Isolation of Polyomavirus Mutants Multiadapted to Murine Embryonal Carcinoma Cells

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Polyomavirus mutants were isolated from PCC4 embryonal carcinoma cells infected with a variant strain of polyomavirus (ev 1001h) and were found to contain a tandem duplication overlapping the enhancers and the origin of replication. These mutants were able to infect several lines of embryonal carcinoma cells, including PCC4, F9, and LT1. The sequence and structure of one of these mutants are presented and compared with those of other PyEC PCC4 mutants previously described.

Wild-type polyomavirus is commonly found in adult mice but is not infectious for early embryos (9). A similar situation is observed with cultured cells: the virus undergoes a full lytic cycle in differentiated cells (17) but is restricted in murine embryonal carcinoma (EC) cells (18). This provides a positive screen for selecting polyomavirus mutants adapted to EC cells (PyEC), and such mutants have been isolated in different laboratories after chronic infection of two EC cell lines, PCC4 Aza and F9 (4, 7, 15, 21).

All these mutants are not phenotypically identical. They express early viral T antigens at 37°C on the EC cell line on which they have been selected. However, if PCC4-adapted mutants are restricted in F9 cells, some F9-adapted mutants express their T antigens with low efficiency in PCC4 Aza cells (20).

Moreover, during in vitro differentiation, F9 cells acquire sensitivity to PCC4-adapted mutants earlier than they do to wild-type polyomavirus (6). This suggests that a "PCC4-like" state is acquired by F9 cells on their way to terminal differentiation. On the other hand, nullipotent LT1 EC cells restrict the expression of polyomavirus T antigens after infection with either wild-type or F9- and PCC4-adapted mutants; no variant able to escape this restriction has ever been described. This leads to the hypothesis that the mechanisms by which EC cells control viral expression may be quantitatively different or qualitatively different or both among EC cell lines. In all cases, the host range phenotype results from genomic rearrangements in the noncoding region which affect the enhancer sequences (7, 8). However, the type and location of these rearrangements are clearly different in both classes of PyEC. F9-adapted mutants exhibit modifications in the enhancer of the *PvuII* 4 fragment (10), usually a single base-pair change (AT to GC at position 5233) combined with various duplications, including the point mutation. PCC4-adapted mutants have a partial deletion in the *PvuII* 4 fragment but bear a duplication in the *BclI-PvuII* fragment, where the second enhancer is localized (22). One would therefore expect the deletion in the first enhancer to prevent further adaptation of a PyEC PCC4 mutant to F9 cells.

In this paper, we describe the selection of mutants from the polyomavirus ev 1001h strain (11), which carries a tandem duplication spanning the enhancers and the origin of

replication. By exerting selection pressure on a strain with four enhancers, our aim was to determine whether new combinations of rearrangements among them could yield new classes of mutants. We describe here PCC4 Aza-adapted polyomavirus mutants which can express their T antigens not only in PCC4 Aza cells but also in F9 cells and, most remarkably, in LT1 cells.

MATERIALS AND METHODS

Cell lines and viruses. The EC cell lines used in this study are listed in Table 1. They were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO Laboratories) on gelatin-coated dishes.

Wild-type polyomavirus A2 was obtained from M. Fried (Imperial Cancer Research Fund, London, England). Strain ev 1001h, a non-hemagglutinating variant of mutant ev 1001 (11), was obtained from F. Cuzin (Centre National de la Recherche Scientifique, Nice, France). All strains were usually propagated by infecting secondary mouse embryo cells at a multiplicity of infection of 10^{-2} PFU per cell. Polyomavirus genomes cloned in pBR328 were excised by restriction enzyme digestion and used to transfect secondary mouse embryo cells with DEAE-dextran (1 mg/ml) for 40 min.

PyEC PCC4-97 and PyEC F9-1, expressing their T antigens, respectively, in PCC4 Aza and F9 cells, have been described previously (7, 21).

Immunofluorescence staining for polyomavirus T antigens. Cells were stained for T antigens by an indirect immunofluorescence technique (21).

