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Genetics

Isolation and characterization of polyoma host range mutants that replicate in nullipotential embryonal carcinoma cells

(viral early gene regulation/viral DNA duplication near origin/developmental control/DNA sequence)

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ABSTRACT **Polyoma wild-type virus replicates in most murine differentiated cells but fails to produce virus in murine embryonal carcinoma cells. Polyoma host range mutants have been isolated that replicate in several nullipotential embryonal carcinoma cell lines but fail to replicate in a pluripotential cell line. The final virus yield of these host range mutants is dependent upon the multiplicity of infection in both differentiated cells and nullipotent embryonal carcinoma cells. Two independently derived host range mutants contain the same single base pair change (A·T to G·C) and a 33- and 67-base pair duplication of those viral DNA sequences containing this point mutation. This duplication of viral DNA is located at 69 map units on the polyoma genome on the late gene side of the origin of viral DNA replication (70.5 map units). This type of mutation suggests several models to explain the polyoma host range restriction in embryonal carcinoma cells.**

Although polyoma virus undergoes a productive infection and simian virus 40 (SV40) expresses its early proteins in most differentiated mouse cells, neither virus produces detectable levels of their early proteins in murine embryonal carcinoma cells (EC cells) (1–3). When pluripotent EC cells are permitted to differentiate, they produce infectious polyoma virus or can be transformed by SV40 and express early viral proteins (4–7). The blockage to polyoma or SV40 gene expression in several different EC cell lines has been investigated (1–3, 8). In nullipotential F9 cells infected with SV40, low levels of viral transcriptional intermediates and RNA have been detected, and the SV40 RNA is not spliced into large and small tumor antigen (T antigen) m-RNAs (3). When F9 cells were stimulated to produce endoderm (9), then SV40 infection resulted in the production of spliced mRNAs, and the viral tumor antigens were synthesized (7). Polyoma infection of the pluripotent EC cell line PCC4-aza-1 (10) results in the production of very small amounts of viral RNA (11) compared to an infection of differentiated cells. Polyoma host range mutants have been isolated that produce infectious virus on PCC4-aza-1 cells (12) but fail to replicate on nullipotent F9 cells. This indicates that the block to polyoma early gene expression in two different EC cell lines, F9 and PCC4-aza-1, may be different.

We report the isolation of polyoma host range mutants that were selected for growth on the nullipotential EC cell line F9. These mutants also replicate on two other nullipotent EC cell lines, MH and FA, but do not produce virus in the pluripotent EC cell line PCC4-aza-1. The yield of infectious host range mutant viruses in both differentiated cells and EC cells was dependent upon the input multiplicity of infection (moi). Two independently derived host range mutants were shown, by restriction enzyme mapping, to contain an insertion in the polyoma genome between map coordinates 67.4 and 70.0. DNA sequence analysis in this region demonstrated that these insertions were 33- and 67-base pair (bp) duplications of viral DNA at 69 map units in the genome. Each duplication contained a single A·T to G·C point mutation at nucleotide 5230 in the Soeda–Griffin numbering system (13). These mutations differ in both type and location from those polyoma host range mutants that replicate exclusively in PCC4-aza-1 cells (11, 12).

MATERIALS AND METHODS

Virus and Cells. The small-plaque Toronto strain of polyoma wild-type virus was obtained from C. Basilico and plaque-purified three times on BALB/c 3T3 cells prior to use in these experiments. EC cell lines PCC4-aza-1 and F9 were obtained from M. Sherman, and nullipotential MA and FH cell lines were from D. Solter. All cells were grown in Dulbecco's modified Eagle's medium with 10% calf or fetal calf serum as described (5, 6).

Isolation of Host Range Mutants. Five independent polyoma plaques were used to make virus stocks, and these were grown in BALB/c 3T3 cells for four undiluted passages. These virus preparations were used to infect F9 cells. The chronically infected F9 cell cultures were passaged every 2 days for 1 mo. At that time F9 cell lysates were assayed (plaque formation) for polyoma virus on BALB/c 3T3 cells. Individual plaques were picked and tested for growth on F9 cells. Three independent PyhrN mutants 1,2,5 (Py = polyoma; hr = host range, N = nullipotential) were isolated.

