoplasmic (lane C; 0.28 μg) and nuclear (lane D; 0.72 μg) polyadenylic acid (+) RNA from Py tsA25E-infected 3T6 (infected to maximize quantity of early RNA according to Favaloro et al. 32). The tsA25E mutant overproduces early RNA (5), and this RNA was used as control RNA. Lanes E and F: autoradiographs of hybridizations of total polyadenylic acid (+) RNA from Py-infected F9 cells. Lane E, F9 cells infected at a multiplicity of infection of 50 for 18 h (75 μg of RNA; 5-day autoradiographic exposure). Lane F, F9 cells grown in the presence of 10^{-6} M retinoic acid for 3 days, then infected at an multiplicity of infection of 50 for 18 h (25 μg of RNA; 2-day autoradiographic exposure). Py DNA sequence data would predict that 1,554 to 1,568 bases of the PstI-1 fragment would be protected from S1 nuclease by spliced early RNA, whereas 1,878 bases would be protected by unspliced RNA.*

Although spliced early RNA could be detected in Py-infected F9 cells, we were unable to detect Py early proteins (T antigens) in undifferentiated F9 cells. Some of the possible explanations concerning this apparent paradox include: (i) Py early RNA is not spliced correctly in undifferentiated F9 cells; (ii) the spliced RNA, for some unknown reason, is not translated in the undifferentiated cell; (iii) our techniques for detecting RNA are more sensitive than those for detecting T antigens, and there may be a low level of T antigens present either uniformly in all of the undifferentiated cells or in a very small subpopulation of differentiated cells in uninduced F9 cultures. We are examining these possibilities.

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