Molecular cloning and DNA sequencing. Polyomavirus genomes were inserted at the *Bam*HI site of pBR328 (3) and transfected in *Escherichia coli* DP 50 (2). The method of Maxam and Gilbert (12) was used for sequencing DNA.

RESULTS

Chronic infections and recovery of mutants. Five separate experiments were run in parallel. PCC4 Aza cells were infected with strain ev 1001h at 100 to 200 PFU per cell. Infected cells were passaged twice a week and checked every week for polyomavirus T-antigen expression. Positive cells were first observed at the passage 3, but stable expression in 20 to 30% of all cells was obtained only by passage 18. Viral stocks were then plaque purified at 31°C on

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TABLE 1. Main characteristics of the three EC cell lines studied

Cell line	Origin		Ability to differentiate		Reference
	Mouse strain	Cell line from:	In vivo	In vitro with inducers	
PCC4	129/Sv	Embryoid body OTT 6050	Yes	Yes	13
F9	129/Sv	Embryoid body OTT 6050	No	Yes	1
LT1	LT	Ovarian cyst 72484	No	No	16

secondary mouse embryo cells with virus dilutions yielding 0 to 1 plaque per plate; a single isolated plaque was set aside in each experiment and named Py-Mg1, Py-Mg2, Py-Mg3, Py-Mg4, and Py-Mg5. This initial harvest was further amplified on secondary mouse embryo cells and then tested for T-antigen expression in various EC cell lines.

T-antigen expression and virus production in EC cells. All five mutants expressed T antigens in PCC4 Aza cells at the same level as any other PCC4-adapted mutant. However, with the exception of Py-Mg1, they also gave positive results in F9 and LT1 cells (Table 2); this had not been observed with the PyEC PCC4 mutants previously described (21). A more thorough analysis of Py-Mg4 is given as an example in Table 3. It shows that T-antigen expression was always enhanced when infected cells were incubated for 72 h at 31°C instead of for 48 h at 37°C. The temperature dependence of viral expression has been reported in Aza-infected PCC4 cells but has not been observed in F9 cells infected by other polyomavirus-adapted mutants (6).

As already noticed with previously isolated mutants (J. Aghion and L. Dandolo, unpublished results), the virus yield in PCC4 and F9 cells was 10- to 20-fold lower than that obtained with the same mutants in secondary mouse embryo cells. However, virus production in M206-infected LT1 cells was about 1 order of magnitude lower than that in F9 and PCC4 cells.

Genome structure. A schematic representation of the noncoding region of the ev 1001h strain is shown in Fig. 1. A single *HpaII* site was found within the duplication. Digestion with this enzyme yielded a fragment (*HpaII*-8) containing

TABLE 2. Expression of T antigens at 37°C 48 h after infection^a

Infecting virus	T-antigen expression in infected cells			
	Secondary mouse embryo	PCC4	F9	LT1
Wild type	+	—	—	—
PyEC PCC4-97	+	+	—	—
PyEC F9-1	+	+	+	—
ev 1001h	+	—	—	—
Py-Mg1	+	+	—	—
Py-Mg2	+	+	+	+
Py-Mg3	+	+	+	+
Py-Mg4	+	+	+	+
Py-Mg5	+	+	+	+

^a A multiplicity of infection of 100 to 200 PFU per cell was used. T antigens were detected by indirect immunofluorescence. PyEC PCC4-97 is a PCC4-adapted mutant (8). PyEC F9-1 is an F9-adapted mutant (7).

the entire duplication (244 base pairs). This fragment does not exist in wild-type polyomavirus A2 and was also lost in all Py-Mg mutants (Fig. 2). Adaptation to PCC4 cells was consistently associated with rearrangements in the noncoding area, as confirmed for at least two mutants (Py-Mg1 and Py-Mg4) by alterations in the *HpaII* 3 fragment and by *PvuII* and *HinfI* digestions (data not shown).

