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Polyoma mutants that productively infect F9 embryonal carcinoma cells do not rescue wild-type polyoma in F9 cells

(mouse teratocarcinoma/mixed infection/*cis* action/DNA replication)

FRANK K. FUJIMURA AND ELWOOD LINNEY

Cancer Research Center, La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT **Mouse embryonal carcinoma cells are refractory to infection by wild-type polyoma virus, the infection process apparently being blocked at a stage after adsorption and penetration but before early protein synthesis. Polyoma virus mutants capable of productive infection of mouse embryonal carcinoma cells have been isolated and these mutants all have DNA sequence alterations in a noncoding region near the origin of replication of the viral genome. PyF101 and PyF441 are two mutants selected for their ability to infect the embryonal carcinoma cell line F9. Here we show that these PyF mutants do not rescue replication of wild-type polyoma during a mixed infection of F9 cells. The mutant and wild-type DNAs were distinguished on the basis of restriction fragments obtained by digestion with *Msp* I or *Bst*NI, and no wild-type DNA was detected in F9 cells coinfected with wild-type polyoma and with either PyF101 or PyF441. The mutant viruses do not appear to inhibit wild-type replication during a mixed infection because both mutant and wild-type DNAs can replicate efficiently in coinfected 3T6 cells which are permissive for both mutant and wild-type viruses. A double mutant having the PyF101 mutation and the ts-25E temperature-sensitive mutation in polyoma large tumor antigen was constructed and found to be temperature-sensitive for replication in F9 cells. This double mutant, designated PyFts-1, can be rescued in F9 cells at the restrictive temperature by coinfection with PyF441. These results suggest that the PyF mutations affect two processes in F9 cells, one involving expression of polyoma early genes and a second involving viral DNA replication.**

Infection of cells in culture by the papovaviruses simian virus 40 (SV40) and polyoma leads to very different responses depending upon the host. Infection of permissive mouse cells by polyoma virus results in a lytic infection whereas infection of nonpermissive rat cells leads initially to abortive infection with expression of viral early proteins but no viral DNA synthesis and, finally, to viral transformation of a small fraction of the infected cells. In some cases, cell differentiation affects the cellular response to papovavirus infection. The mouse teratocarcinoma stem cell, called the embryonal carcinoma (EC) cell, is refractory to infection by polyoma or SV40 (1). No viral early proteins can be detected in infected EC cells. Differentiation of EC cells can lead to cells that are sensitive to polyoma and SV40 (1–3). Upon infection of these differentiated cells, papovavirus early gene expression occurs with synthesis of viral tumor (T) antigens and, in the case of polyoma, productive infection can take place.

The block to infection of EC cells by SV40 and polyoma is not at the level of virus adsorption, penetration, or transport to the nucleus (4). In the case of polyoma-infected EC cells, viral gene expression is obtained by fusing the infected EC cells with permissive mouse cells (5). Only a low amount of viral early RNA can be detected in SV40- or polyoma-infected EC cells. The viral early RNA present in SV40-infected F9 EC cells has been reported to be unspliced (6), whereas that present in polyoma-infected F9 cells has been reported to be predominantly spliced (7). Viral T antigens have never been detected in SV40- or polyoma-infected EC cells. Thus, it appears that EC cells are defective for papovavirus development at a stage preceding early protein synthesis, possibly at the level of initiation of early transcription or of early RNA splicing.

Recently, we (8) and others (9–12) have isolated mutants of polyoma virus capable of productive infection of EC cells. All of these mutants have DNA sequence alterations within a noncoding region of the polyoma genome near and to the late side of the postulated origin of DNA replication (13, 14). The DNA sequence alterations range from a point mutation (8) to extensive rearrangements with deletions, duplications, and translocations (10). The polyoma mutants PyF441 and PyF101 were selected by their ability to infect F9 cells (8). PyF441 is a point mutant having an A·T-to-G·C transition at 69.6 map units. In addition to this point mutation, the PyF101 mutant DNA contains a tandem duplication of 54 base pairs (bp) of sequences encompassing the point mutation with both copies of the duplication having the point mutation. We now report that these PyF mutants cannot rescue wild-type polyoma virus in F9 cells coinfected with mutant and wild-type viruses. This *cis*-acting effect appears to be at the level of viral DNA replication and is not due to the inability of polyoma large T antigen to complement in *trans* in F9 cells.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Details for cell culture and virus infection of the mouse EC cell line F9 (15) and of mouse 3T6 cells have been described (8). The wild-type polyoma virus used in these studies is the large-plaque strain 3 whose DNA sequence has been determined (16). The polyoma mutants PyF101 and PyF441 that productively infect F9 cells were described in a previous paper (8). All virus stocks were prepared by low-multiplicity infection of 3T6 cells.

