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Genetics

# Isolation and characterization of polyoma virus mutants able to develop in embryonal carcinoma cells

(mouse teratocarcinoma/PCC4 cells/origin of replication/duplication of DNA sequences)

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**ABSTRACT** **Embryonal carcinoma (EC) mouse cells have been shown to be resistant to infection by retroviruses and small oncogenic DNA viruses, including simian virus 40 and polyoma. When allowed to differentiate, *in vitro* or *in vivo*, EC cells become as susceptible to these viruses as differentiated mouse cell lines are. In order to study the relationships between differentiation of EC cells and viral expression, we have isolated and characterized several polyoma mutants that can express early and late functions in undifferentiated EC cells. These mutants, which arose spontaneously during high-multiplicity infection of PCD3 cells (a differentiated fibroblast-like cell line derived from PCC3 EC cells), were selected on PCC4 cells (undifferentiated EC cells) and twice plaque purified. Restriction enzyme analysis of the DNA from several mutants has shown that they all exhibit an additional sequence located in the *Pvu* II endonuclease fragment 4, close to the junction between *Hpa* II endonuclease fragments 3 and 5. The size of the insertion varies from 10 to 50 base pairs. The biological properties, including oncogenicity, transforming ability, host range, and burst size of the mutants so far analyzed are similar to those of wild-type virus.**

The close relationships between murine teratocarcinoma stem cells and early embryonic mouse cells are well established, and many of these cell lines are widely used as tools for the study of differentiation (1–3). Among the modifications involved in the differentiation process, the ability of the cell to support the expression of some oncogenic viruses is affected. The undifferentiated murine teratocarcinoma stem cells are refractory to infection with polyoma (Py), simian virus 40 (SV40) (4, 5), minute virus of mouse (6), and ecotropic C type viruses (7, 8). During differentiation, the cells become susceptible to these viruses; Py can develop a complete lytic cycle, and SV40 can express early functions, as detected by immunofluorescence. Previous studies have shown that the embryonal carcinoma (EC) stem cells are capable of adsorbing Py and SV40 virions and that the virus penetrates into the cell, is transported to the nucleus, and is normally uncoated (9). No tumor (T) antigen can be detected after penetration of either virus into EC cells. Fusion experiments of permissive cells with EC cells show that Py virus is expressed in the heterokaryons (10). Moreover, when EC cells are fused with BHK (hamster) cells that have been infected by Py virus, one can observe the appearance of viral capsid (V) antigen in the heterokaryon—i.e., EC cells possess factors required for late expression of the virus (10). Microinjection of SV40 or Py DNA into the nucleus of EC cells did not yield any expression of T antigen (M. Boccara and A. Graessman, personal communication).

It appears that the resistance of the murine teratocarcinoma stem cells to infection with SV40 and Py involves a block early in the infectious cycle, after adsorption and penetration but before T antigen expression.

Analysis of mRNA in F9 infected with SV40 at high multiplicity of infection (moi) has revealed the presence of SV40 RNA, transcribed on both early and late viral DNA strands (11). The viral RNA is unstable and produced at a low level, and the posttranscriptional processing is not achieved as in normal mouse cells: neither cytoplasmic nor nuclear RNA is spliced. In contrast, infection of PCC4 cells with Py virus leads to the production of a low level of viral mRNA (unpublished results) (about $10^{-2}$ of the amount usually found in differentiated mouse cells) that is normally spliced (F. Kelly and R. Kamen, personal communication).

In order to elucidate the molecular mechanism responsible for the block in virus expression, we looked for Py variants able to develop in nondifferentiated multipotential teratocarcinoma stem cells. We describe here the selection and some biological and physical properties of this class of variants.

