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Negative Regulation of Early Polyomavirus Expression in Mouse Embryonal Carcinoma Cells†

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Embryonal carcinoma cells are resistant to infection by polyomavirus (Py). We showed that this block was partially removed by inhibiting protein synthesis temporarily. The block was also partially removed when Py was coinfected with simian virus 40. Cycloheximide treatment of cells infected with Py mutants able to grow on PCC4 embryonal carcinoma cells led to 3- to 10-fold increases in the production of T-antigen-positive cells. At 31°C, Py T-antigen expression was enhanced when the cells were treated with cycloheximide. We suggest that a negative labile regulatory protein(s) is synthesized in PCC4 cells, preventing the initiation of early Py transcription by binding to the noncoding sequence, especially the enhancer element B and perhaps also element A, and that the Py mutants retained a binding site(s).

Differentiated mouse cells are permissive for polyomavirus (Py) infection. They therefore replicate Py DNA and produce virus. Such cells are also sensitive to simian virus 40 (SV40) and undergo an abortive infection, in which only T antigen is produced. In contrast, undifferentiated mouse embryonal carcinoma (EC) cells, such as PCC4 and F9 cells, fail to express any Py or SV40 functions (1, 25). It was demonstrated that Py penetrates into such undifferentiated cells and that their genomes can be recovered uncoated in the cell nucleus (24). After infection of PCC4 cells with wild-type Py, a very low level of early, normally spliced mRNA was found by Dandolo et al. (7). They concluded that the block is probably located at the transcriptional level.

Several Py mutants able to grow on PCC4 EC cells were isolated (17, 26). These mutants, PyEC PCC4 97 and PyEC PCC4 204, contain a deletion and a duplication on the late side of the origin of replication, i.e., within the enhancer sequences (17).

A study of heterocaryons produced by the fusion of undifferentiated EC cells preinfected with Py with differentiated EC cells showed that Py can be expressed in EC cell nuclei (2). This suggests that a diffusible *trans*-activating factor(s) required for early gene expression and synthesized in differentiated cells can supply the functions lacking in EC cells. The gene(s) coding for this factor(s) would be repressed in EC cells. This idea was reinforced by a study involving 5-azacytidine treatment of EC cells. 5-Azacytidine is a cytosine analog known to prevent DNA methylation (16), and undermethylation has been shown to be important in generating a chromosome structure permissive for transcription (5, 11). In earlier work, we isolated undermethylated clones of EC cells and showed that they are partially permissive to Py infection (6). Subsequently, we showed that PCC4 cells differentiate spontaneously at 31°C and become permissive to Py at this temperature (C. Cremisi and P. Duprey, J. Cell Physiol., in press). By studying the mechanism by which Py permissiveness is established at 31°C, we suspected that PYV expression is also negatively regulated in EC cells.

In this work, we therefore set out to test the following hypothesis of negative regulation of Py expression. A labile protein(s) binds to viral chromatin and prevents the initiation of early gene transcription. By inhibiting the synthesis of this hypothetical labile protein(s), we hoped to release the transcription block. Since we knew nothing about the turnover of this protein(s) or the length of life of its messenger RNA, our sole purpose was to upset the equilibrium between its association with viral chromatin and the other factors necessary for the transcription process and thus to allow the residual expression of T antigen.

To test this model, we used cycloheximide to inhibit protein synthesis. If cycloheximide treatment lasts long enough and if the protein(s) is labile enough, viral chromatin could be obtained without this DNA-binding protein, which would allow the transcription of early Py genes. Thus, in the absence of protein synthesis, early mRNA would accumulate. Reversal of the effect of cycloheximide would allow the translation of the mRNA accumulated during the treatment. For the reasons described above, any expression of T antigen would necessarily be of low amplitude but would constitute the qualitative expression of a negative type of gene regulation.

PCC4 cells grown on cover slips were infected with wild-type Py strain A2 at a multiplicity of infection of 50 to 100 PFU per cell. After 2 h of adsorption, the cells were placed in medium containing 0.5 μg of cycloheximide per ml. After 24 h, the medium was removed and replaced with drug-free medium. At 48 h after infection, the cells were fixed and examined by indirect immunofluorescence staining for the presence of Py T antigen, as described by Gorman et al. (10) and Vasseur et al. (27).

The PCC4 cells did not seem to suffer from the cycloheximide treatment. At a concentration of 0.5 μg/ml, inhibition of protein synthesis was not total (about 85%) and the cells did not stop growing. In each experiment, about 5×10^4 to 5×10^5 cells were carefully examined. The results are summarized in Table 1. After cycloheximide treatment, a small but significant percentage of PCC4 cells (1 to 3%) was stained by the Py T antigen, 50 to 150 times more than the percentage of untreated cells stained (0.02%). In contrast, an 80% decrease in the proportion of Py T-antigen-positive cells was observed when the same cycloheximide treatment was

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TABLE 1. Py T-antigen expression in PCC4 cells under different conditions of infection[a]

T-antigen-positive PCC4 cells	Conditions of infection								
	37°C							31°C[b]	
	Py wild type	Py wild type + cycloheximide	Py wild type + SV40	PYEC PCC4 97	PYEC PCC4 97 + cycloheximide	PYEC PCC4 204	PYEC PCC4 204 + cycloheximide	Py wild type	Py wild type + cycloheximide
No. for one expt (%)	18 (0.02)	1,280 (1–3)	655 (0.5–1.2)	2,640 (2–5)	27,000 (6–25)	2,650 (2–5)	9,000 (6–15)	2,600 (2–3)	6,600 (7)

[a] When mouse fibroblast 3T6 cells were infected with wild-type Py and treated with cycloheximide, there was an 80% decrease in the proportion of T-antigen-positive cells.