Isolation of Viral DNA. Low molecular weight DNA was isolated from infected cultures by the procedure of Hirt (17). The Hirt supernatant was treated with proteinase K followed by RNase A as described by Favaloro *et al.* (18). Where specified, supercoiled polyoma DNA was purified by equilibrium centrifugation in CsCl with ethidium bromide (19).

Restriction Endonucleases and Gel Electrophoresis. All restriction endonucleases were purchased from New England BioLabs and used under conditions specified by the supplier. The one exception was *Bst*NI, which was used at 30°C rather

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Abbreviations: SV40, simian virus 40; EC, embryonal carcinoma; T antigen, tumor antigen; bp, base pairs(s); moi, multiplicity of infection; pfu, plaque-forming units.

than at 60°C. The lower temperature was required to obtain complete digestion at the two adjacent *Bst*NI (*Eco*RII) sites at 69.6 map units of PyF mutant DNAs. Electrophoresis of DNA fragments was performed in 1.4% agarose or 7.5% acrylamide (acrylamide/bisacrylamide, 20/1) gels with Tris acetate buffer (20) as described (8). Preparative purification of DNA fragments from agarose gels by using KI and hydroxyapatite was as described (8). Fluorography of gels containing ^{3}H-labeled DNA was performed by impregnating the gels with EN^{3}HANCE (New England Nuclear) and then exposing the dried gel against preflashed Kodak X-Omat AR film at −70°C.

Blot Hybridization. DNA fragments in agarose gels were blotted onto nitrocellulose filters by the Southern procedure (21). Blots were hybridized to ^{125}I-labeled wild-type polyoma DNA (≈2 × 10^6 cpm/μg) as described (7). Washed blots were exposed at −70°C against preflashed Kodak X-Omat AR film with a Du Pont Cronex Hi-Plus intensification screen.

[^{3}H]Thymidine Labeling of Infected Cells. F9 cells, plated 24 hr previously at 5 × 10^5 cells per 10-cm dish, were infected with the viruses specified and cultured at 37°C in Dulbecco's modified Eagle's medium containing antibiotics (50 units of penicillin and 50 μg of streptomycin per ml) and 5% horse serum. The total multiplicity of infection (moi) was 50 plaque-forming units (pfu) per cell; for mixed infections, the moi for each virus was 25 pfu per cell. Thirty-nine hours after infection, each culture was incubated with 5 ml of medium containing 2.5% horse serum and 50 μCi of [^{3}H]thymidine (ICN; 73.5 Ci/mmol; 1Ci = 3.7 × 10^{10} becquerels). Supercoiled DNA was purified as described above from the infected culture at 45 hr after infection. Labeling of infected 3T6 cells was identical except that cells were initially plated at 1 × 10^6 cells per 10-cm dish, calf serum was used in place of horse serum, and the total moi was 25 pfu per cell.

Construction of PyFts-1 Double Mutant. A seed stock of the polyoma tsA mutant ts-25E (22) was obtained from W. Eckhart. The mutant was plaque-purified and propagated on 3T6 cells at the permissive temperature of 32°C. The purified form I DNAs of PyF101 and ts-25E (22) were digested with *Bgl*I followed by *Bcl* I, and the DNA fragments were purified by agarose gel electrophoresis. This combination of enzymes cleaves polyoma DNA into two fragments, the smaller fragment containing the PyF mutations (8) and the larger fragment covering the rest of the polyoma genome including the ts-25E mutation (23–24). The small *Bgl* I/*Bcl* I fragment of PyF101 DNA was ligated to the large *Bgl* I/*Bcl* I fragment of ts-25E DNA and the resulting ligated DNA was used to infect F9 cells at 32°C by the DEAE-dextran procedure (25) as described (8). The virus arising from the DNA infection was plaque-purified on 3T6 cells. The plaque-purified virus, designated PyFts-1, contained the 54 bp increase in the *Hpa* II DNA fragment 3 characteristic of PyF101 DNA and was temperature-sensitive for replication in F9 cells. Mutant stocks were propagated by low moi passage in 3T6 cells. For Fts-1 stocks used in these experiments, the ratio of pfu on 3T6 cells at the restrictive temperature (39°C) relative to the permissive temperature (32°) was less than 10^{-5}.