## MATERIAL AND METHODS

**Cell Lines.** PCC4 aza1 is an EC cell line derived from the transplantable mouse teratocarcinoma OTT6050 and selected for resistance to azaguanine (12). When injected into strain 129 mice, these cells give rise to trigerminal teratocarcinoma. They grow *in vitro* as undifferentiated stem cells. F9 is a nullipotential EC cell line also derived from OTT6050 (13). PCD3 is a fibroblast-like cell derived from multipotential PCC3 EC cell line (13). Medium and cell culture conditions for these cells are according to Jakob *et al.* (12). NIH 3T3, BALB 3T3, and UC1B (gift from T. Benjamin) have been described (14, 15). FR-3T3 rat cell line is a gift from F. Cuzin.

**Virus.** Py wild-type (wt) A2, large plaque, obtained from M. Fried, was usually propagated by infecting secondary mouse embryo cells (2°ME) at a moi of 0.01 plaque-forming unit (PFU) per cell.

**Immunofluorescent Staining for Py T and V Antigens.** Cells were stained for T and V antigens by the indirect immunofluorescent technique (16), using antiserum against Py T antigen from BN rats bearing tumors produced by injection of Py-transformed BN cells (gift from T. Benjamin) and sheep anti-V antiserum (gift from D. Paulin).

**Transformation.** Infection of FR-3T3 cells and selection of transformants were performed as described (17).

**Preparation of Viral DNAs.** 2°ME cells were infected with Py wt A2 or with clonal isolates of Py EC mutants at a moi of 5 PFU per cell. The viral DNA was extracted, 4 days after infection, by the selective procedure of Hirt (18), treated with phenol, and purified by equilibrium centrifugation in ethidium bromide/cesium chloride gradients.

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Abbreviations: Py, polyoma; SV40, simian virus 40; EC, embryonal carcinoma; 2°ME, secondary mouse embryo cells; wt, wild type; moi, multiplicity of infection; PFU, plaque-forming units; T antigen, tumor antigen; V antigen, viral capsid antigen.

**Restriction Endonuclease Digestion.** Restriction endonucleases *Eco*RI, *Hae* III, *Pst* I, *Pvu* II, and *Hpa* II were purchased from New England BioLabs. Enzyme digestions were performed as described by Griffin (19).

**Slab Gel Electrophoresis.** The products obtained by restriction enzyme digestion were fractionated by electrophoresis on 1.4% agarose or 5% polyacrylamide/bisacrylamide gels made in 40 mM Tris·HCl, pH 7.8/20 mM sodium acetate/2 mM EDTA. DNA fragments were located by visualization of the ethidium bromide-stained gel in ultraviolet light.

## RESULTS

**Selection of Mutants of Polyoma Virus Able to Develop in PCC4 Cells.** Infection of permissive cells with Py virus at high moi leads to the production of virus particles that contain DNA molecules heterogeneous in size. The viruses are mostly defective and contain reiterated viral sequences or host sequences covalently linked to the viral genome (20).

In order to obtain such heterogeneous viruses, PCD3 cells were infected with Py wt A2 at a moi of 1000 PFU per cell. Forty-eight hours after infection, the virus was recovered and two subsequent multiplications of the stock obtained were performed on the same cells. The resulting stock of Py virus was used to infect PCC4 cells. Thirty-six hours after infection, the EC cells were fixed and stained for T antigen. In four independent infections, using virus produced by four different series of passages at high moi, 0.2–0.4% of the infected PCC4 cells were shown to express T antigen. After infection with Py wt A2, under the same conditions, only an occasional cell was found to be positive for T antigen (less than 0.01%). By this procedure, we have therefore obtained some virus able to overcome the block for the expression of early functions. Because fusion experiments of PCC4 cells with Py-infected BHK cells have shown that EC cells are able to express the late functions of Py virus (10), some virus of the heterogeneous stock, which express T antigen on PCC4 cells, should be able to develop on these cells. In order to select and purify such viruses, PCC4 cells were infected with heterogeneous stocks of Py and then passaged as usual every other day. Twice a week, some cells were fixed and stained for T antigen. After 2 weeks no more T antigen could be detected. Two weeks later, some T-positive cells (about 0.1%) appeared in the culture. In subsequent passages, an increase of the percentage of T antigen-positive cells to 5–10% was observed. A similar proportion of cells were shown to express V antigen. From this time (i.e., 2 months after infection with the heterogeneous stock) the cells were multiplied and the culture medium was stored after each change. Virus was recovered from medium by centrifugation. Using this virus stock to infect PCC4, we have detected by immunofluorescence 10–30% T antigen-positive cells 36 hr after infection (Fig. 1) and 24 hr later 10–15% V antigen-positive cells. It seems, therefore, that by means of a persistent infection of PCC4 we have selected Py viruses able to develop a lytic cycle on EC cells. These viruses have been plaque purified twice on 2°ME cells. From several hundred clones recovered, 50 have been multiplied and titrated. PCC4 cells were infected at a moi of 100 PFU per cell with these 50 clones of Py mutant; in all cases, 36 hr after infection, 5–50% T antigen-positive cells have been detected, depending on the virus stock used. V antigen detected 60 hr after infection was expressed at the same level. In the same conditions of infection, Py wt A2 did not produce more than 0.01% T antigen-positive cells.

