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Physical and Biological Features of Polyoma Virus Mutants Able to Infect Embryonal Carcinoma Cell Lines

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Three new polyoma mutants were selected for their ability to grow on the embryonal carcinoma cell line F9. These mutants share in common an insertion of two nucleotides, a thymine and an adenine, in the noncoding region located on the late side of the origin of replication. We have found that these insertions exist in all of the other polyoma virus mutants able to grow on F9 cells (Fujimura et al., Cell **23**:809–814, 1981; Katinka et al., Nature (London) **290**:720–722, 1981; K. Sekikawa and A. J. Levine, Proc. Natl. Acad. Sci. U.S.A. **78**:1100–1104, 1981). The region containing these insertions could be folded into a stable secondary structure which included a guanine plus cytosine (G+C)-rich stem. The adenine and thymine were inserted in such a way that they maintained the palindrome in the G+C-rich stem and were complementary in the putative secondary structure that we present here. Another class of polyoma virus mutants selected on a multipotential carcinoma cell line (PCC4-Aza) were characterized by a more complex rearrangement (deletion and duplication) which occurred in the same region. This arrangement preserved the G+C-rich palindrome and also yielded a sequence which still allowed the folding of another type of stable secondary structure. The significance of these findings is discussed.

Polyoma virus (Py) is a mostly silent and permanent host of the mouse (29). In most differentiated murine cell cultures, Py completes a lytic cycle with the liberation of mature viral particles. In semipermissive rodent cells, such as hamster (15) or rat (25), only the early viral functions are expressed, without apparent DNA replication, and some of the cells may acquire a transformed phenotype (31). Murine embryonal carcinoma (EC) cells isolated from teratocarcinomas are similar to the pluripotent cells of the embryo (7). As early embryonic cells, EC cells are refractory to infection by Py and a number of other viruses (14). The virus penetrates these cells and is uncoated normally in the nucleus but does not express either early or late functions (5). Boccara and Kelly (6) have shown that the block to viral development probably resides in the expression of the early functions. In our quest to better understand the virus cell interactions, we sought Py variants capable of growth on two EC cell lines: PCC4-Aza (11) and F9 (4). PCC4 cells differ from the F9 cells in their ability to differentiate both in vivo and in vitro, whereas the F9 cells only differentiate in vitro when induced with retinoic acid (11). We previously reported the isolation and characterization of Py mutants capable of growth on PCC4-Aza (13) and on F9 cells (12). We report here the isolation and characterization of three new Py EC F9 mutants. We analyzed the region where the different point mutations and rearrangements occurred in the mutants and detected an extremely limited region of the Py genome involved in all of the modifications observed in the PCC4 and F9 mutants. This same region included the DNase I-sensitive region observed in the chromatin of Py wild type and Py EC PCC4-97 (9). The importance of the local sequence changes and the unusual chromatin structure will be discussed in respect to their possible effect on transcription or replication of the virus and the host range of this type of mutant.

MATERIALS AND METHODS

Virus and cells. The large-plaque A2 strain of Py that is considered the wild type (w.t.), obtained from M. Fried, ICRF, London, was propagated by infecting secondary mouse embryo cells at a multiplicity of infection (MOI) of 0.01 PFU per cell. Py EC PCC4-97 and PCC4-204 and Py EC F9-1 and F9-5 were obtained and propagated as previously described (12, 34).

PCC4-Aza 1 is an EC cell line derived from the transplantable mouse teratocarcinoma OTT6050 and selected for resistance to azaguanine (11). F9 is a

blocked EC cell line also derived from OTT6050 (4). PCD3 is a fibroblast-like cell derived from the EC PCC3 cell line (20). The other EC cell lines with their references are as follows: PCC7 (18), PCC3 (11), PCC3 T 1090 (unpublished data), PSA-2 (16), C 17S1 1003 (18), and LT1 (28).

Medium and cell culture conditions for these cells were those described by Nicolas et al. (20). FR-3T3 rat cells were a gift from F. Cuzin, Centre universitaire, Nice, France.

Immunofluorescence assay. Indirect immunofluorescence detection of tumor (T) and viral capsid (V) antigens was done as previously described (34).

Preparation of viral DNAs. Secondary mouse embryo cells were infected with either A2 w.t. Py or clonal isolates of the Py EC mutants. At 4 days after infection, the viral DNA was extracted by the Hirt procedure (10) and, after phenol treatment, submitted to cesium chloride-ethidium bromide equilibrium centrifugation.