RESULTS

Analysis of Mutant and Wild-Type Polyoma DNA Replication During Mixed Infection of F9 Cells. The polyoma mutant PyF101 has a tandem duplication of 54 bp located within *Hpa* II DNA fragment 3 (8), making this DNA fragment distinguishable by electrophoresis from the corresponding wild-type fragment. F9 EC cells were infected with wild-type polyoma or with PyF101. Low molecular weight DNAs were isolated from the infected cells, digested with *Msp* I (*Hpa* II), and visualized by ethidium bromide staining after electrophoresis in a 1.4% agarose gel (Fig. 1). No viral DNA fragments were detectable in DNA from wild-type-infected F9 cells (Fig. 1, lane e); viral DNA fragments were clearly detectable in DNA from F9 cells infected with PyF101 (Fig. 1, lane a). *Msp* I digests of DNA from F9 cells coinfected with wild-type and PyF101 indicated the presence of the *Hpa* II fragment 3 corresponding to only the mutant DNA (Fig. 1, lanes b–d). Varying the multiplicity of infection of the coinfecting viruses in F9 cells did not lead to detection of the wild-type *Hpa* II fragment 3. In the experiment shown in Fig. 1, the wild-type multiplicity was varied from 25 to 100 pfu per cell; the PyF101 multiplicity was 50 pfu per cell. In other mixed infection experiments (data not shown), multiplicities between 10 and 500 pfu per cell were examined and, in all cases, no wild-type *Hpa* II fragment 3 was seen. In contrast, mixed infection of mouse 3T6 cells, which are permissive for both wild-type and PyF101, led to the efficient recovery of

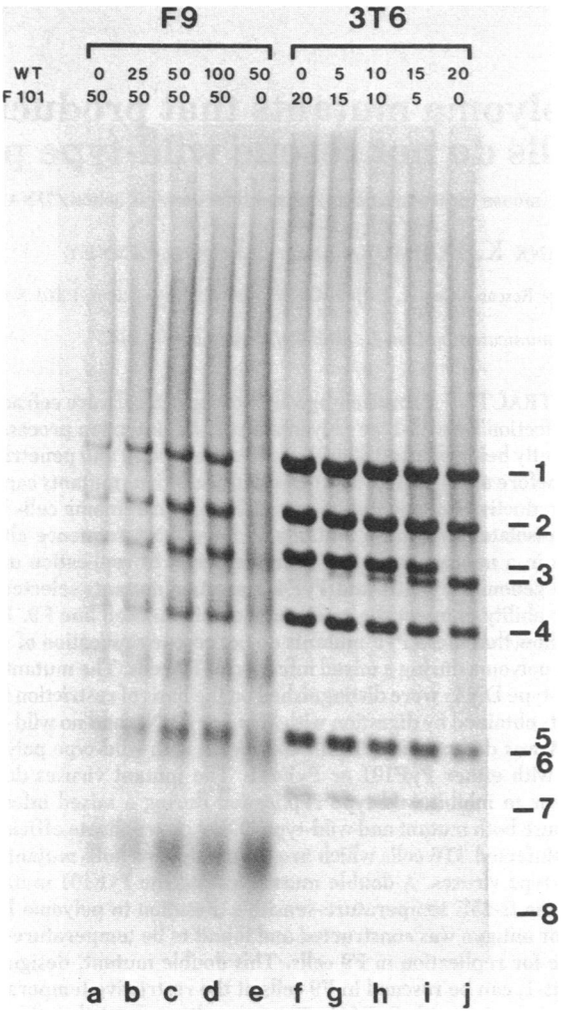

FIG. 1. *Hpa* II restriction fragments of low molecular weight DNAs from wild-type and mutant polyoma-infected cells. F9 and 3T6 cells, plated 24 hr prior to infection at densities of 5 × 10^5 and 2 × 10^6 cells per 10-cm tissue culture plate, respectively, were infected with wild-type or PyF101 mutant at the moi indicated at the top. Forty-eight hours after infection, low molecular weight DNA was isolated by the Hirt procedure (17), and aliquots of the DNA samples corresponding to 33% of each F9 culture and to 17% of each 3T6 culture were digested with *Msp* I and fractionated in a 1.4% agarose gel. The gel was stained with ethidium bromide. A negative print of the gel is shown. The locations of the eight *Msp* I fragments (which are identical to the *Hpa* II fragments) of polyoma DNA are indicated. Wild-type *Hpa* II fragment 3 is 54 bp smaller than the PyF101 *Hpa* II fragment 3; thus the wild-type fragment migrates more rapidly in the gel.

both wild-type and mutant *Hpa* II fragment 3 DNAs, indicating that the presence of PyF101 did not inhibit replication of wild-type virus (Fig. 1, lanes g–i).