Cloning and sequencing of a polyvalent mutant. The genome of Py-Mg4 was cloned in the *Bam*HI site of pBR328. DNA from individual clones was digested with *Bam*HI and transfected on secondary mouse embryo cells. Viral harvests were then tested for T-antigen production in PCC4 Aza, F9, and LT1 cells. Clone M206 generated a polyvalent viral stock (Fig. 3). By in vitro reconstruction, the small *Bam*HI-*Bgl*II fragment was shown to carry the modifications responsible for the mutant phenotype. The sequence of M206 was determined from nucleotide 5073 through the origin to nucleotide 93 and compared with those of the original ev 1001h and wild-type A2 strains (Fig. 1A).

Clone M206 is derived from ev 1001h by two deletions which affect both copies of the duplicated sequence. (i) A deletion from nucleotide 5204 to nucleotide 32 removes one origin of replication and part of the *PvuII* 4 fragment but leaves intact the simian virus (SV40)-like core enhancer sequence TGTGGTTTGT (22). (ii) A deletion from nucleotide 5122 to nucleotide 5228 removes the second copy of the SV40-like enhancer but does not alter the adenovirus early region 1A (Ad5 E1A)-like core enhancer sequence CAG-GAAGTGAC (22). This deletion is associated with a duplication of a CTAG sequence at its origin proximal boundary.

As a result of these two deletions, the general structure of M206 closely resembles that of two other mutants which were selected on PCC4 cells from wild-type polyomavirus A2 and which have been described previously (8). This resemblance includes the ability of the noncoding region to be folded into a stable secondary structure, as previously proposed (20). However, the polyvalent clone M206 exhibits distinctive features (see below).

DISCUSSION

The use of a chronic infection to select polyomavirus host range mutants adapted for growth in EC cells is a valuable tool; F9- and PCC4 Aza-adapted mutants have been recovered and analyzed. In both cases, the phenotypic changes are associated with genotypic rearrangements involving one of the two transcriptional enhancers identified in the noncoding region of the viral genome (22). PyEC PCC4 mutants (8) exhibit a duplication of sequences, including the Ad5 E1A-like enhancer, accompanied by a deletion which removes the SV40-like enhancer present in the *PvuII* 4 frag-

TABLE 3. Effect of temperature on T-antigen expression in EC cells^a

Infecting virus	% of infected cells expressing T antigen at indicated temp (°C)					
	PCC4		F9		LT1	
	31	37	31	37	31	37
Wild type	3-10	0.01	0.1	0	0.01	0
ev 1001h	1-2	0.1	0.1	0.1	0.01	0
Py-Mg4	30-40	20-30	15-20	3-10	25-40	10-15

^a Infected EC cells were incubated either for 48 h at 37°C or for 72 h at 31°C and checked for T-antigen expression by indirect immunofluorescence.

FIG. 1. Sequence rearrangements in mutant M206. (A) Schematic drawing of the control regions in three strains of polyomavirus: wild-type A2 (wt), ev 1001h (ev 1001), and M206 (M 206). ev 1001h bears a tandem duplication from nucleotide 5100 to nucleotide 48. The duplication has a single point mutation, G $\rightarrow$ A, at position 5228 (G. Magnusson, personal communication) ($\blacktriangle$). M206 is derived from ev 1001h by two deletions which leave intact the nucleotide 48-nucleotide 5100 junction and by a CTAG addition at the end of the second deletion. The alternating dark blocks indicate the region of a large potential secondary structure in the *Pvu*II 4 fragment. Ori, Origin of replication. (B) Detailed sequence and potential secondary structure. The heavy arrows indicate inverted repeats which limit the large secondary structure proposed for the wild-type strain. Symbols: $\blacksquare$, SV40-like core enhancer sequence; $\blacktriangledown$, Ad5 E1A-like core enhancer sequence; $\bullet$, AGAGGC sequence found in T-antigen-binding sites. Pu, Stretch of 18 purines. CTAG direct repeats are overlined. Numbering is according to Tyndall et al. (19).

ment. On the other hand, most PyEC F9 mutants (4, 7, 15) have a single point mutation which creates, in the *Pvu*II 4 fragment, a second copy of the SV40 enhancer consensus sequence; in some cases, there is also a tandem duplication which contains the point mutation. As a consequence of such a difference in the two types of rearrangements, mutants simultaneously adapted to both PCC4 and F9 cells have never been described. Furthermore, LT₁ cells, which have been shown to be representative of a third class of EC cells (6), are resistant to both PCC4- and F9-adapted mutants.