Molecular cloning: ligation, transformation, transfection, and plasmid purification. Ligation of Py w.t. or EC F9 mutants to pBR322 and the construction of hybrid viral genomes were done by standard procedures as previously described in detail (13). Transformation of *Escherichia coli* K-12 DP-50 or *E. coli* K-12 C600 was done as described elsewhere (13). Chloramphenicol-amplified plasmids were extracted by lysing bacteria with 1 mg of lysozyme (grade I; Sigma Chemical Co.) per ml, 50 mM EDTA, and 0.05% Triton X-100, heated immediately for 10 min at 70°C. After ultracentrifugation of the plasmids at 40,000 rpm for 30 min, the DNA was precipitated with 10% polyethyleneglycol 6000 in 0.5 M NaCl and submitted to cesium chloride-ethidium bromide equilibrium centrifugation. Cell transfection was done by the method of McCutchan and Pagano (19).

Enzymes and electrophoretic systems. Restriction enzymes were either purified by the standard procedures or purchased from Bethesda Research Laboratories, New England BioLabs, or Boehringer Mannheim Corp. T_4 polynucleotide kinase and ligase were purified by the methods of Richardson (22) and Tait et al. (30), respectively. Slab gels of 0.7 to 2% agarose (Sigma; type II) or of 4 to 10% acrylamide (29:1, acrylamide:bisacrylamide) were run in a buffer of 90 mM Tris-borate (pH 8.45)–2.5 mM EDTA as previously described (12).

DNA sequencing. DNA was 5′ end labeled with [γ-^{32}P]ATP (Amersham Corp.; 3,000 Ci/mmol) and T_4 polynucleotide kinase by the exchange reaction, as previously described (12). The nucleotide sequence was determined by the partial chemical degradation procedure of Maxam and Gilbert (17). After fractionation of the products on a 0.35-mm-thick, 6 or 20% polyacrylamide–7 M urea gels as described by Sanger and Coulson (23), the gels were submitted to autoradiography.

Marker rescue of the Py EC F9 phenotype. Py w.t. DNA was linearized with restriction endonuclease *Bcl*I and further digested with S1 nuclease to prevent reannealing of the four-nucleotide cohesive ends. DNA from Py EC F9-5 was digested by restriction endonucleases *Bam*HI and *Hpa*II, and the *Hpa*II-*Bam*HI fragment of 659 base pairs (bp) containing the thymine and adenine insertions was isolated (see Fig. 1; the *Bam*HI site is not represented, but is positioned 659 nucleotides downstream from the *Hpa*II site towards the late region). The w.t. DNA and the DNA from Py EC F9-5 were mixed at a ratio of 1:1 (wt/wt), denatured with 0.2 M NaOH at 20°C, neutralized with Tris-hydrochloride (pH 7.5), and allowed to renature for 10 min at 68°C. The mixture was then used to transfect secondary mouse embryo cultures, and the virus burst was tested for its infectivity on F9 cells.

Computer analysis of nucleotide sequences. The search of secondary structures and their relative stability was done with a computer program developed by J. Ninio and J. P. Dumas following the principles described by Ninio (21).

RESULTS

Isolation of Py EC F9 mutants. When permissive PCD3 differentiated mouse cells were infected with w.t. Py at very high MOIs of 1,000 to 10,000 PFU per cell, a very heterogeneous population of viral genomes was recovered. This population was used to infect EC F9 cells at an MOI of 10 to 100 PFU per cell. Alternatively, the F9 cells were infected at a very high MOI (5,000 PFU per cell) with a mostly homogeneous viral stock of w.t. Py. This procedure was used to determine whether naturally occurring EC-type mutants may be found in regular stocks of Py propagated at low MOIs. In both experiments, by 72 h after infection less than 0.01% of the F9 cells were T antigen positive as monitored by immunofluorescence. This value was much lower than that obtained with w.t. Py on PCC4-Aza, (1% T antigen-positive cells) described elsewhere (34). Nevertheless, that we obtained T-positive F9 cells after infection with either heterogeneous or regular Py stocks implies that the EC mutation occurs easily and may be present in most stocks of Py prepared at low MOIs. The infected cells were passaged every 2 days, and after 2 months 30 to 40% became T and V antigen positive. The virus recovered from the chronically infected EC F9 cells was plaque purified twice on secondary mouse embryo cells. When used to reinfect F9 cells at an MOI of 50 PFU per cell, by 48 h postinfection 60% of the cells were T antigen positive. However, when assayed by plaque formation, the viral yield on F9 cells was 10 times lower than that obtained on secondary mouse embryo cells. The five Py EC F9 mutants isolated (12; this work) showed the same percentage of T antigen-positive cells when used to infect F9 cells.