Like PyF101, mutant PyF441 has a point mutation at 69.6 map units. Unlike PyF101, PyF441 DNA contains only the point mutation and has no duplication of sequences encompassing the point mutation. Because replication of wild-type DNA was not rescued by coinfection of F9 cells with PyF101, we wanted to determine whether this *cis*-acting property was also shared by the point mutant. In the PyF mutants, the wild-type DNA sequence of 5′ C-C-A-G-G-C-C-T-A-G 3′ is altered to 5′ C-C-A-G-G-C-C-T-G-G- 3′ (8). The mutation creates a new restriction site for the enzyme *Bst*NI (*Eco*RII) which cleaves at the sequence C-C-$\frac{T}{A}$-G-G. The mutation is in *Eco*RII DNA fragment 16 (16) of polyoma DNA, the wild-type fragment being 75 bp and the mutant fragment being 70 bp.

In order to distinguish replicated from input viral DNA, infected F9 cells were labeled from 39 to 45 hr after infection with [^{3}H]thymidine. Supercoiled DNA was isolated, digested with *Bst*NI, fractionated by electrophoresis, and analyzed by fluorography. The results, shown in Fig. 2, indicated that no wild-type DNA replication was detected in F9 cells during mixed infection with PyF441 or PyF101 (lanes d and e). As before, efficient replication of both wild-type and mutant DNAs was observed in coinfected 3T6 cells (lanes j and k).

Rescue of Polyoma Large T Antigen Function in F9 Cells. Francke and Eckhart (26) have shown that polyoma large T antigen is required for initiation of viral DNA replication. The question arising from the present results is why large T antigen synthesized by the PyF mutants did not rescue efficient replication of wild-type polyoma DNA in coinfected F9 cells. To pursue this question, we constructed a double mutant containing the PyF101 mutation and the ts-25E mutation. The ts-25E lesion is in complementation group A of polyoma (22) and consists of a single base change in the coding sequence corresponding to the carboxyterminal region of large T antigen (23, 24). The double mutant, PyFts-1, was temperature sensitive for replication in F9 cells (Fig. 3, lanes o and p), showing that PyF mutant DNA replication in F9 cells requires large T antigen. Mixed infection of F9 cells at the restrictive temperature with PyFts-1 and PyF441 led to replication of both viral DNAs (Fig. 3, lane r), indicating that the functional large T antigen of PyF441 rescued replication of PyFts-1 DNA. Note that in F9 cells the ts-25E parent did not replicate efficiently at either the permissive or the restrictive temperature (Fig. 3, lanes i and j) and that ts-25E replication could not be rescued by coinfection with PyF101 (Fig. 3, lanes k and l).

