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Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells

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The genomic rearrangements of six polyomavirus mutants selected on PCC4 embryonal carcinoma cells have been compared and their common characteristics pointed out. All mutants show a duplication which includes at least the adenovirus type 5 (Ad5) E1A-like enhancer core sequence plus a deletion of variable size and location. The presence of the second enhancer core sequence, the SV40-like enhancer, is not required for expression of the PyEC PCC4 phenotype. Two of these mutants are also able to express polyomavirus T antigen on F9 and LT1 cells. Multiadaptation seems to require the duplication of the Ad5 E1A-like core sequence, the maintenance of the SV40-like core sequence and a local change in DNA stability.

Key words: polyomavirus/host-range/teratocarcinoma

Introduction

We and others (Vasseur *et al.*, 1980, 1982; Katinka *et al.*, 1980, 1981; Fujimura *et al.*, 1981; Sekikawa and Levine, 1981) have described the isolation and DNA sequence of polyomavirus mutants able to express early and late viral genes on PCC4 and F9 teratocarcinoma stem cells which usually restrict virus growth at the level of early transcription (Swartzendruber *et al.*, 1977). While PyEC PCC4 mutants are still restricted in F9 and LT1 embryonal carcinoma (EC) cells, PyEC F9 mutants, restricted in LT1 EC cells, exhibit a low level of expression in PCC4 EC cells (Vasseur *et al.*, 1982). The host-range phenotype results from modifications involving the non-coding region of polyoma DNA which has been shown to contain regulatory signals for both replication and transcription (Tyndall *et al.*, 1981; Luthman *et al.*, 1982; de Villiers *et al.*, 1984), and includes two separate enhancer domains A and B (Herbomel *et al.*, 1984).

Mutants selected on F9 cells were shown to undergo genomic rearrangements which, as in the case of PyEC PCC4 mutants, affect the polyoma enhancer region but are clearly distinct both in nature and localization. Since 10 of these PyEC F9 mutants have now been sequenced, it has been possible to determine their common features and to draw some conclusions about the minimum genome modification, an AT→GC transition at nucleotide 5233 (Fujimura *et al.*, 1981; F.Mélin, unpublished data), responsible for their host-range phenotype. Some mutants also harbor a tandem repeat, both copies of which contain the single base pair change.

Sequence rearrangements of the two previously isolated PyEC PCC4 mutants (Katinka *et al.*, 1980) consist of a tandem duplication accompanied by a deletion of non-coding sequences on the late side of the origin of replication. To understand better the modifications responsible for the PyEC PCC4 phenotype, three additional independent mutants were selected from wild-type polyomavirus A2 strain and a fourth one (Mélin *et al.*, 1985) from variant strain ev1001 (Magnusson and Nilsson, 1982). Their sequence and host-range phenotype were compared. The results indicate that all PyEC PCC4 mutants share identical types of rearrangements and that, except for two of them, they can express T antigen only in PCC4 or PCC4-like (Georges *et al.*, 1982) EC cells.

Results

Isolation and cloning of PyEC PCC4 mutants

Three independent experiments, using separate wild-type polyomavirus A1 plaques, were carried out to isolate PyEC mutants from PCC4 cells according to the selection procedure already described (Vasseur *et al.*, 1980). Following plaque purification on secondary mouse embryo cells, three isolates, which were shown to be infectious for PCC4 cells, were amplified on secondary mouse embryo cells. Viral DNA was isolated from each virus stock and cloned in the *Bam*HI site of a pBR vector. Cloned viral genomes (A, 500 and 5000) were excised with *Bam*HI endonuclease and used to transfect secondary mouse embryo cells which produced viruses still expressing early functions in PCC4 cells.

Host-range phenotype of PyEC PCC4 mutants

It can be seen in Figure 1 that, with the exception of mutant A, which can express T antigen in the three classes of EC lines previously characterized (Georges *et al.*, 1982), the other four mutants, including mutants 97 and 204 already described (Katinka *et al.*, 1980), are restricted to PCC4 or PCC4-like cells. No clear differences in the efficiencies of infection could be detected among the five mutants. Possible explanations for the multiadapted phenotype of mutant A will be supplied in the Discussion.