Preparation of Viral DNA and Restriction Enzyme Analysis. Polyoma was grown in BALB/c 3T3 cells, and viral DNA was selectively extracted by the procedure of Hirt (14). After extraction with phenol and precipitation with ethanol, the DNA was purified by ethidium bromide/CsCl density gradients. Restriction enzymes were purchased from New England BioLabs, and the DNA was cut and analyzed on 1.4% (wt/vol) agarose gels or polyacrylamide gels as described (15). DNA sequence determination was carried out by the procedure of Maxam and Gilbert (16). The DNA sequence was determined between the *Eco*RI site at 66.7 map units and the *Hpa* II site at 70.5 map units (188 bases).

RESULTS

Isolation of Polyoma Host Range Mutants that Replicate on F9 Cells. Three independent polyoma host range mutants were isolated by serial passage in chronically infected F9 cells over a period of a month. The three mutants, PyhrN-1, PyhrN-2, and PyhrN-5 along with the wild-type virus were used to infect BALB/c 3T3 cells, PCC4-aza-1, and F9 EC cell lines at a moi of 1 plaque-forming unit (PFU) per cell (2×10^5 cells per 60-mm

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Abbreviations: bp, base pair(s); SV40, simian virus 40; EC cells, embryonal carcinoma cells; PFU, plaque-forming units; moi, multiplicity of infection; T antigen, tumor antigen.

Table 1. Replication of polyoma wild-type and host range mutant viruses on 3T3 and EC cell lines

Virus	Virus yield, PFU/ml		
	BALB/c 3T3	PCC4-aza-1	F9
Pywt	2.4×10^7	$<10^2$	$<10^2$
PyhrN-1	5×10^5	$<10^2$	7.0×10^4
PyhrN-2	1.2×10^7	$<10^2$	9.0×10^4
PyhrN-5	2.3×10^7	$<10^2$	2.2×10^5

Each cell culture was infected with wild-type (wt) or mutant polyoma at a multiplicity of 1.0 PFU per cell. The virus yield was measured by plaque assay in BALB/c 3T3 cells after 5 days at 37°C in each of the cell lines.

culture dish) for 5 days at 37°C. Virus yields were then assayed by plaque formation in BALB/c 3T3 cells at 37°C (Table 1). Polyoma wild-type virus replicated well in BALB/c 3T3 cells but not at all in the two EC cell lines. The three PyhrN mutants grew in BALB/c 3T3 cells and, with varying efficiencies, in F9 EC cells but not at all in the PCC4-aza-1 EC cell line. Growth curves of polyoma wild-type virus and the PyhrN-5 mutant in BALB/c 3T3 cells; in nullipotential EC cell lines F9, MH, and FA; and in pluripotential EC cell line PCC4-aza-1 are shown in Fig. 1. The PyhrN-5 mutant replicated to higher titers in BALB/c 3T3 cells than in any of the EC cell lines. This mutant, selected for replication in a nullipotential F9 EC cell line, also replicated in two (nonselected phenotype) other nullipotential EC cell lines (MH and FA) albeit to a slightly lower titer than in F9. The PyhrN-5 mutant and the wild-type virus failed to replicate in the pluripotent PCC4-aza-1 EC cell line. Although this is a small sampling of EC cell lines, the host range mutants appear to be able to replicate in nullipotential EC cell lines and not in a pluripotential cell line.

Each of the host range mutants were tested for replication on BALB/c 3T3 and F9 cells at 32°C, 37°C, and 39.5°C, and no temperature-sensitive properties of these mutants were detected.

Dependence of Virus Yield on Multiplicity of Infection. BALB/c 3T3 or F9 EC cells were infected with wild-type polyoma, PyhrN-2, and PyhrN-5 at input moi of 1, 10, and 100 PFU per cell to determine whether the input moi affected the final yield of viruses in these cells. After 3 days at 37°C, the cells were lysed and the extracts were assayed on BALB/c 3T3 cells