Biological properties of the Py EC F9 mutants. The biological properties of the newly isolated Py EC F9 mutants were tested. Five independent clones of Py EC F9 mutants obtained by two different procedures were used in this study. Their ability to transform rat 3T3 fibroblasts in vitro was noticeably the same as that found for their parental w.t. Py. They were likewise capable of inducing tumors in newborn hamsters and

TABLE 1. Detection by immunofluorescence of T antigen-positive EC cells after infection with Py EC F9 mutants

EC cell line	T antigen-positive cells (%)[a]
F9	40–60
PCC7	40–60
PCC4-Aza	5–10
PCC3	5–10
PCC3 T 1090	5–10
PSA-2	5–10
C 17S1 1003	1–5
LT1	0.5–1

[a] Percentage of cells shown to be T antigen positive at 48 h postinfection. MOI, 50 PFU per cell.

of growing on a variety of differentiated mouse cell lines without any detectable restriction. The Py EC F9 mutants were checked for their ability to grow on different EC cell lines. Mutants previously isolated on PCC4-Aza cells were found to be totally negative when tested for T and V antigen on F9 cells. In contrast, Py EC F9 mutants were efficient in infecting PCC4-Aza cells: at 48 h postinfection at an MOI of 100 PFU per cell, 6 to 10% of the cells were T and V antigen positive. The growth efficiencies of the F9 mutants were tested on other EC cell lines by immunofluorescence detection of T antigen-positive cells. All EC cell lines were found to be positive but to different extents (Table 1). These results were independent of the F9 mutant used for infection. The EC strains studied were different in their differentiation potencies (PCC3 versus PCC3 T 1090), in their mouse strain origin (PCC3 and PCC4 arise from a teratoma of the 129/Sv strain, while C 17S1 arises from strain C3H), or in the teratocarcinoma from which they were derived (PCC3 and PCC4 originate from OTT6050, whereas PSA-2 is derived from OTT5568). Thus, in spite of the differences, these cells seemed to restrict viral expression in the same way. This restriction could be overcome by the same type of mutation.

Physical characterization of mutants. DNA from the five different mutants was prepared on secondary mouse embryo cells and submitted to restriction endonuclease analysis. All mutants conserved their unique sites (*Bgl*I, *Bam*HI, *Eco*RI, and *Bcl*I) as well as the two *Knp*I and *Hinc*II sites of the Py w.t. genome (data not shown). Further restriction analysis, particularly around the origin of Py DNA replication, was performed. Unlike the Py EC PCC4 DNA, no common feature for the five Py EC F9 mutant DNAs could clearly be found. One mutant (Py EC F9-1) had the same restriction pattern as did the w.t. Py when restricted with *Hpa*I, *Hae*III, or *Pvu*II; another (Py EC F9-5) had additional *Pvu*II and *Hpa*II fragments of about 180 bp; yet another (Py EC F9-5000) had its *Pvu*II-4 fragment shortened by about 20 bp; a fourth mutant (Py EC F9-HM) had its *Pvu*II-4 fragment increased by about 90 bp; and the fifth (Py EC F9-i) had an additional *Pvu*II fragment of 280 bp. All of these genomic modifications could be localized around the origin of DNA replication (Fig. 1).

To determine the genomic piece which allowed new growth capacities, we conducted a marker rescue experiment, as described above. The virus obtained on secondary mouse embryo cells was found to be infectious on F9 cells.

Molecular cloning of the Py EC F9 DNAs. Although the viral DNA obtained from the mutants that were plaque purified twice was homogeneous as judged by gel electrophoresis, we found it worthwhile to clone the viral genomes in *E. coli* K-12 C600. This was done to exclude any possibility that a minor population was solely, or in complementation with the major one, responsible for the new host range capacities of the virus.

The *Bam*HI-linearized Py genomes were inserted into the unique *Bam*HI site of pBR322. DNA from 10 to 15 recombinant plasmids of each mutant was purified and subjected to endonuclease restriction analysis. About 90% of the clones had mutant viral DNA restriction patterns homologous to the initial viral genomes.

It must be noted that whenever a Py genome (PCC4 or F9 mutants) contained a duplicated sequence, the right orientation was preferred to the left one for insertion into pBR322 (8 or 9 clones out of 10). We defined the right or left orientation as the longer or shorter distance, respectively, between the *Eco*RI site of pBR322 and the origin of replication of Py. When no duplicated sequences were present (i.e., Py EC F9-1 or Py EC F9-5000), the distribution of right or left recombinant plasmids was roughly equal. We have no satisfactory explanation for this fact.