From the 50 plaque-purified virus clones, 6 were selected for further studies. These viruses are referred to as Py EC (for embryonal carcinoma, to recall the way they have been selected) followed by the clone number.

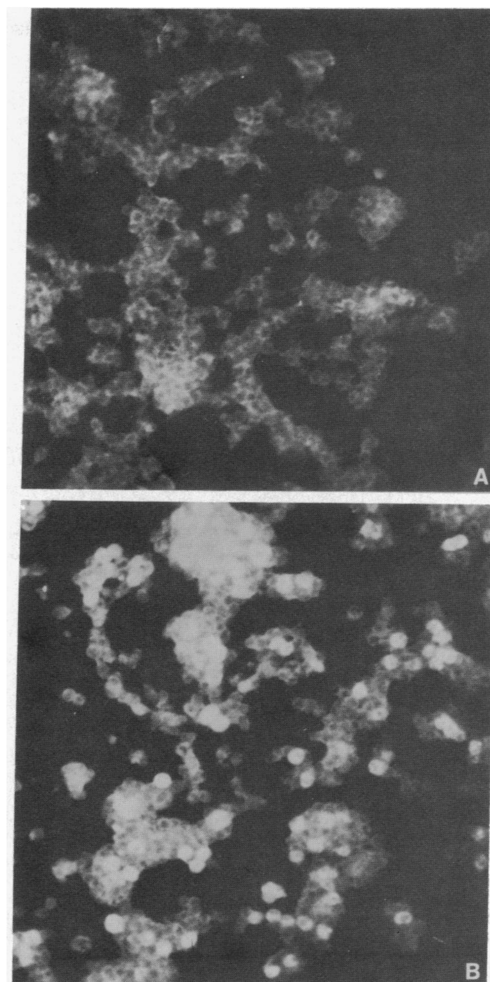


FIG. 1. T antigen immunofluorescence in PCC4 cells, monitored 36 hr after infection with Py wt A2 (*A*) or Py selected on PCC4 (*B*), at a moi of 100 PFU per cell. (×40.)

**Restriction Enzyme Analysis.** Six Py EC mutants were submitted to a detailed analysis with restriction enzymes *Eco*RI, *Hpa* II, *Hae* III, *Pst* I, and *Pvu* II. The cleavage sites of these enzymes (19) are indicated on the physical map of polyoma (Fig. 2).