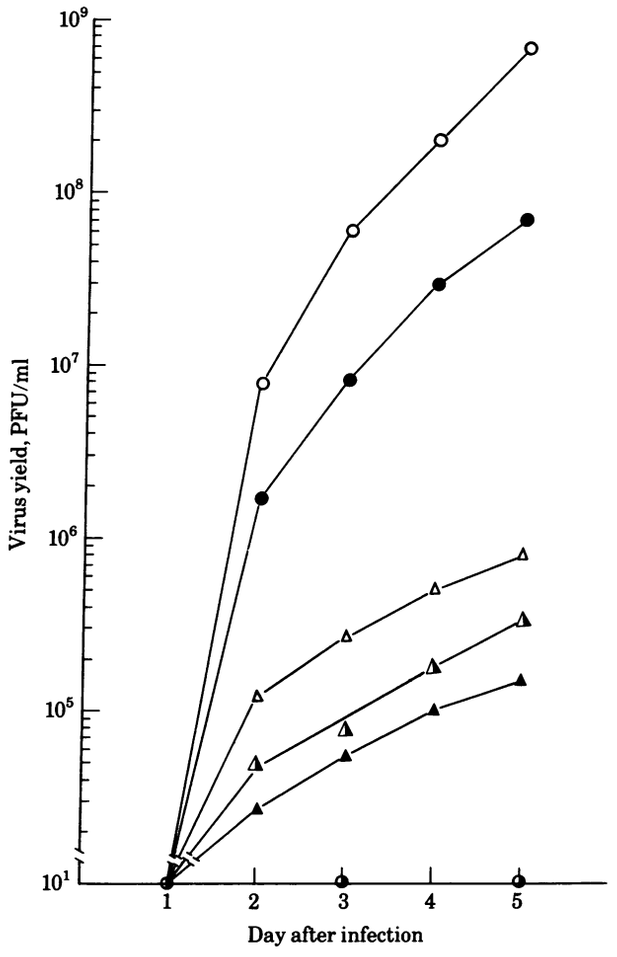

FIG. 1. Growth curve of polyoma wild type and host range mutants in 3T3 and EC cell lines. BALB/c 3T3 and EC cell lines F9, MH, FA, and PCC4-aza-1 were infected with either polyoma wild type or PyhrN-5 mutant at a moi of 1 PFU per cell at 37°C. At each day for 5 days, a cell culture was lysed and the virus titer was determined by plaque assay on 3T3 cells. ○——○, Polyoma wild type in 3T3 cells; ●——●, PyhrN-5 in 3T3 cells; △——△, PyhrN-5 in F9 cells; ▲——▲, PyhrN-5 in MH cells; ▲——▲, PyhrN-5 in FA cells; ◑——◑, polyoma wild type in F9, MH, FA, and PCC4-aza-1 EC cell lines and PyhrN-5 in PCC4-aza-1.