Nucleotide sequence of the Py EC F9 DNAs. Restriction mapping of the Py EC F9 mutants suggested that all of the rearrangements were localized near the origin of DNA replication on its late side. By sequencing, we found that the early side of the genome was identical to w.t. Py for about 200 nucleotides into the early region from the junction of *Hpa*II fragments 3 and 5 (Fig. 1). To characterize the late side, we sequenced three of the F9 Py mutants presented in this paper from three sites on the cloned genomes: the junction of *Hpa*II fragments 3 and 5 into the late region; the *Bgl*I site towards the late region; and *Bcl*I towards the origin (Fig. 1). Sequences were done in parallel on the cloned and uncloned viral genomes, and no difference

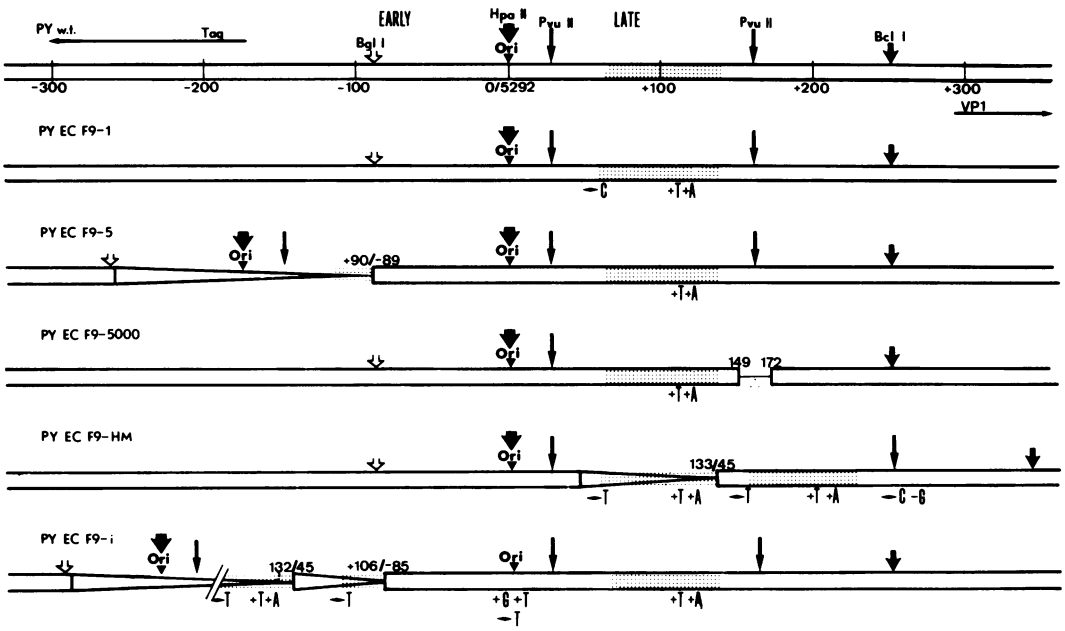

FIG. 1. Scheme of the genomic organization around the origin of DNA replication in five Py EC F9 mutants. Shown in the upper line is the organization of the 600 bp around the origin (Ori) of DNA replication in w.t. Py, including the N-termini of the early T antigen and the VP2 late capsid protein. The region of the genome around +100 that includes the putative secondary structure shown in Fig. 3 is dotted. Aligned with the origin of DNA replication are the five F9 mutants with their additional (+), deleted (−), or transitive nucleotides (▲), and the duplicated sequences and deletion in Py EC F9-5000 (△). The sites for the restriction endonucleases are shown.

could be detected in the nucleotide sequence around the origin of DNA replication. Py EC F9-5000 had a 21-bp deletion from nucleotide 150 to nucleotide 171 (the numbering system follows that described in reference 27). Otherwise, the mutant had a sequence identical to that of w.t. Py except for the insertion of a thymidine between nucleotides 106 and 107 and an adenine between nucleotides 120 and 121 (Fig. 2). In Py EC F9-HM, the sequence between nucleotides 45 and 132, which contained the thymine and adenine insertions, was duplicated. In both duplicated segments there was also a transition of nucleotide 63 from adenine to thymine. Two additional differences were found between the sequences in Py EC F9-HM and w.t. Py: deletion of the guanine at position 166 and replacement of the cytosine at position 165 by a thymine (Fig. 2). Py EC F9-i presented an even more complex picture (Fig. 1). The sequence revealed two duplications; the first stretching from nucleotides 85 to 132, the second from nucleotides 45 to 106. The insertion of a thymine between 106 and 107 and an adenine between 120 and 121 was present in each duplicated sequence. The transition of an adenine to a thymine at nucleotide 63 occurred twice in the three times that nucleotide 63 was present and on the two left-hand duplicated sequences (Fig. 1 and 2). Moreover, in the right-hand copy of the origin of replication at the *Hpa*II site there was an insertion of a guanine between nucleotides −6 and −5, an insertion of a thymine between −2 and −3, and a transition of a cytosine into a thymine at −1, thus destroying the *Hpa*II site (Fig. 2).