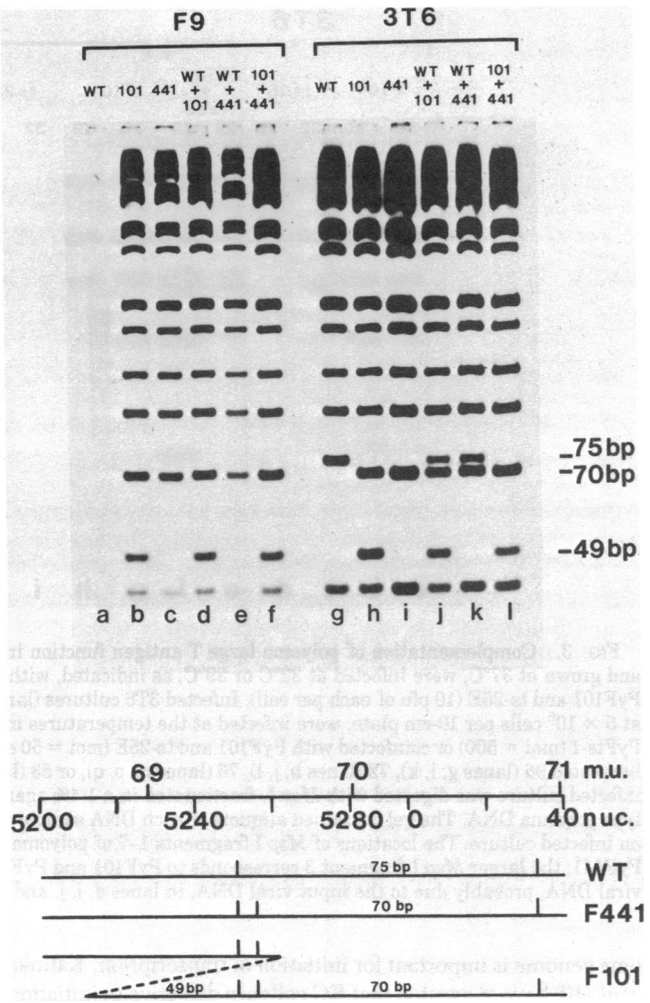

FIG. 2. *Bst*NI fragments of [^{3}H]thymidine-labeled form I DNAs from wild-type and mutant polyoma-infected cells. Form I DNAs, purified by equilibrium centrifugation in CsCl/ethidium bromide, were isolated from cultures infected as shown and labeled with [^{3}H]thymidine from 39 to 45 hr after infection. Aliquots of the form I DNA samples corresponding to 15% of each infected culture were digested at 30°C with *Bst*NI and fractionated in a 7.5% polyacrylamide gel. The radiolabeled DNA fragments in the gel were visualized by autofluorography. The lower part of the figure illustrates the *Bst*NI restriction sites distinguishing wild-type and PyF mutant DNAs. The point mutation in the PyF mutant DNAs creates a new *Bst*NI restriction site at nucleotide (nuc.) position 5258 (8) according to the numbering system of Deininger *et al.* (16). This mutation is located at 69.6 map units (m.u.). The point mutation is located in *Bst*NI fragment 16, the 75-bp wild-type fragment being cleaved by *Bst*NI into a 70-bp fragment and an undetected 5-bp fragment. The tandem duplication in PyF101 DNA leads to an additional 49-bp *Bst*NI fragment.

DISCUSSION

One block to productive infection of EC cells by wild-type polyoma virus occurs after virus adsorption and penetration but before viral early protein synthesis (4, 5). Because the present results indicate that the PyF mutants are unable to rescue wild-type polyoma virus during mixed infection of F9 EC cells, we suggest that synthesis of viral early proteins, by itself, is not sufficient for replication of wild-type polyoma in F9 cells. The PyF101 and PyF441 mutations apparently have two effects in F9 cells: (*i*) permitting synthesis of early viral proteins, particularly large T antigen, and (*ii*) allowing replication of viral DNA in a large T antigen-dependent process. Because PyF441 DNA has only a point mutation which has been shown by marker rescue to be sufficient for productive infection of F9 cells (8), a single base pair transition apparently can affect two viral functions in F9 cells.

The genome of wild-type SV40 has a 72-bp tandem duplication near and to the late side of the origin of DNA replication (27, 28). One copy of the tandem duplication apparently is sufficient for virus viability (29, 30), but deletion of both copies leads to a defect not only in early transcription but also in late transcription and possibly DNA replication (30). Thus, the region of the SV40 genome analogous to that of the polyoma genome containing the PyF mutations may regulate initiation of both transcription and DNA replication.

The location of the PyF mutations is approximately 150 bp upstream from the sites corresponding to the 5′ ends of both early (31) and late (32) polyoma mRNAs. The requirement of specific sequences about 150 bp upstream for initiation of transcription of cellular (33) and viral (30, 34) genes has been reported, supporting the possibility that this region of the poly-