Nucleotide sequence of the PyEC PCC4 DNAs

Prior to sequencing, it was established by *in vitro* reconstruction, that, in all cases, the mutant phenotype resulted from modifications of the small *BclI-BglI* fragment of the polyomavirus genome. The nucleotide sequence of the three mutants was determined from nucleotide 5022 through the origin of replication up to nucleotide 90. The genetic maps of the modified region of the five mutants selected on PCC4 cells is shown in Figure 1. The structure of another multiadapted mutant, M 206, isolated in PCC4 cells (Mélin *et al.*, 1985) from a variant of polyomavirus, ev1001, which carries a tandem duplication (nucleotides 5100–48) overlapping the two enhancer domains and the origin of replication (Magnusson and Nilsson, 1982), is also included in Figure 1 for comparison with mutant A. Analysis of the different rearrangements leads to the following obervations.

(i) None of the mutants exhibit point mutations but all present a duplication of part of the A enhancer domain found in the polyomavirus genome (Herbomel *et al.*, 1984).

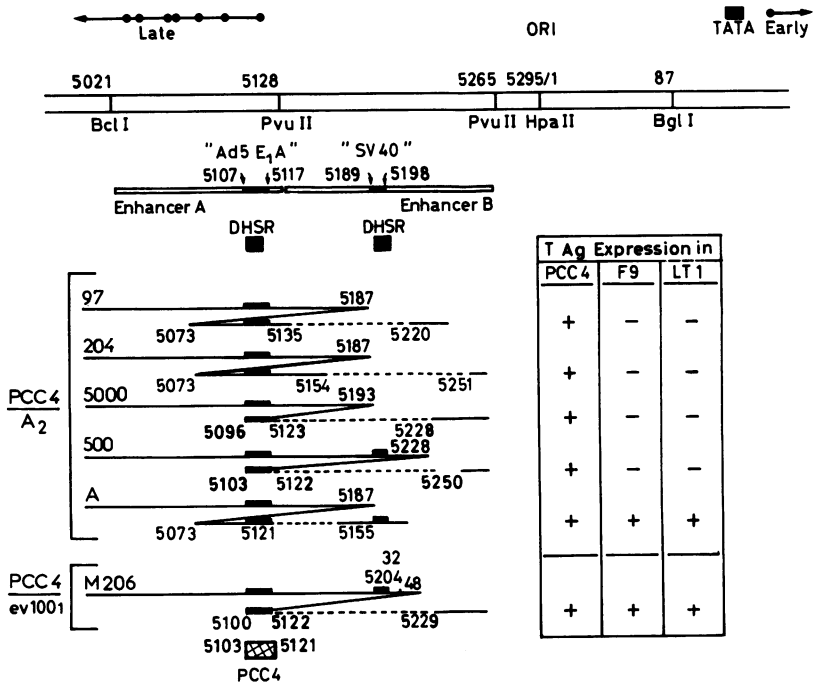

Fig. 1. Schematic comparison of the sequences of polyomavirus mutants isolated on PCC4 cells and their phenotype on the different EC cell lines. The top line shows a restriction map of wild-type A2 polyoma strain around the origin of replication (the numbering is according to Tyndall *et al.*, 1981). Above it are represented the early and late caps and the direction of transcription as dotted arrows (Cowie *et al.*, 1981, 1982), the early TATA box as a black box marked TATA (Friedmann *et al.*, 1979), the origin of replication as ORI (Friedmann *et al.*, 1979). Below are indicated the two enhancer domains A and B (Herbomel *et al.*, 1984) and their two core sequences as black boxes in the open boxes of the enhancer domains, numbered and named according to their sequence homology with the 'Ad5 E1A' or 'SV40' consensus sequence (Herbomel *et al.*, 1984), the two DNase I-hypersensitive sites as two black squares named DHSR (Herbomel *et al.*, 1981). The mutant sequences are schematized by the different lines. The name of each mutant is written at the beginning of each line. The numbering gives the endpoints of deletions and duplications; dashed lines indicate deleted sequences and black boxes on each line the core enhancers. In front of the brackets are indicated the cell line on which the mutants were isolated (PCC4) and the polyoma virus strain they are derived from (A2 or ev1001). ⊠ is the minimal sequence duplicated in PyEC PCC4 mutants. On the right, is indicated the ability (+) or inability (−) of each mutant to express T antigen on the three EC cell lines: PCC4, F9 and LT1.