Computer analysis of the potential secondary structure. To analyze the different rearrangements observed, we systematically investigated the potential secondary structures along the late noncoding region (nucleotides 26 to 230). The computer program designed by Ninio and Dumas was used, which selects the most probable secondary structure of RNAs by both thermodynamic and statistical criteria (21). In the w.t. sequence a very prominent potential secondary structure thus appears, from nucleotide 62 to nucleotide 130, with a ΔG as high as −34 kcal/mol (−142 kJ/mol). When inserting the F9 rearrangements into this structure, a very peculiar feature stands out: in all five mutants, an adenine and a thymine are inserted into the major stem of this secondary structure, stem A, so that the adenine and the thymine match each other to add an extra A·T base pair into the stem, although at two possible places (Fig. 3). This defined the only feature common to all of the F9 mutants.

In the PCC4 mutants, half of the sequence

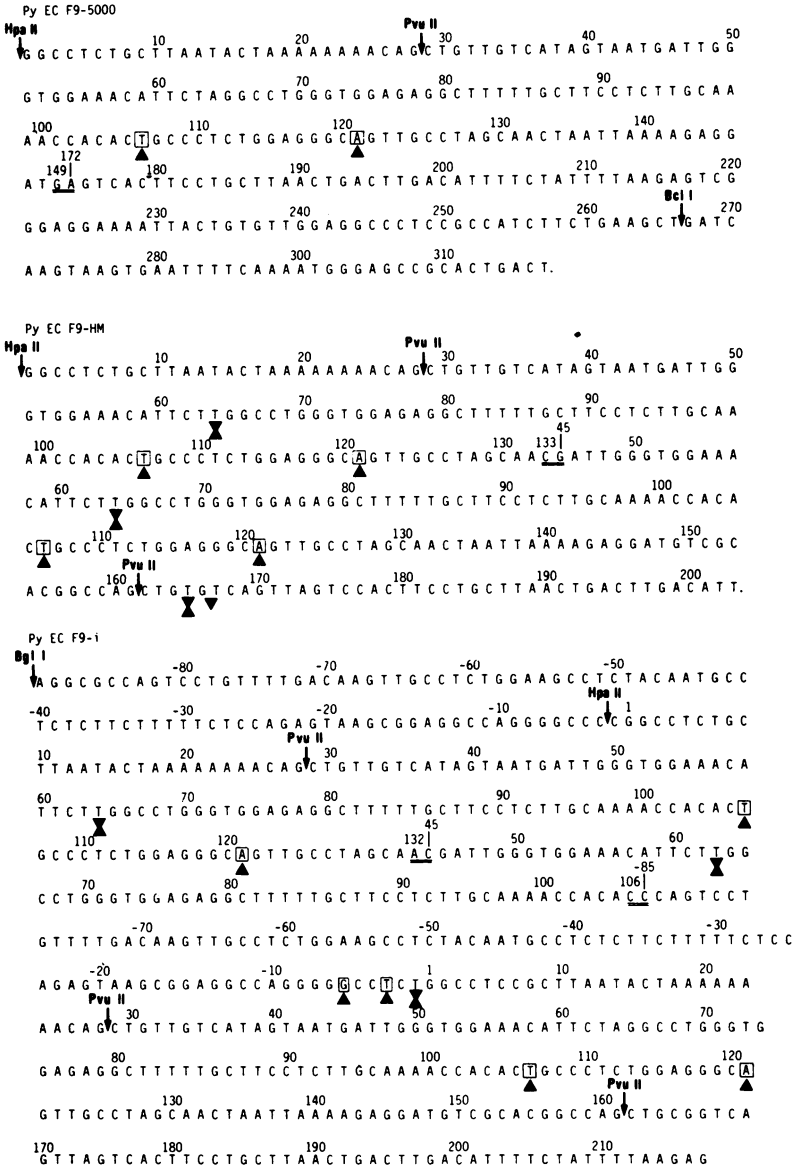

FIG. 2. Nucleotide sequence of the Py EC F9 mutants. The sequences presented here start at the junction of *Hpa*II fragments 3 and 5 at the origin of DNA replication and proceed toward the late region of the genome. For Py EC F9-i, the sequence starts at the *Bgl*I site on the early side at nucleotide −90 and proceeds toward the origin of DNA replication. Inserted (▲), deleted (▼), or transitive nucleotides (⧗), as well as the duplication or deletion junction nucleotides (double underlining), are indicated. The numbering system follows that described by Arrand et al. (1).

involved in the putative secondary structure was deleted, precisely removing the portion up to the initial A·T pairing of palindrome A. However, a very stable alternative secondary structure was still possible (ΔG, −26.7 kcal/mol [−111 kJ/mol]) due to the insertion of a sequence situated downstream (nucleotides 157 to 220) which includes a long dyad symmetry (Fig. 4, stem B).