All the Py EC mutants so far analyzed have conserved the *Eco*RI cleavage site (data not shown). Fig. 3 shows the *Hpa* II patterns of Py EC 97 and Py EC 204 compared to wt A2. The two mutants show a *Hpa* II-3 fragment longer than that of wt. No other difference could be detected. The size increase varies from 0.3% (Py EC 204) to 1% (Py EC 97) of genome length, as calculated from electrophoretic mobility. All the Py EC mutants so far analyzed yield a modified *Hpa* II-3 fragment. Py EC 97 has the longest insertion observed and Py EC 204 the smallest. This localization has been confirmed by digestion with *Pst* I (data not shown): as expected, modification involved only an increase in the length of the *Pst* I-2 fragment. Fig. 4 shows the *Hae* III and *Pvu* II restriction patterns of Py EC 204 and Py EC 97. *Hae* III restriction patterns were submitted to microdensitometric analysis (Fig. 5). All *Hae* III fragments are present in Py EC 97 and Py EC 204, but only one copy of fragment 14 is found as compared to wt, in which two fragments (14 and 14′) with identical electrophoretic mobilities have been detected (19). In Py EC 97, a new fragment is seen migrating behind fragment 11 and in Py EC 204 the new fragment migrates between fragments 12 and 13. One of the two

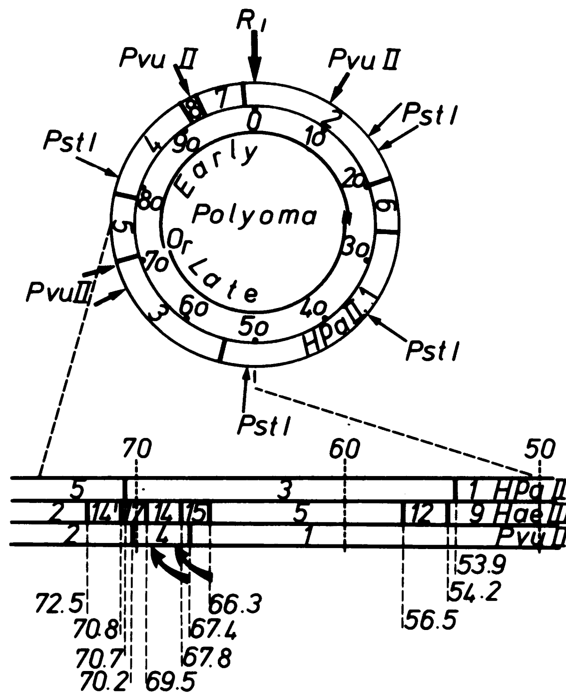


FIG. 2. Physical map of Py A2. Cleavage map of Py DNA by *Hpa* II is shown in the annulus. Cleavage sites by *Pst* I and *Pvu* II are shown by arrows. The 50–75 region has been amplified and sites for *Hpa* II, *Pvu* II, and *Hae* III indicated precisely. Black curved arrows indicate possible sites for sequence insertion in the DNA of Py EC mutants.

14 or 14′ fragments has disappeared and has been replaced by this new fragment. *Hpa* II digestion shows that only the *Hpa* II-3 fragment is modified. This fragment contains *Hae* III-14 but not *Hae* III-14′ (Fig. 3). Therefore, modification has affected the *Hae* III-14 fragment. The *Pvu* II restriction pattern shows that fragment 4 has disappeared and been replaced by two new fragments. This implies that a new *Pvu* II site has been created. The *Pvu* II-4 fragment of Py EC 97 has been replaced by two new fragments (2.3% and 1.4% of genome length). In

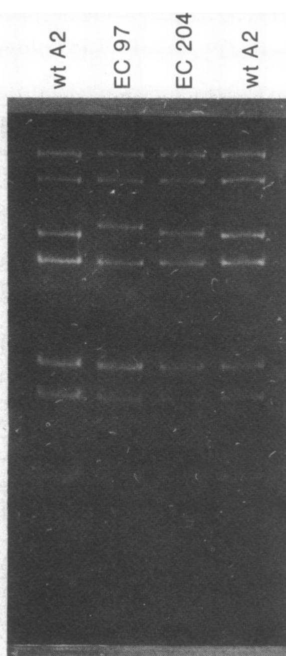


FIG. 3. *Hpa* II fragment pattern analysis on 5% polyacrylamide gel electrophoresis.