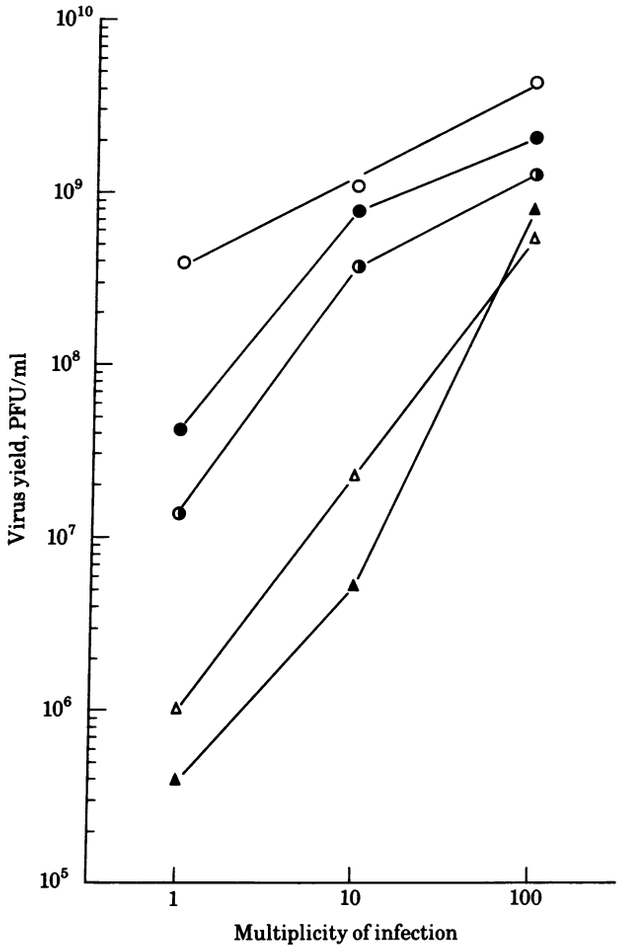

FIG. 2. The dependence of the final yield of virus upon the input moi. BALB/c 3T3 or F9 cells were infected with polyoma wild type, PyhrN-2, or PyhrN-5 at an input moi of 1, 10, or 100 PFU per cell. After 3 days at 37°C each cell culture was lysed and titered in BALB/c 3T3 cells. ○——○, Polyoma wild type in 3T3 cells; ●——●, PyhrN-2 in 3T3 cells; ◑——◑, PyhrN-5 in 3T3 cells; △——△, PyhrN-2 in F9 cells; ▲——▲, PyhrN-5 in F9 cells.

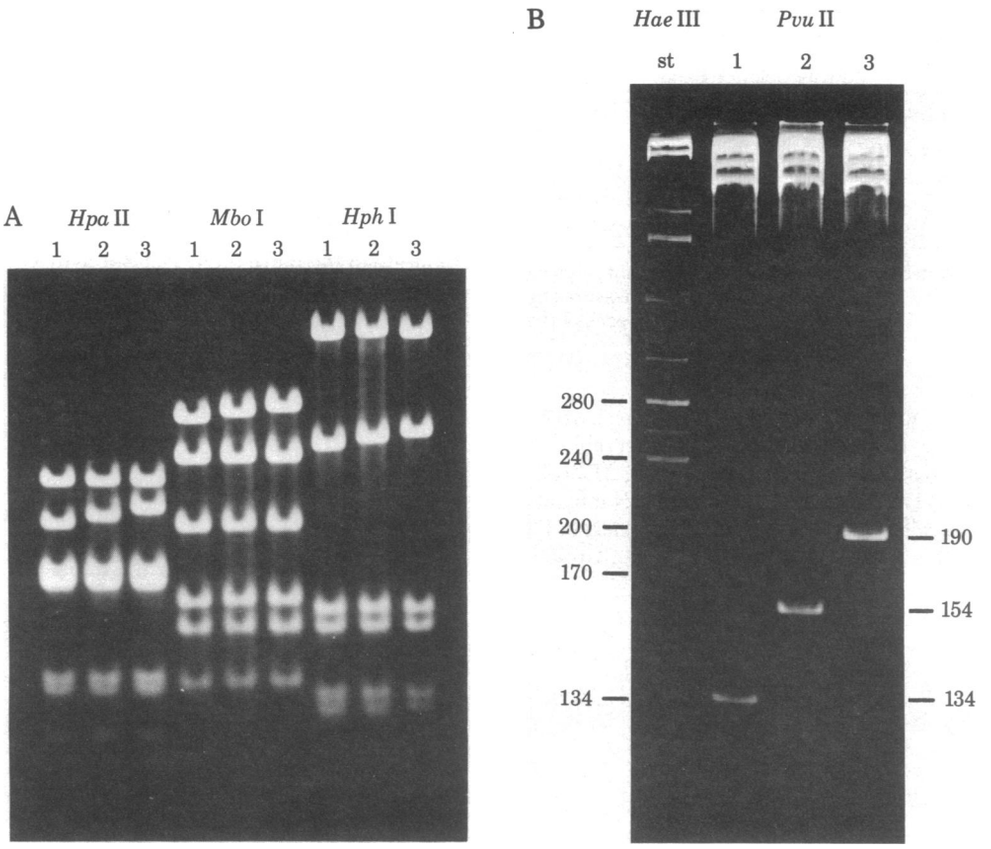

FIG. 3. Ethidium bromide-stained DNA fragments derived from polyoma wild-type virus and PyhrN-2 and PyhrN-5 mutants. (*A*) Isolated DNAs from wild type (lanes 1), PyhrN-2 mutant (lanes 2), and PyhrN-5 mutant (lanes 3) were cut with restriction enzymes *Hpa* II, *Mbo* I, and *Hph* I and analyzed in 1.4% argarose gel. (*B*) Analysis in 17% polyacrylamide gel of a *Hae* III digest of plasmid pMZ 71 DNA (lane st) as a molecular weight marker and of *Pvu* II restriction fragment 4 from wild type (lane 1), PyhrN-2 mutant (lane 2) and PyhrN-5 mutant (lane 3).