No other structure of similar stability could be found over the rest of the genome, and the next most stable putative secondary structures, at the origin of DNA replication, had a stability of −21 kcal (−87.9 kJ).

DISCUSSION

Py does not develop in EC cell lines. Having isolated Py mutants capable of growth in two EC cell lines, PCC4-Aza and F9, we established that

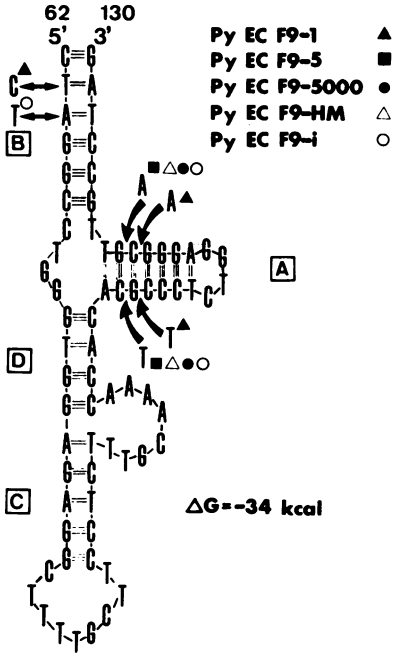

FIG. 3. Putative secondary structures in w.t. Py and the Py EC F9 mutants. The structure on the early coding strand in w.t. Py is shown with the modifications brought by the insertions (▲) or transitions (▼) in the five Py EC F9 mutants. A–D: Decreasing order in which each double-stranded fold contributes to the total stability of the structure.

both groups of mutants retain all the properties of w.t. Py. Restriction analysis of their genomes showed no detectable alteration of the coding regions. The sole but persistent difference from w.t. Py could be located on the late side of the origin of DNA replication in the noncoding region, roughly covering nucleotides +10 to +230. The genome of all of the Py EC PCC4 mutants showed a rearrangement by which a deletion stretching from a variable left-hand border (+45 or +77) to a constant right-hand border (+107) was replaced by a duplication of a downstream sequence with, again, a variable left-hand start (+138 or +157) and a constant right-hand stop at +220 (Py EC PCC4-204 and Py EC PCC4-97, respectively [15]). On the contrary, the five Py EC 79 mutants did not show a homogeneous pattern of rearrangement. However, four of them displayed an insertion of a thymine between nucleotides 107 and 108 and an adenine between nucleotides 121 and 122. In Py EC F9-1 (12), the thymine is inserted between 108 and 109 and the adenine between 120 and 121.

Two recent papers by Sekikawa and Levine (26) and Fujimura et al. (8) also describe the isolation and characterization of Py EC F9 mutants. Although they used different Py strains (small-plaque Toronto and large-plaque A3, respectively), their mutants had in common a unique nucleotide transition: a thymine at position 63 was changed to a cytosine. We found this transition in only one mutant (Py EC F9-1). Fujimura et al. noted that only 0.1% of the F9 cells are T antigen-positive when infected with mutants containing only one copy of this transition, whereas 5 to 10% of the cells are positive when the sequence containing this transition is duplicated. With our Py EC mutants originating from the A2 strain, the percentage of the T antigen-positive cells attained 40 to 60% with or