	PCC4	F9	LT1
97	+	−	−
204	+	−	−
5000	+	−	−
500	+	−	−
A	+	+	+
M206	+	+	+

(ii) In three out of five mutants (A, 97 and 204) the boundary of the first repeat is located at nucleotide 5187 and the second copy starts at nucleotide 5073. However, no significant sequence homology is observed which could account for a recombination event between these two residues.

(iii) Although the extent of the duplication is variable, it always includes the region between nucleotides 5103 and 5121. This region, containing sequences identified as essential for early transcription (Luthman *et al.*, 1982), is included in the tandem duplications found in several polyomavirus strains (Ruley and Fried, 1983) and exhibits a strong homology with a sequence repeated twice in the enhancer of the E1A gene of adenovirus type 5 (Ad5) (Hearing and Shenk, 1983).

(iv) All mutants also display a deletion, variable in size and location, which affects the B enhancer domain. In four cases (A, 500, 5000 and M 206) the deletion has a common boundary around nucleotide 5121−5123, but no sequence homology is observed at the other variable boundary. It should be noticed that the deletion in mutant A (nucleotide 5121−5155) is overlapped by the duplication in mutant 204 (nucleotide 5073−5154). Hence, there exists no common sequence deleted in all mutant genomes.

(v) The activator core sequence of the B domain, TGTGGTTTTG (nucleotide 5189−5198), which is found in various transcriptional enhancers (Gluzman and Shenk, 1983) has been deleted in mutants 97 204 and 5000. By contrast, a GC-rich palindrome (nucleotide 5171−5188), next to this B core sequence, is conserved in all mutants and duplicated in mutant A.

Discussion

Analysis of five independent mutants selected on PCC4 cells from wild-type polyomavirus A2 strain and of one mutant derived from variant strain ev1001 confirms that the rearrangements leading to this phenotype are different and more complex than those observed after adaptation to F9 cells. They are also remarkably similar among the different PyEC PCC4 mutants and, as in all PyEC mutants, they involve the non-coding region of the viral genome, on the late side of the origin of replication.

Essential features of the polyoma regulatory region

This region exhibits a number of essential elements involved in the regulation of transcription and/or replication of polyomavirus (Tyndall *et al.*, 1981; Luthman *et al.*, 1982; de Villiers *et al.*, 1984).

Two enhancer domains, A and B, have been identified (Herbomel *et al.*, 1984); each contains its own core sequence. The A core enhancer is homologous to an essential sequence described by Hearing and Shenk (1983) for the Ad5 E1A enhancer sequence. It is surrounded by two inverted repeats (*a* and *a'* in Figure 2) which create a potential hairpin. The B core sequence matches the SV40 consensus sequence pointed out by Weiher *et al.* (1982).

Several inverted repeats give the B domain the potentiality to adopt a 'clover leaf' structure as described by Soeda *et al.* (1980). These include a GC-rich palindrome and elements *a*, *a'*, *b*, *b'*, *c* and *c'*, as shown in Figure 2.