[b] After 2 h of adsorption at 37°C, the infected PCC4 cells were shifted to 31°C for 24 h.

applied to mouse fibroblast 3T6 cells, which are permissive to Py infection. These data suggest that a negative regulator(s) controls early Py transcription in PCC4 cells. It could interact directly with Py DNA or indirectly, for example by decreasing the level of cellular factors required for Py transcription. To choose between these two possibilities, we subjected these cells to mixed infection with both Py and SV40 virus. Each was used at the same multiplicity of infection of 50 to 100 PFU per cell. At 48 h after infection, the cells were examined for Py T antigen and 0.5 to 1.2% were positive for Py T antigen, i.e., 25 to 60 times more than the control cells infected with Py only (Table 1).

PCC4 cells infected with PyEC PCC4 97 and PyEC PCC4 204 were also treated with cycloheximide to see if its effect was the same as on wild-type Py and enhanced the production of T antigen. We observed that when mutant-infected cells were treated with cycloheximide, 3 to 10 times more were T-antigen positive than when they were not so treated (Table 1).

It was recently shown that PCC4 cells differentiate in epitheliallike cells when cultured at 31°C and that they become permissive to Py infection (8; Cremisi and Duprey, in press). To determine whether Py permissiveness at 31°C was due to the synthesis of a new protein(s) or, on the contrary, to a decrease in the protein(s) already present, we performed the following experiment. Immediately after infection, the PCC4 cells were transferred to a temperature of 31°C for various periods in the presence or absence of cycloheximide and then returned to 37°C in a drug-free medium until 48 h after infection. The cells were then fixed for immunofluorescence staining. For infected cells, 24 h at 31°C allowed some expression of Py T antigen and this expression was enhanced three- to fourfold when these cells were treated with cycloheximide (Table 1).

All these results are compatible with the negative regulation model described above and argue in favor of the hypothesis that, in PCC4 cells, a labile protein(s) prevents the initiation of early gene transcription. Mixed infection with Py plus SV40 suggests that (i) the hypothetical negative regulatory protein(s) also has an affinity for SV40 DNA and consequently does not act indirectly by decreasing the level of cellular factors required for Py transcription, but probably acts directly by interacting with Py DNA and (ii) the binding site(s) of the hypothetical protein(s) is in the noncoding sequence located between the early and late genes, since it is the only homologous DNA sequence in these DNAs. This region was analyzed in detail by several laboratories (9, 14, 26), and two distinct nonoverlapping enhancer elements A and B were defined in the enhancer region (3, 13). The core of element B, whose sequence is the consensus sequence described by Weiher et al. (29), is homologous to the SV40 core enhancer, whereas the core of element A is homologous to the E_{1a} enhancer (12, 13). Consequently, the negative regulatory protein(s) seems able to bind at least to element B. The fact that SV40 at 50 to 100 PFU per cell allowed a slight expression of early Py genes suggests that the regulatory protein(s) has more affinity for SV40 than for Py DNA. It is of interest that a higher Py multiplicity of infection did not allow significant early Py expression (7, 8, 27; unpublished results); i.e., it is very difficult to titrate out this regulator with Py DNA, whereas it seems possible to do so with SV40 DNA.

This protein(s) might also bind to sequences other than those of element B. This hypothesis is supported by the results obtained with the mutant-infected cells treated with cycloheximide, which showed that despite DNA rearrangement the mutants also underwent negative regulation. The Py mutants can be partially expressed in PCC4 cells either because the rearrangement of the DNA sequence (deletion of element B and duplication of element A) allows more efficient binding of a *trans*-acting positive factor(s), shown to be necessary for early papovavirus expression (19, 21, 23), or the rearrangement reduces but does not abolish the affinity of the repressor protein for Py DNA. A combination of these two possibilities can also be envisaged. In both cases, however, it should be assumed that the negative regulatory protein(s) also has an affinity for sequences other than element B, possibly element A, since PyEC PCC4 mutants have a duplication of element A.

The results obtained at low temperature suggest that at 31°C, the synthesis of the negative regulatory protein(s) is significantly reduced; thus it is at least partially responsible for Py expression at that temperature. When the cells are permanently cultured at 31°C, they differentiate into epitheliallike cells (Cremisi and Duprey, in press). It is conceivable that this differentiation is due to the derepression of other genes.

It was recently shown that the viral enhancers SV40, Py, and adenovirus E_{1a} can be negatively regulated in HeLa cells by E_{1a} proteins (4, 28) and that the same applies to murine sarcoma virus in F9 cells (10). This last study showed that early SV40 genes can be expressed in F9 cells in an enhancer-independent fashion (20) and depending on DNA concentration.

It is of interest to note that F9 cells contain a cellular E_{1a}-like activity (15). Therefore, it is not impossible that this protein(s) is responsible for negative regulation of Py in EC cells. E_{1a}-like activity might also be responsible for repressing other cellular genes not expressed in EC cells, such as major histocompatibility complex class 1 antigens (18, 22).

The negative regulatory protein(s) synthesized in PCC4 cells must be inactivated or greatly reduced in differentiated mouse cells, such as 3T6 cells. EC cells must also contain some *trans*-acting positive factors, since both the wild-type Py after cycloheximide treatment and the mutants could be expressed in these cells. However, the amounts of these

factors probably increase upon differentiation (23). Taken together, the results discussed in this study suggest that both negative and positive regulators control the activity of early Py genes in EC cells.

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