(Fig. 2). The final yield of wild-type virus in 3T3 cells showed a slight dependence upon input moi. The final yield of both PyhrN-2 and -5 mutants in 3T3 cells had a greater dependence upon input moi than did wild-type virus, but the most extreme example of dependence on moi was shown by the mutant viruses in F9 cells. A 100-fold increase in the input moi resulted in a 1000-fold increase in the final yield of mutant virus on F9 cells. Polyoma wild-type virus failed to replicate (less than 10^2 PFU/ml) in F9 cells at any of these input moi.

Restriction Enzyme Analysis of the Polyoma Host Range Mutants. The block to expression of SV40 early genes in F9 cells is, at least in part, the result of a failure to convert the early viral RNA into large and small T antigen mRNAs (3). To examine whether the PyhrN mutants contained genomic rearrangements that might facilitate RNA processing, restriction enzyme maps were constructed for the polyoma wild-type virus and the PyhrN-2 and -5 mutants. Viral DNA was cleaved with *Hpa* II, *Hph* I, *Mbo* I, and *Pvu* II, and the DNA was analyzed by agarose gel electrophoresis (Fig. 3). The *Hpa* II 2 fragment was larger than the corresponding wild-type fragment by about 20 bp in the PyhrN-2 mutant and about 60 bp in the PyhrN-5 mutant. Similarly the *Hph* I 2 fragment was larger in the PyhrN-2 and -5 mutants than in the corresponding wild-type fragment. A further refinement of the position of these insertions in PyhrN-2 and -5 derives from the analysis with *Mbo* I where the *Mbo* I 1 fragment of these mutants was slightly larger than the corresponding *Mbo* I fragment of the wild-type virus. With these three enzymes, all other DNA fragments in the wild-type and PyhrN-2 and -5 genomes were identical. This analysis placed the insertions in PyhrN-2 and -5 mutants between 70.5 and 65.4 map units (*Hpa* II 5/2 fragment junction and the *Mbo* I 6/1 fragment junction) as shown in Fig. 4. To refine this analysis, the wild-type and mutant DNA were cut with *Pvu* II, which cuts out a fragment of 134 bp between 67.4 and 70.0 map units on the polyoma wild-type genome map. Fig. 3*B* shows that the insertion is contained within the *Pvu* II fragment 4, with the PyhrN-5 mutant containing the larger insert when compared to PyhrN-2.

The map of these insertions in the PyhrN mutants is shown in Fig. 4. The wild-type polyoma virus employed in this study differs in several ways from the A-2 large-plaque Dulbecco strain previously characterized (13). There is one extra *Eco*RI site at 66.7 map units in addition to the one at 0.0 and an extra *Hpa* II site (4a, 4b) at 40.0. The *Hph* I site at 2.5 map units in the A-2 strain is not present in this wild-type polyoma virus. The *Hin*dIII, *Pst* I, *Bam*HI, and *Pvu* II sites in the A-2 strain and the polyoma strain under study here were identical. The position of the insertions in the PyhrN-2 and -5 mutants lies between 67.4 and 70.0 map units (the two *Pvu* II sites) on the late gene side of the origin of DNA replication (70.5 map units at the *Hpa* II 5/2 fragment junction).

DNA Sequence of the Polyoma Wild-Type Virus and Host Range Mutants. The DNA sequence of the polyoma wild-type virus and the PyhrN-2 and PyhrN-5 mutants were determined for the region between the *Eco*Ri site at 66.7 map units and the *Hpa* II site at 70.5 map units (188 bases) by the Maxam–Gilbert procedure (16). Fig. 5 presents a portion of that DNA sequence from the wild-type virus and the alterations found in the mutants. The wild-type DNA sequence between bp 5213 and 5247 contains an 8-bp direct repeat (in the boxed area) separated by