Recently a feature consisting of two short direct repeats

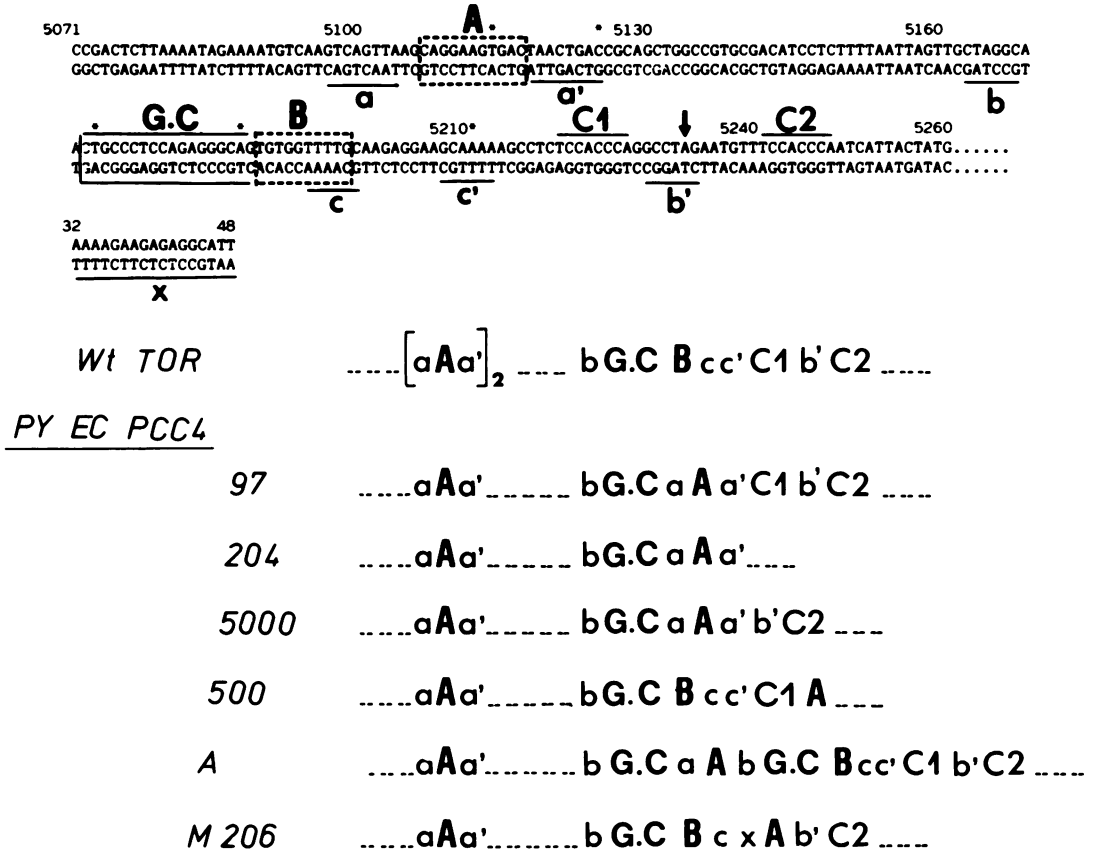

Fig. 2. Sequence and some essential features of the non-coding region of polyomavirus wild-type strains A2 and TOR and PyEC PCC4 mutants. On top is shown the nucleotide sequence of polyomavirus A2 strain. Nucleotides which differ from the sequence published by Soeda *et al.* (1980) are indicated by stars. The two sequences, A and B, boxed with dotted lines, represent respectively the Ad5 E1A and SV40-like core sequences of the two enhancer domains shown in Figure 1. C1 and C2 refer to the direct repeat (TCCACCCA) homologous to direct repeats found in the SV40 and BPV enhancers. Inverted repeats are underlined and designated by couples of small letters (a−a', b−b', c−c'). The GC-rich palindrome is boxed with solid lines. X indicates a stretch of 17 nucleotides from the early side of the origin of replication which is duplicated and transposed to the late side in mutant M 206. The arrow points to the location of the AT−GC transition observed in PyEC F9 mutants. Below, the structures of wt TOR and PyEC PCC4 mutants are schematized using the elements pointed out in the wild-type A2 sequence.

separated by 10−20 bp has been identified in the polyomavirus, BPV and SV40 activators (Lusky *et al.*, 1983). In polyomavirus, these two sequences (C1 and C2 in Figure 2), composed of the octanucleotide TCCACCCA, are located in the B domain and surround the point mutation observed in PyEC F9 mutants.