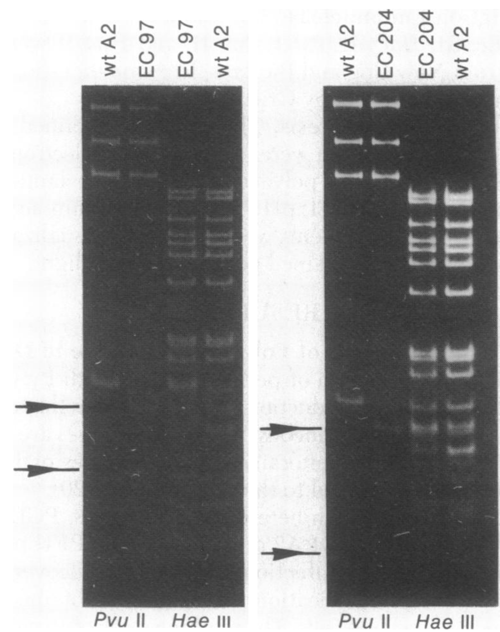


FIG. 4. *Pvu* II and *Hae* III fragment pattern analyses on 5% polyacrylamide gel electrophoresis. Arrows indicate the positions of the new *Pvu* II fragments.

Py EC 204 the sizes of these two fragments have been estimated to be 2.3% and 0.7% of genome length. Table 1 summarizes the results of genome modifications observed in restriction fragment analysis. Taken together, these data give a reasonably accurate localization and size estimation of the additional sequences. It is notable that, of the two fragments that have replaced the *Pvu* II-4 fragment, one is constant in size (2.3%) in all Py EC mutants analyzed so far (Table 1 and data not shown). The other additional fragment is variable in size. This could be explained by assuming that the insertion site is always the same.

These restriction enzyme analyses were performed with viral DNA extracted from 2°ME cells. Identical results were observed when Py EC DNA was prepared from infected PCC4 cells.

**Biological Properties of Py EC.** Py EC variants and wt A2 were compared with respect to their biological properties, including tumorigenicity in new-born hamster, transforming efficiency of rat FR-3T3 cells, and virus burst size in several mouse cell lines. As shown in Table 2, no significant difference between Py EC mutants and wt virus could be found. In addition, the time course of lytic development on differentiated mouse cells is similar for both Py wt and Py EC mutants (data not shown). Therefore, acquisition of the capacity to develop in undifferentiated murine teratocarcinoma stem cells does not confer any selective advantage on the mutant, nor does it lead to the appearance of any other new biological property.

Table 1. Size of restriction fragments modified in Py EC mutants compared to wt as calculated from electrophoretic mobility

| Restriction fragment | Fragment size, % of genome length | | |
|---|---|---|---|
| | wt A2 | Py EC 97 | Py EC 204 |
| *Hpa* II-3 | 16.8 | 17.8 (+1.0) | 17.1 (+0.3) |
| *Hae* III-14 or new fragment | 1.7 | 2.8 (+1.1) | 2.1 (+0.4) |
| *Pvu* II-4 or new fragments | 2.8 | 2.3 and 1.4 (+0.9) | 2.3 and 0.7 (+0.2) |

Sizes of insertions are given in parentheses. Py wt A2 fragment sizes are quoted from ref. 19. Total genome length is 5292 base pairs.