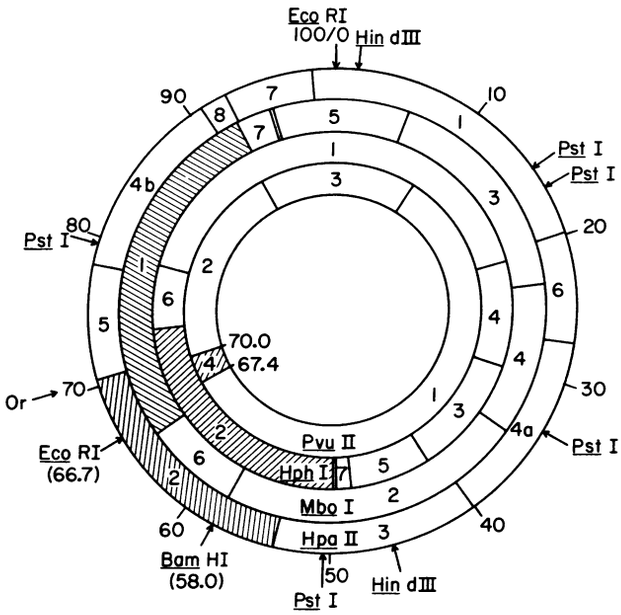

FIG. 4. Restriction enzyme map of Polyoma wild-type DNA and host range mutant DNA. The restriction enzyme fragments were ordered by double digestion of polyoma DNA from wild type and the host range mutants. The shaded DNA fragments were found (Fig. 3 *A* and *B*) to be 20–60 bp larger with the PyhrN-2 and -5 mutants than with wild-type DNA. The mapping places the insertion found in the PyhrN mutants were between 67.4 and 70.0 on the genome.

11 bp. In each of the two mutants, the A·T bp (no. 5230) that is equidistant between the two repeats was mutated to a G·C bp. In PyhrN-2 there was then a tandem duplication of the viral DNA sequences between bp 5214 and 5246 (33 bp), with each duplication containing the new G·C point mutation. In PyhrN-5 there was a tandem duplication of viral DNA sequences between bp 5182 and 5246 (67 bp), with each duplication containing the new G·C point mutation. Neither mutant contained a deletion of polyoma DNA beyond the 188-bp sequence. Thus, both independently derived mutants contained three common features: an A·T to G·C bp change at nucleotide 5230, a duplication of the region containing this point mutation, and a common break point between nucleotides 5246 and 5247 at one end of the duplication.

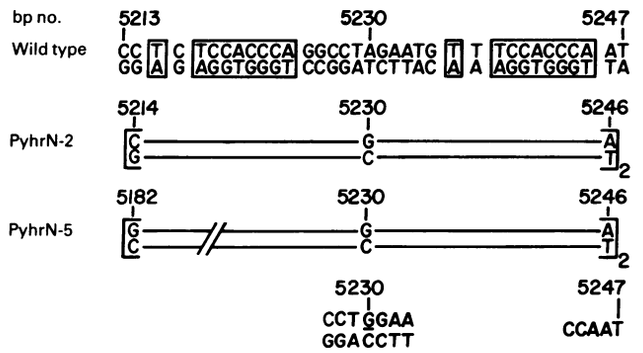

FIG. 5. DNA sequence of polyoma wild type and mutants PyhrN-2 and PyhrN-5. The DNA sequence was determined by the Maxam–Gilbert method (16). The three DNA's were sequences between the *Eco*RI site at 66.7 map units and the *Hpa* II site at 70.5 map units (188 bp). A portion of this sequence is presented between nucleotides 5213 and 5247 (numbering system in ref. 13). The PyhrN-2 and -5 mutants both contain an A·T to G·C point mutation at nucleotide 5230. In PyhrN-2 there is also a tandem duplication of nucleotides 5214 to 5246 which contains the point mutation. In PyhrN-5 there is a tandem duplication of nucleotides 5182 to 5246 also containing the point mutation twice. No deletions were detected in the 188 bp sequence.