Sequences involved in the host-range phenotype of PyEC mutants

F9 phenotype. A single base pair change in the B domain, common to most F9-adapted mutants, is sufficient to create the F9 phenotype (Fujimura *et al.*, 1981; Mélin, unpublished data). This modification generates a second copy of the SV40-like core enhancer (Fujimura *et al.*, 1981) in the *b'* sequence (Figure 2) and increases the efficiency of the B domain in F9 cells as shown by a CAT enhancement assay (Linney and Donnerly, 1983).

PCC4 phenotype. The rearrangements observed in all PyEC PCC4 mutants affect several elements of the regulatory region. They result in a duplication of part of the A enhancer domain combined with a deletion of part of the B enhancer domain. To distinguish the nature and relative position of the structural features required for the PCC4 phenotype, a scheme of the non-coding region has been drawn (Figure 2) in which sequences of wild-type polyomavirus known or thought to be important for early transcription and replication, or involved in a potential secondary structure, are represented by letters.

The following features are common to all mutants.

(i) The duplication always includes and, in some mutants (500, 5000 and M 206) contains almost exclusively, the A core enhancer sequence (nucleotide 5107−5117). One copy of the A core is kept in its original context, the second is transposed into the deleted B domain. This transposition seems to be important since the tandem repeat (nucleotide 5096−5139), present in polyomavirus Toronto strain (Ruley and Fried, 1983), which also duplicates the A core, does not allow T antigen expression in PCC4 cells.

(ii) The GC-rich palindrome and the *b* sequence in the B domain are conserved in all mutants, suggesting that these elements might be essential.

(iii) The size and location of the deletion vary exceedingly depending on the mutant. It can be seen in Figures 1 and 2 that it affects the other elements of the B domain (the B core enhancer, *b', c, c'*, C1 and C2) and in one case (mutant 204) removes all of them. This implies that these sequences do not need to be preserved or can be replaced in the rearranged genome to maintain virus viability in PCC4 and in secondary mouse embryo cells. Due to the variable size of the duplication and to the variable size and location of the deletion, no constant spacing of key sequences is observed in the rearranged genomes. It cannot be concluded whether the deletion is necessary for, or unrelated to, the expression of the PyEC PCC4 phenotype.

(iv) Since the deletion removes some inverted repeats, we assessed, using a computer program elaborated by Ninio (1979), the potentiality and stability of a secondary structure in the re-