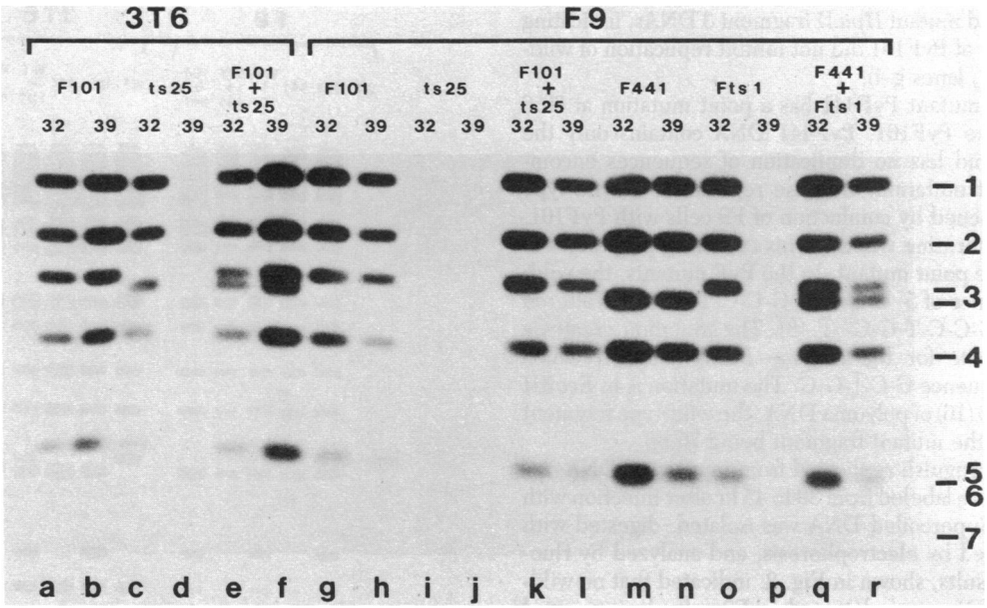

FIG. 3. Complementation of polyoma large T antigen function in F9 cells. 3T6 cells, plated 24 hr previously at 2×10^6 cells per 10-cm plate and grown at 37°C, were infected at 32°C or 39°C, as indicated, with PyF101 (moi = 10 pfu per cell) or ts-25E (10 pfu per cell) or coinfected with PyF101 and ts-25E (10 pfu of each per cell). Infected 3T6 cultures (lanes a–f) were harvested 55 hr after infection. F9 cells, plated 24 hr previously at 5×10^5 cells per 10-cm plate, were infected at the temperatures indicated with PyF101 (moi = 50), ts-25E (moi = 50), PyF441 (moi = 500), or PyFts-1 (moi = 500) or coinfected with PyF101 and ts-25E (moi = 50 each) or with PyF441 and PyFts-1 (moi = 500 each). Infected F9 cultures were harvested 96 (lanes g, i, k), 72 (lanes h, j, l), 76 (lanes m, o, q), or 53 (lanes n, p, r) hr after infection. Low molecular weight DNA isolated from each infected culture was digested with *Msp* I, fractionated in a 1.4% agarose gel, blotted onto nitrocellulose, and hybridized against ^{125}I-labeled wild-type polyoma DNA. The gel contained aliquots of each DNA sample corresponding to either 6% (lanes a–f), 12% (lanes g–l), or 15% (lanes m–r) of an infected culture. The locations of *Msp* I fragments 1–7 of polyoma DNA are indicated. The smaller *Msp* I fragment 3 corresponds to ts-25E and PyF441; the larger *Msp* I fragment 3 corresponds to PyF101 and PyFts-1. The original fluorogram of the blot in this figure showed faint bands of viral DNA, probably due to the input viral DNA, in lanes d, i, j, and p.

oma genome is important for initiation of transcription. Katinka *et al.* (10) have suggested that EC cells are defective in initiation of early transcription on wild-type polyoma DNA and that the DNA sequence changes of polyoma mutants that productively infect EC cells create sites that are functional for initiation of early transcription in EC cells.

The present results showing that the PyF mutants are unable to rescue replication of wild-type polyoma DNA in F9 cells suggest that these EC cells may be defective for replication of wild-type viral DNA even in the presence of functional large T antigen. By showing that the PyFts-1 double mutant can be rescued by PyF441 at the restrictive temperature, we have ruled out the possibility that, for some reason, large T antigen function cannot be complemented in F9 cells. Another trivial explanation is that wild-type DNA is inactivated or degraded in F9 cells. We consider this explanation unlikely because both early and late viral protein synthesis can be rescued in wild-type polyoma-infected EC cells either by fusion with permissive cells (5) or by inducing the infected EC cells to differentiate (ref. 3; unpublished data).

Although suggestive, our results do not prove that F9 cells, even in the presence of functional large T antigen, are defective for replication of wild-type polyoma DNA. Furthermore, it is interesting that polyoma mutants isolated on one EC cell line do not necessarily infect another EC cell line (11, 12), suggesting that different EC cell lines may differ in the mechanisms by which they block wild-type polyoma development. This could mean that wild-type polyoma can be rescued by the appropriate mutant viruses during mixed infection of some other EC cell lines.

Whatever the mechanism(s) that block wild-type polyoma infection of EC cells may be, differentiation can lead to cell types that no longer are refractory to polyoma infection. It would be useful to determine whether these changes in the cellular processes regulating viral gene expression and genome replication during EC cell differentiation are relevant for regulation of cellular gene expression and cellular DNA replication. Further characterization of polyoma mutants that infect EC cells might be helpful in addressing this question.

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