DISCUSSION

The host range restriction of papovaviruses in murine EC cells has been analyzed at both the genetic (11, 12) and biochemical (3, 7, 11) levels. In SV40-infected F9 EC cells, early viral RNA was synthesized in reduced levels and was not processed into functional mRNAs (3, 7). In polyoma-infected PCC4-aza-1 cells, the levels of viral RNA were severely depressed, indicating a transcriptional block may exist in these cells (11). Thus, the restriction to early viral gene expression in different EC cell lines may be different. In support of this concept is the isolation of the PyhrN mutants in this study. These mutants replicate in F9 cells and in two other nullipotential EC cell lines, FH and MA, but failed to replicate in PCC4-aza-1 EC cells. Blangy and his colleagues (11, 12) have isolated polyoma host range mutants that replicate in PCC4-aza-1 EC cells but not in F9 cells. These PCC4 mutants contain a duplication of viral DNA derived from the region of the polyoma genome at 67 map units. One of the duplicated copies of this DNA was then translocated into a deletion of DNA sequences at 69 map units of the viral genome (11).

The PyhrN mutants isolated in this study are quite different. They contain a tandem repeat (of 33 and 67 bp) of polyoma DNA sequences containing an A·T to G·C bp point mutation (at nucleotide 5230) located at 69 map units in the genome. The Blangy mutants (11, 12) that replicate in PCC4-aza-1 EC cells do not contain this A·T to G·C bp change (11) observed in the PyhrN mutants that replicate only in F9 EC cells. Taken together, these results strongly suggest that the block to early gene expression in different EC cell lines (3, 7, 11, 12) is different and that these cell lines could represent different developmental stages of gene expression. Cells derived and differentiated from EC cells do support the replication of polyoma virus and the expression of SV40 gene products (1–9), suggesting that these viruses can be used to explore cellular control mechanisms operative during developmental changes.

The DNA sequence analysis of the PyhrN mutants suggests some alternative ways in which these mutants may overcome the F9 EC cell restriction to polyoma replication. The sequence 5′C-C-T-G-G-A-A3′ found in both mutants but not in the wild-type virus (Fig. 5) is complementary to the sequence 5′T-T-C-C-A-G-G3′ which is present at the donor splice site (T-T-C-C-A-G ↓ G-T, nucleotides 404–411, with the arrow indicating the proposed splice site) (13, 17) of polyoma large T antigen. The acceptor splice site is thought to be 5′C-A-G ↓ G3′ (nucleotides 792–795) for large T antigen. Indeed, one possible set of donor splice sites for the small and middle T antigen 5′T-C-C-A-A-G3′ (nucleotides 742–747) are similar to that of large T antigen (13, 17). Thus, transcripts across the altered region (at 69 map units) in the PyhrN mutants would produce a RNA that might hydrogen bond (inter or intramolecular bonding) with the splice junctions for polyoma early proteins. Small molecular weight RNA molecules have been implicated in splicing reactions, and it remains possible that the altered regions in these PyhrN mutants may encode a small RNA transcript.

The nucleotide sequence in the region of the PyhrN mutations contains the sequence C-C-A-A-T (Fig. 5, nucleotides 5243–5247) which is found on the 5′ side of a variety of eukaryotic genes (18). A similar sequence also has been observed near the 5′ end of the Herpes virus thymidine kinase gene, and in that case deletions that remove this sequence reduce the levels of the thymidine kinase gene mRNA in microinjected *Xenopus* oocytes (S. McKnight, personal communication). It is curious

that both PyhrN mutants have one of their duplication break points in this sequence (C-C-A-A↓T). Thus, in both mutants the tandem duplication results in the restoration of a single C-C-A-A-T site at one end of the duplication.

SV40 appears to be restricted in its expression of early proteins in F9 cells because of a failure to splice or process the early RNA properly (3, 7). If polyoma virus has a similar defect in F9 cells, the point mutation and tandem duplications in these mutants might permit a low level of splicing to occur. The PyhrN mutants isolated here do not replicate as efficiently in F9 cells as they do in 3T3 cells. It is also possible that the mutation and duplication provide a stronger promoter for the early genes or a new splice site that stabilizes the precursor RNA. Either one of these alternatives could increase the levels of viral RNA in the cell, and this in turn could permit some splicing of this RNA to occur. Clearly additional information will be required before an understanding of the restriction to papovavirus gene expression in EC cells will be forthcoming. This type of information could well lead to a better understanding of developmental regulation as well as virus gene expression.

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