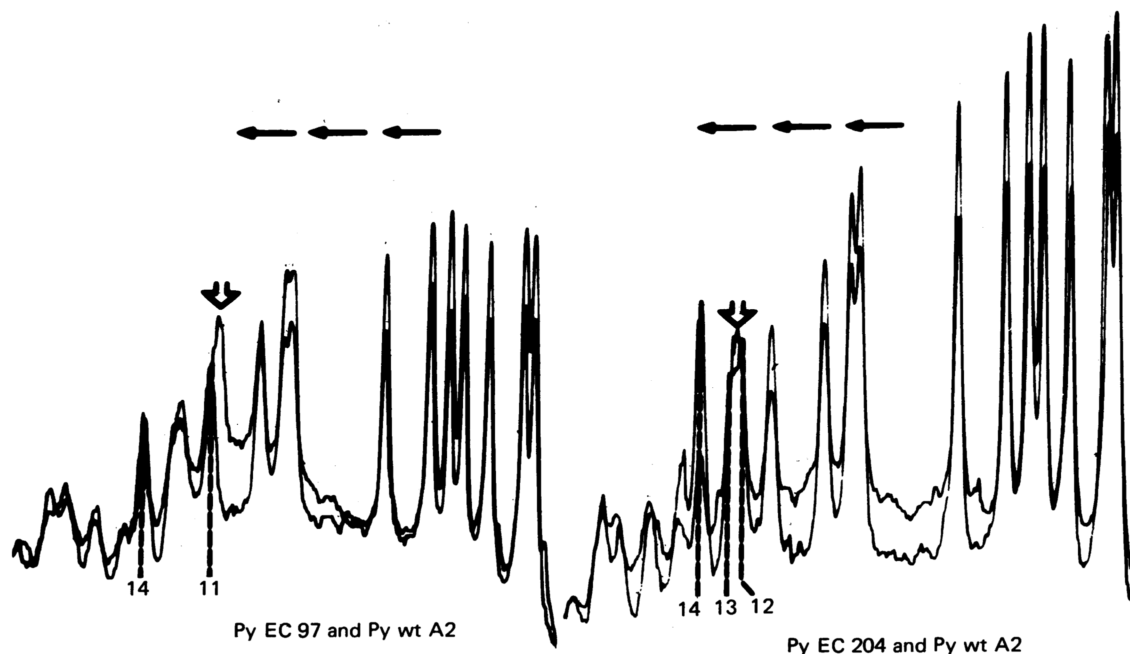


FIG. 5. Microdensitometric scanning of the *Hae* III patterns (shown in Fig. 4) of Py EC 97 and Py EC 204 compared to wt. The differences between Py EC and Py wt are shown by vertical arrows indicating the positions of new fragments. Electrophoretic migration was from right to left.

## DISCUSSION

Py virus mutants were selected for their ability to multiply on PCC4 cells. As revealed by restriction enzyme analysis, the DNA of all mutants exhibited a size increase in the *Hpa* II-3 fragment, mapping close to the *Hpa* II-3/5 junction, in the *Hae* III-14 fragment. This region has been shown to be frequently rearranged and reiterated in Py virus stocks passaged at high moi (20). The size of the additional sequence ranges from 0.3% to 1% of genome length. *Pvu* II digestion indicates that the insertion always contains an additional recognition site for this enzyme, which is always located at 2.3 map units from the *Pvu* II 67.4 or 70.2 site. This result strongly suggests that the insertions observed in all mutants analyzed share a common sequence located at the same site. The insertion might have resulted either from partial duplication of viral sequences or from recombination with the host genome. Because duplications of the *Hpa* II-3/5 junction have been widely observed (20), the additional *Pvu* II site could have arisen by duplication of a portion of the *Hae* III-17 fragment, including the *Pvu* II 70.2 site. Although acquisition of cellular sequences is rarely demonstrated during early high moi passages of Py virus (20), such an event might have taken place during the course of selection on PCC4 cells. DNA sequence analysis of the mutants should elucidate this point.

Size increase of the *Hpa* II-3 fragment is the only rearrangement observed by restriction enzyme analysis, but one cannot exclude that other undetected modifications (point mutations, small deletions or additions) may be responsible for the ability of mutants to develop on PCC4 cells. This hypothesis seems, however, unlikely in view of the striking physical similarities shared by the genomes of independent mutants obtained through an identical selection procedure. Some available strains of Py virus exhibiting a longer *Hpa* II-3 fragment than wt A2 strain (strain NG59 RA, described in ref. 21) or containing a duplication of the DNA sequences around the *Hpa* II-3/5 junction (strain Py M41, described in ref. 22), were shown to be unable to express T antigen on PCC4 cells (unpublished results). This confirms that selection on EC cells has led to the emergence of a highly specific genome modification.