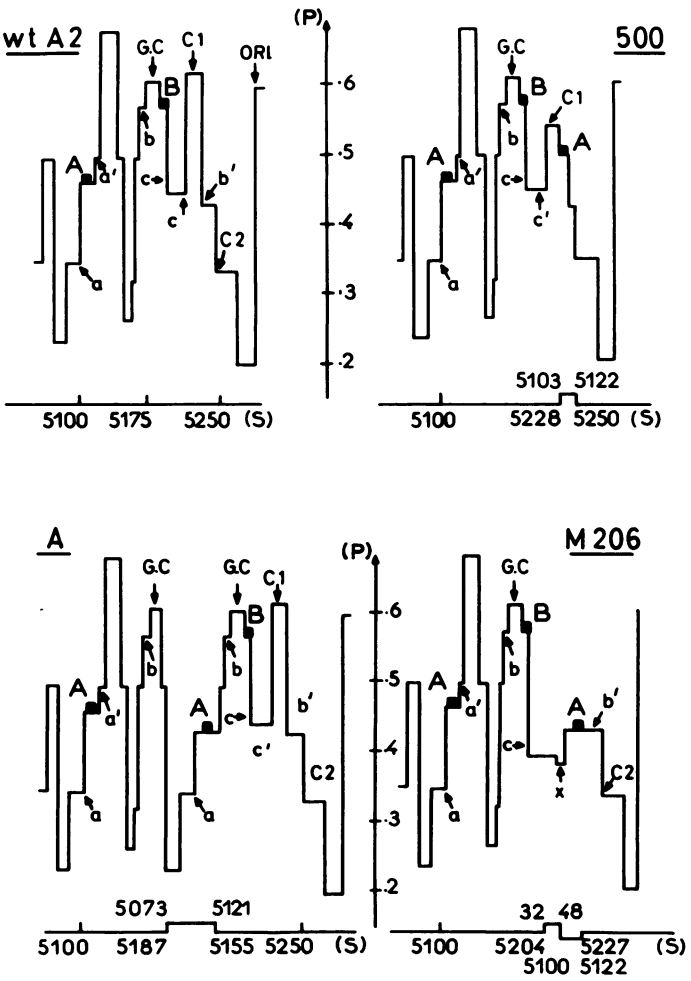

Fig. 3. Stability profiles [stability (P) *versus* DNA sequence (S)] of the non-coding region of polyomavirus wild-type A2 and PyEC PCC4 mutants 500, A and M 206. The profiles were computed according to Gabarro-Arpa and Michel (1982) for a constant environment (W = 2.5). Letters on the profiles refer to the different sequence features described in Figure 2. Arrows associated with letters point to the approximate center of each sequence. Core sequences A and B are indicated by black squares. Nucleotide numbers on the abscissa indicate the endpoints of deletions and duplications shown in Figure 1.

arranged B domain. As previously reported for mutants 97 and 204 (Vasseur *et al.*, 1982), the deletion-duplication event allows, for all mutants, an alternative secondary structure which has a stability very similar to that obtained for the B domain of wild-type DNA ($\triangle G$ = −34 kcal; −142 kJ).

Threfore, adaptation of polyoma virus to PCC4 cells requires the transposition of a copy of the A core enhancer sequence in the vicinity of elements of the B domain and the presence of the GC-rich palindrome, but other sequences, thought to be involved in early transcription, appear to be dispensable.

PyEC F9 mutants can express T antigen at a low level in PCC4 cells (Vasseur *et al.*, 1982). This suggests that duplication of the B core, as a result of a point mutation, is a possible but relatively inefficient substitute to the A core duplication for increasing the efficiency of the polyomavirus enhancer in these cells.

Multiadapted phenotype. All sequenced mutants do not exhibit the same phenotype: mutants A and M 209 (Mélin *et al.*, 1985) also express T antigens on two other EC cell lines, F9 and LT1. Adaptation to all three EC cell lines is a more complex phenomenon. Duplication of the A core enhancer into the B domain and conservation of *b* and of the GC-rich palindrome are still observed, but other sequences also seem to be involved in the polyvalent phenotype.

Comparison of the genome structure of polyvalent mutants A and M 206 with that of mutant 500, which is adapted only to PCC4 cells, indicates that the presence of a B core sequence is necessary but not sufficient. In addition, the relative positions of the B and duplicated A cores are different in the two multiadapted mutants.

In an attempt to correlate the extended host-range phenotype with a particular conformation of the enhancer domains, we analyzed the stability of the DNA of wild-type A2 and of mutants 500, A and M 206 between nucleotides 5070 and 50. For that purpose, stability profiles [stability (P) *versus* DNA sequence (S) at constant environment (W)] were computed by the Gabarro-Michel algorithm (Gabarro-Arpa and Michel, 1982) as already reported in an analysis of the local stability of eukaryotic promoters (Bensimhon *et al.*, 1983). Comparison of the four profiles (Figure 3) shows that, in mutant 500, the duplication-deletion event does not significantly alter the local stability around the B core enhancer. By contrast, in mutants A and M 206 the rearrangements lead to the appearance of a large destabilization of the DNA, either to the left (mutant A) or to the right (mutant M 206) of the conserved B core sequence. We would like to suggest that this local change in stability results in a more efficient enhancing activity of the B core sequence in F9 cells. Adaptation to these cells can therefore be achieved by two types of modification: either by creating a second copy of the B core as a result of a point mutation in the *b'* sequence, or by increasing the efficiency of the existing B core as a consequence of a change in DNA conformation. Both types of rearrangement point out that the B core is the major active enhancer sequence of polyomavirus in F9 cells, without excluding a cooperation with the A enhancer domain.