By selecting on PCC4 cells mutants from a viral strain which carries a tandem duplication overlapping the two enhancers and the origin of replication (11), a new phenotype has been obtained; although initially adapted for growth in PCC4 cells, these mutants can also express their T antigens in both F9 and LT₁ cells. This is the first time that such a polyvalent adaptation has been reported.

The rearrangements which generate the PyEC M206 polyvalent clone from ev 1001h lead to a general structure which is very similar to that previously described for PCC4-adapted mutants (Fig. 1): (i) duplication of a sequence overlapping the Ad5 E1A-like enhancer (nucleotides 5100 to 5122); (ii) deletion of part of the *Pvu*II 4 fragment in the tandem duplicated sequence, leading to the transposition of the adenovirus-like enhancer into the environment of the SV40-like enhancer; and (iii) no alteration of the G $\cdot$ C-rich palindrome (nucleotides 5170 to 5187).

FIG. 2. *Hpa*II fragment pattern analysis on 4% acrylamide gels. The *Hpa*II 8 fragment (indicated by an 8) of ev 1001h, which contains the second origin of replication, was lost in all Py-Mg mutants, and the *Hpa*II 3 fragment (indicated by a 3) was modified. Lanes: a, Py-Mg2; b, Py-Mg5; c, Py-Mg4; d, ev 1001h; e, Py-Mg3; f, Py-Mg1; g, wild-type polyomavirus A2.

FIG. 3. Phenotype of viral clone M206. At 48 h after infection with M206 virus, cells were fixed and analyzed for T-antigen expression. After being stained with fluorescein-labeled immunoglobulin, cells were washed once with a solution of ethidium bromide (1 μ g/ml) in phosphate-buffered saline and then washed twice with phosphate-buffered saline alone. Therefore, under UV lighting, positive nuclei appeared yellowish green, and negative ones appeared red. Color slides were duplicated in black and white with a filter to increase the relative intensity of positive nuclei. (a) PCC4 Aza cell line; (b) F9 cell line; (c) LT1 cell line.

On the other hand, clone M206 does not display the typical point mutation of F9-adapted mutants or any of the more complex rearrangements found in other mutants of this class (20). It does, however, exhibit distinctive features (Fig. 1B) which have not been seen in any of the mutants previously described: (i) the SV40-like enhancer of the *Pvu*II 4 fragment is conserved upstream from the duplicated adenovirus-like enhancer; (ii) 17 base pairs from the early side of the origin of replication (nucleotides 32 to 48), including an AGAGGC sequence found in the T-antigen-binding sites (5), are inserted close to the transposed adenovirus-like enhancer; and (iii) the deletions have generated a sequence between nucleotides 5200 and 5234 which contains 41 purines out of 57 nucleotides, including a stretch of 18 purine residues in a row.

It should be pointed out that the mere presence of two copies of the Ad5 E1A-like enhancer and of one or two copies of the SV40-like enhancer is not sufficient to generate the polyvalent phenotype. This is illustrated by the inability of strain ev 1001h and of spontaneous variants of polyomavirus containing duplications of the enhancer (14) to express their T antigens in any EC cell line. We do not know presently which of the new features or combinations of them is responsible for the polyvalent phenotype. Other similar mutants which have already been isolated need to be sequenced to determine what their common characteristics are.

Although M206 expresses T antigens at a comparable level in all three EC cell lines, virus production in LT1 cells is 10-fold lower than that in PCC4 or F9 cells. It should be pointed out that attempts to isolate PyEC mutants with polyomavirus A2 by chronic infection of LT1 cells have always failed. This observation, together with the low virus production observed with M206, suggests that full viability in LT1 cells requires additional modifications of the viral genome. In fact, this is reminiscent of the differences in genome rearrangements leading to the PCC4 or F9 phenotype. A new approach for isolating mutants on LT1 cells which has permitted us to obtain a new class of mutants exhibiting full adaptation to all EC cell lines has now been devised (H. Pinon and F. Mélin, unpublished results).

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