Sequences located between 67 and 71 map units have been shown (*i*) to contain the origin of DNA replication (23, 24), (*ii*) to encode the 5′ termini of both early and late viral mRNAs (25, 26), (*iii*) to contain a binding site specific for eukaryotic RNA polymerase II (27). According to these data and because expression of early viral RNAs is regulated by large T antigen (28), one can assume that a promoter for transcription of early mRNAs is located in that region. The modification observed in Py EC mutants may somehow affect the mechanism of

Table 2. Biological properties of Py EC mutants

| | Tumorigenicity* | | | Transforming efficiency,† colonies or dense foci as % of input | | Virus production,‡ PFU × $10^{-6}$ per culture | | | |
|---|---|---|---|---|---|---|---|---|---|
| Virus strain | $10^3$ PFU | $10^4$ PFU | $10^5$ PFU | In soft agar | On plastic | BALB 3T3 | NIH 3T3 | UC1B | 2°ME |
| Py EC 97 | 3/10 | 6/9 | 2/2 | 0.05–0.1 | 0.05–0.1 | 13 | 50 | 40 | 30 |
| Py wt A2 | 2/5 | 6/11 | 5/7 | 0.05–0.1 | 0.05–0.1 | 8 | 20 | 30 | 50 |

* Tumors were scored up to 3 months after subcutaneous injection of virus into 2- to 3-day-old baby hamsters. Numbers are animals with tumors/animals injected at each of three doses, PFU per animal.

† FR-3T3 cells were infected at a moi of 100–200 PFU per cell; 24 hr after infection, 5 × $10^4$ cells were seeded in soft agar or on plastic dishes and incubated at 33°C. Colonies were scored 15–20 days later.

‡ Cells were infected at a moi of 0.1 PFU per cell; 72 hr later, cells and medium were collected and virus was extracted by freezing–thawing–sonicating and low-speed centrifugation. Virus production was measured by plaque assay on 2°ME.

transcription initiation. This hypothesis is supported by the fact that viral RNA synthesis in PCC4 cells, infected with Py wt virus, is about $10^{-2}$ of the amount observed early after infection of permissive mouse cells (M. Vasseur, unpublished results; F. Kelly and R. Kamen, personal communication). Although, under normal tissue culture conditions, PCC4 cells maintain only a single morphology typical of EC cells, one cannot exclude that viral RNA is synthesized by a few cells that have undergone differentiation. In F9 cells infected with SV40, low levels of unstable and unspliced viral RNA can be detected (11). It suggests that, in these cells, the block for expression of SV40 early functions is correlated with a defect in mRNA maturation. On the other hand, no such defect has been observed in PCC4 cells infected with Py wt virus (F. Kelly and R. Kamen, personal communication). In addition, Py EC mutants do not exhibit any detectable modification in the 78–86 map units region, which is involved in the splicing of early mRNAs (29). It is therefore unlikely, although not excluded, that the sequence rearrangement in Py EC mutants leads to a modification of the splicing mechanisms. F9 cells were infected with some Py EC mutants (Py EC 97, 105, and 204): none of them expressed T antigen in these cells (data not shown). It should be pointed out that F9 cells are nullipotent *in vivo* and *in vitro,* whereas PCC4 cells are capable of multipotential differentiation *in vivo.* The mechanism involved in viral restriction might therefore be different in both cell lines and (or) for Py and SV40.

Selection for the ability to develop on PCC4 cells did not suppress or alter the main biological functions of the virus: Py EC mutants have retained the transforming, oncogenic, and host-range properties of wt virus. The modification observed in Py EC mutants is strictly relevant to the new property acquired—i.e., growth on multipotential EC cells.

Our working hypothesis is that the genome rearrangement observed in Py EC mutants involves a modification of regulation of viral DNA transcription in EC cells. Fusion experiments between EC cells and differentiated cells (10) have shown that undifferentiated teratocarcinoma stem cells lack factors required for viral expression; these factors may be involved in the control of transcription and be related to differentiation. Because Py EC mutants, as opposed to wt virus, do not require these factors for viral expression, this class of mutants provides a model system to study the molecular mechanism of regulation of gene expression during embryonic differentiation.

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