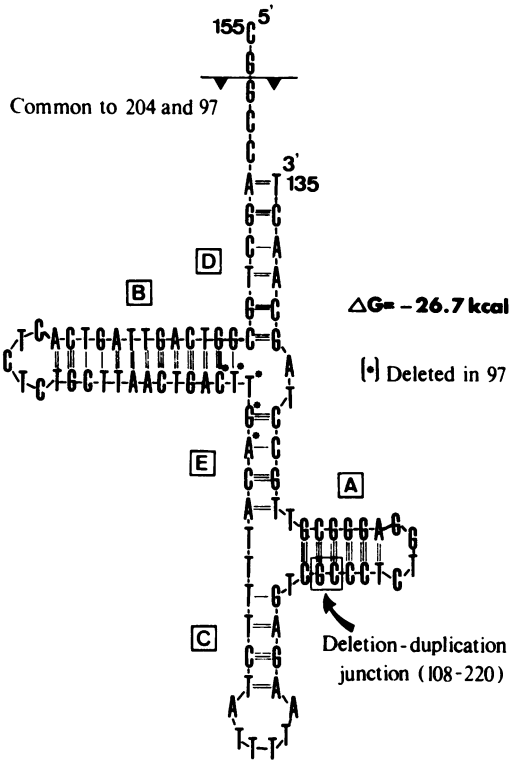

FIG. 4. Putative stable secondary structures in Py EC PCC4-204 and PCC4-97 mutants. The Py EC PCC4 mutants, unlike the F9 ones, have a complex rearrangement on the late side of the genome near the origin of DNA replication in the same noncoding region. The rearrangement consists of a deletion between nucleotides 45 and 108 (Py 204) or nucleotides 76 and 108 (Py 97), replaced by a duplication of nucleotides 138 to 120 (Py 204) or 107 to 220 (Py 97). The secondary structure presented on the early coding strand includes the region common to both mutants from nucleotide 157 to the constant duplication deletion junction at nucleotides 108-220 and further on to nucleotide 135. *, Nucleotides missing in Py 97. A–E: Decreasing order in which each double-stranded fold contributes to the total stability of the structure. The free energy value of −26.7 kcal (−112 kJ) given for Py 204 is very similar to the value for Py 97.

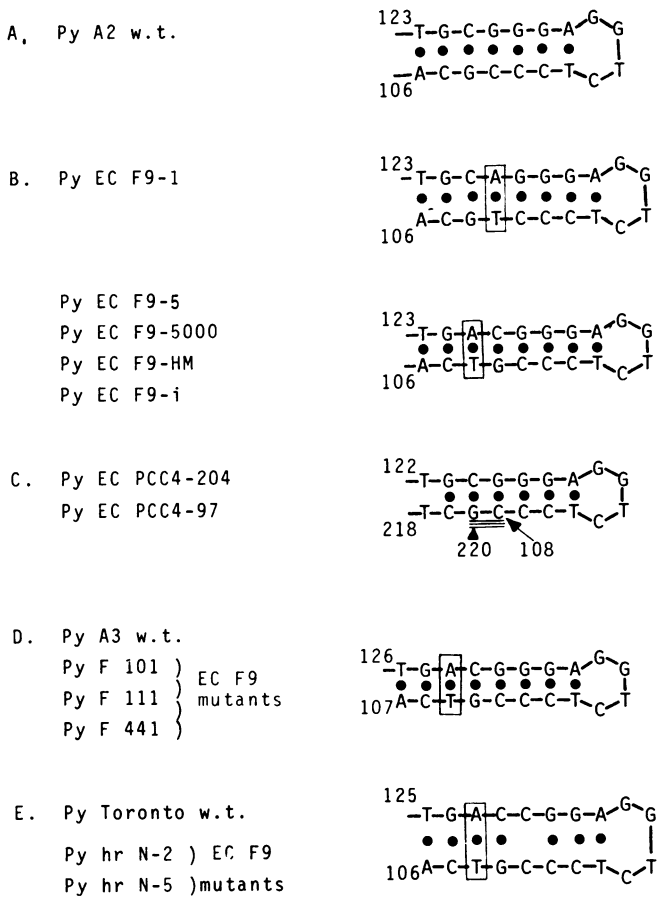

FIG. 5. Major symmetry element on different Py genomes. The folded palindrome on the early coding strand between nucleotides 106 and 123 is shown for w.t. Py (A), and its equivalents in the Py EC F9 mutants (B), Py EC PCC4 mutants (C), Py A3 EC F9 mutants (D), and the Py Toronto EC F9 mutants (E). The thymine and adenine insertions are boxed. The duplication-deletion junction of the Py EC PCC4 mutants is underlined. The numbering system is the same as in Fig. 1–4 (1).

without the transition at position 63. However, the w.t. A3 strain already contains the thymine and adenine insertions found in our A2-derived Py EC F9 mutants. The only known difference between the A2 and A3 strains is a deletion of 13 bp in the A3 strain between nucleotides −48 and −36 in the noncoding early side of the origin (Fig. 2, Py EC F9-i). This sequence includes seven nucleotides (GCCTCTC) complementary to a sequence between nucleotides +75 and +81 (GAGAGGC) situated inside the proposed secondary structure (Fig. 3). Sekikawa and Levine (26) used the small-plaque Toronto Py strain, which differs in a number of sequences from the A2 or A3 Py strains. The Toronto strain has an imperfect palindrome (Fig. 5), which may explain the small plaques obtained upon infection with it (see below).