No polyomavirus mutants adapted to PCC4 and F9 cells but still restricted in LT1 cells, or adapted only to LT1 cells, have been isolated. Either the combination of rearrangements leading to the simultaneous adaptation to F9 and PCC4 cells also results in the ability for the mutant genome to be expressed in LT1 cells; or more subtle and as yet undetected modifications are specifically involved in the PyEC LT1 phenotype.

Tissue-specificity of polyoma enhancer domains

Other types of polyomavirus mutants able to grow on trophoblast cells (Tanaka *et al.*, 1982), on Friend erythroleukemic cells (Delli Bovi *et al.*, 1984) and neuroblastomas (P.Amati, personal communication) have been isolated; they are also affected in the enhancer region and the modifications, which are specific of the cell type they are derived from, consist mainly of duplications and deletions. Rearrangements of this small region of 200 bp are therefore able to confer to the virus a variety of tissue specificities, pointing to the essential role of enhancer sequences in the control of gene expression during differentiation.

Since PyEC mutants have been selected for efficient expression of the viral genome in different EC cell lines, they provide a useful tool to study cell type specificity of the two polyomavirus enhancer domains in their original genome context. However, up to now, the selection procedure of these mutants has always included plaque purification and amplification of the virus on differentiated secondary mouse embryo cells. Therefore only viruses which have kept the ability to replicate both in EC and differentiated cells have been isolated. Recently (H.Pinon *et al.*, in preparation), viral mutant DNA was cloned directly

from EC cells and virus stocks prepared in the same cells. This led to the selection of PyEC mutants which exhibit a lytic effect on all EC cell lines. Whether these mutants are preferentially adapted to EC cells rather than to differentiated cells is under investigation.

Materials and methods

Cell lines and viruses

EC cell lines used are PCC4 (Nicolas *et al.*, 1976), F9 (Berstine *et al.*, 1973) and LT1 (Stevens and Varmus, 1974). Medium and cell culture conditions for these cells are according to Jakob *et al.* (1973). Wild-type polyomavirus A2, large plaque, was obtained from M.Fried (Imperial Cancer Research Fund, London). Mutants PyEC PCC4 97 and 204, have been previously isolated by chronic infection of PCC4 cells using polyoma A2 strain (Vasseur *et al.*, 1980) and mutant M 206 (F.Mélin *et al.*, 1985) using polyoma ev1001 strain (Magnusson and Nilsson, 1982) obtained from F.Cuzin (CNRS, Nice). Viruses were propagated by infecting secondary mouse embryo cells at a multiplicity of 10^{-2} plaque forming units (p.f.u.) per cell.

After molecular cloning, viral stocks were obtained by transfection of secondary mouse embryo cells with *Bam*HI-cleaved plasmid DNA, using DEAE-dextran (1 mg/ml) for 40 min.

Immunofluorescence staining for T antigen

Cells were stained for polyoma T antigens, 48 h post-infection, by indirect immunofluorescence as previously described (Vasseur *et al.*, 1980).

Molecular cloning and DNA sequencing

Mutant DNAs were cloned in the *Bam*HI site of pBR328 for PyEC PCC4 A and of pAT153 for PyEC PCC4 500 and 5000, in the *Escherichia coli* strain HB101 (Maniatis *et al.*, 1982). The small *Bam*HI-*Bgl*I mutant polyoma fragment was cleaved with *Hinf*I and *Hpa*II or with *Pvu*II and *Sau*3A and end-labeled at its 5′ ends with T4 polynucleotide kinase (New England Nuclear) using [γ-^{32}P]-ATP (6000 Ci/mmol, Amersham). The method of Maxam and Gilbert (1980) was used for DNA sequencing.

Computer analysis of nucleotide sequence

The search for secondary structures and their relative stability was done with a computer program developed by Ninio and Dumas (Institut Jacques Monod, Paris) following the principles described by Ninio (1979). Stability profiles (denaturability) of the non-coding region were computed from the nucleotide sequence by the Gabarro-Michel algorithm (Gabarro-Arpa and Michel, 1982). The profiles (stability P *versus* sequence S) have been calculated for a constant value of the environment parameter (W = 2.5).

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