We have previously noted (12) that the adenine and thymine are inserted in such a way that they are complementary in the tRNA-like structure proposed by Soeda et al. (27). Using the topological model of Ninio (21) and the optimized computer program derived from it as described above, we found that the most stable secondary structure that can be constructed for w.t. Py is not the cloverleaf (−30 kcal; −126 kJ), but that presented in Fig. 3 (−34 kcal; −142 kJ). The values found for Py EC F9-I (−31 kcal; −130 kJ), Py EC F9-5 (−37 kcal; −155 kJ), and the other three Py EC F9 mutants were very similar. As stated above, a similar secondary structure with a free energy value of −27 kcal (−112 kJ) can be proposed for the two EC PCC4 mutants in the same region (shown in Fig. 4) in spite of the rearrangement (deletion and duplication) that occurred in the genome. This value again comes very near that of w.t. Py.

Even more striking in these mutants was the conservation of a palindrome between nucleo-

tides 106 and 123 in w.t. Py as shown in Fig. 5. This secondary structure exposed the thymine and adenine insertions in the EC F9 mutants and the deletion-duplication borders of the EC PCC4 mutants. This palindrome contributed most to the stability of the putative structures shown in Fig. 3 and 4. It must be noted that the rearrangement in the EC PCC4 mutants conserved the guanine plus cytosine (G+C)-rich content of this w.t. Py palindrome and that these mutants did not grow on F9 cells. The insertion of an A·T complementary base pair permitted growth on F9 as well as on PCC4 cells, although to a lower extent. It may be proposed that the important feature for growth on PCC4 cells is a rearrangement safeguarding the palindrome, which is also necessary for growth in differentiated cells. This rearrangement is not sufficient for growth on F9 cells, whereas the A·T insertion, which also enhances growth on PCC4 cells, is required. Such an insertion may be sufficient, at least theoretically, for a change in the DNA secondary structure from B-DNA (right handed) to Z-DNA (left handed) (E. M. Lafer, personal communication).

The noncoding region of the Py genome on the late side of the origin of DNA replication is a hot spot for mutation and rearrangement, not only in the selection of Py EC mutants, but frequently in spontaneous Py mutants (unpublished data). It was previously shown that SV40 chromatin had a region that was very sensitive to DNase I digestion (33), and electron microscopy observation showed this region to be devoid of nucleosomes (24). This region is about 350 bp long and is located around the origin of replication asymmetrically towards the late region (24). Similar experiments with Py show an analogous DNase I-sensitive region stretching from the origin of DNA replication up to nucleotide +275 in the late region (9). This naked chromatin may be more apt to undergo mutation and rearrangement. The DNase I-sensitive region is not homogeneous. A differential sensitivity with two hypersensitive sites of about 30 nucleotides centered at nucleotides +85 (±5) and +195 (±5) coincides with a protected region of about 25 nucleotides centered at nucleotide +115 (±5). The protected region is thus located around the G+C-rich palindrome. The fine mapping of the DNase I-sensitive region of Py EC PCC4-97 (9) shows that the sensitive region seems to have the same boundary at about +265 nucleotides towards the late region, with persistence of the same two hypersensitive sites and the protected region now surrounding the constant deletion-duplication (nucleotides 108-220) junction of the mutant. No experimental evidence as yet can explain the existence of the hypersensitive or protected region in the viral chromatin. A hypothetical protein could specifically bind to a local secondary structure or palindrome and prevent accessibility to DNase I.

Recently, Tyndall et al. (32) showed that the noncoding region on the late side of the genome between nucleotides +50 and +250 is essential for early transcription, DNA replication, or both. Moreover, the noncoding sequences on the late side of the Py origin of DNA replication and their SV40 counterparts were recently shown to enhance transcription of a heterologous gene such as rabbit β_1-globin (3, 35). These sequences are implicated in all of the modifications of the Py EC mutants. Thus, this region might be involved in the control of expression of both early and late Py genes with respect to the differentiated state of the cell.

The symmetry elements and particularly the palindrome shown in Fig. 5 might be necessary for viral expression in differentiated or embryonal cells. In all of the characterized Py EC mutants the palindrome was preserved, but the modifications in this palindrome (Py EC F9) or around it (Py EC PCC4) were obligatory for growth in the corresponding EC cells. These modifications may either create a site for interaction with an effector intervening in the transcription/replication of the virus, or, on the other hand, abolish a site for an inhibitor of these processes. In either case the factor would be present in the EC cells but not in the differentiated ones. Yet another possibility is that a factor absent in the EC cells but present in differentiated cells becomes dispensable by the sequence modifications in the Py EC mutants. We are currently looking for proteins which interact specifically with w.t. or EC Py sequences.

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