Reports

Molecular Cloning of Polyoma Virus DNA in *Escherichia coli*: Plasmid Vector System

Abstract. *A series of recombinant plasmids containing polyoma virus (PY) DNA were constructed, and their biological activity was evaluated in mice and in cultured mouse cells. While all of the recombinants studied contain the complete, potentially infectious viral DNA, in no case was the intact recombinant PY-plasmid DNA, or live* Escherichia coli *containing the recombinant plasmids, capable of inducing PY infection of mice, either by feeding or by parenteral injection.*

To evaluate certain postulated mechanisms of possible biohazard stemming from recombinant DNA research with *Escherichia coli*, we have examined the ability of *E. coli* carrying a recombinant plasmid or phage (*1*) containing the entire polyoma virus (PY) genome to cause PY infection in mice (*2*). This is a unique experimental system in that mice of all ages are highly susceptible to infection with PY, and infected mice develop a high titer serum antibody against the viral capsid protein. This antibody response is the basis of the mouse antibody production (MAP) test for PY virus (*3*). We have used this assay to study the infectivity of PY DNA itself, and found that picogram amounts of both circular and linear PY DNA are infectious when injected into weanling mice (*4*). A DNA preparation can register as positive in the MAP test only by initiating a productive PY infection—that is, the synthesis of viral protein, the encapsidation of viral DNA, and the subsequent spread of virus, which stimulates production of antiviral antibody. Thus, the question of whether mice can acquire polyoma infection from bacteria carrying the viral genome in recombinant DNA molecules can be studied by testing exposed mice for serum antibody response.

Since PY replicates only in cells of certain rodents and replicates efficiently only in mouse cells, cloning of this virus provides an exceptionally safe system for laboratory study.

PY DNA was cloned with the plasmid vector system pBR322–*E. coli* χ1776, a certified EK2 host vector system (*5*), under P4 physical containment (*6*). The pBR322 DNA has a molecular size of 2.6×10^6, contains single recognition sites for five different restriction endonucleases, including Eco R1 and Bam H1, and confers both ampicillin and tetracycline resistance on the *E. coli* which harbor it (*5*). The PY DNA [3.4×10^6 daltons (*7*)], contains a single Eco R1 site (*8*), located in the early region of the genome (*9*), an area known to code for the viral tumor (T) antigen, and a single Bam H1 site (*10*), located in the late region of the viral DNA, which codes for the structural polypeptides of the virion (*11*) (Fig. 1). Therefore, insertion of the PY genome into the plasmid vector at the Eco R1 site leaves the entire late region of the viral genome intact, while insertion at the Bam H1 site leaves the entire early region intact.

Mouse cells (3T6) were infected at low multiplicity with a large plaque variant of PY virus (*12*), and supercoiled PY DNA was prepared from cell extracts by differential salt precipitation and subsequent cesium chloride–ethidium bromide equilibrium density centrifugation (*4*). Closed circular pBR322 DNA was extracted by a modification of the clear lysate procedure (*13*) from *E. coli* RR1 (*14*) after chloramphenicol amplification, and was purified by cesium chloride–ethidium bromide dye density centrifugation (*15*). Equal amounts by weight of the two DNA's were mixed and cleaved with Eco R1 or Bam H1. After inactivation of the restriction endonuclease by heating at 68°C for 6 minutes, the mixtures of plasmid and viral DNA's were ligated, with T4 ligase, at a total DNA concentration of 50 μg/ml for 30 minutes. Electrophoresis of portions of these reaction mixtures in 0.7 percent agarose slab gels indicated that approximately 90 percent of the full-length linear PY DNA had been incorporated into larger molecular species. Portions of the ligation mixtures containing 0.125 μg of vector DNA were used to transform *E. coli* χ1776 (*16*).

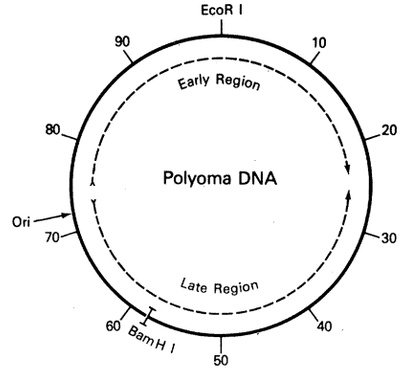

Fig. 1. Organization of the PY genome and location of Eco R1 and Bam H1 endonuclease cleavage sites. The dashed lines indicate the polarity and approximate extents of the early and late transcripts. The origin of DNA replication (*Ori*) is shown at approximately 71 MAP units from the Eco R1 site (*22*).

Bacteria transformed by the Eco R1 ligation mixtures were screened for PY DNA by filter hybridization (*17*). We examined approximately 300 colonies transformed to ampicillin resistance; seven contained DNA sequences that hybridized to ^{32}P-labeled PY DNA.

Bacteria containing a recombinant plasmid with an insert at the Bam H1 site are characterized by ampicillin resistance and tetracycline sensitivity, since the inserted DNA interrupts the tetracycline resistance determinant. We examined 200 colonies of *E. coli* χ1776 transformed to ampicillin resistance by pBR322 ligated to PY DNA at the Bam H1 site; nine were found to be tetracycline sensitive. Each of these contained the PY genome.

Plasmid DNA was prepared as described above from chloramphenicol-amplified bacterial cultures of seven clonal isolates containing PY DNA sequences inserted at the Eco R1 site. Analysis by agarose gel electrophoresis before and after restriction endonuclease cleavage indicated that all seven clones contained supercoiled recombinant plasmid DNA (6×10^6 daltons), consisting of pBR322 (2.6×10^6 daltons) joined to full-length PY DNA (3.4×10^6 daltons) at the Eco R1 site (Fig. 2, gel I, lanes 2 and 3). To verify that the entire viral genome was present, each of these recombinant plasmid DNA's was cleaved by Eco R1 plus Hpa II, and the products were separated by electrophoresis in a composite gel consisting of 3 percent acrylamide and 0.5 percent agarose. Of the bands generated, nine comigrated with the nine fragments derived from comparable cleavage of authentic PY

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DNA (data not shown), a confirmation that the complete genome was present.

Orientation of the PY DNA insert in the recombinant plasmid molecule was ascertained by digestion with Hind III, which cleaves pBR322 DNA once and PY DNA twice. Cleavage of recombinant plasmids pPR18 and pPR21 with Eco R1 yielded two fragments corresponding in size to full-length plasmid and PY DNA's (Fig. 2, gel I, lanes 2 and 3). However, cleavage with Hind III yields distinctly different patterns (Fig. 2, gel I, lanes 5 and 6), compatible with insertion of the viral DNA in the two possible orientations (Fig. 2, models A and B). These DNA restriction fragments, after denaturation in situ and transfer to a nitrocellulose sheet (*18*), were hybridized to ^{32}P-labeled PY DNA (Fig. 2, panel II) (*19*). As anticipated, the 1.55×10^6 dalton fragment of PY DNA generated by Hind III cleavage of both pPR18 and pPR21 (Fig. 2, lanes 5′ and 6′) is present in both clones and comigrates with a marker cleaved from authentic viral DNA (Fig. 2, lane 4′). The Hind III cleavage products of a PY-pBR322 recombinant plasmid oriented as shown in model A (Fig. 2) should yield two additional DNA bands (4.6 and 0.1×10^6 daltons) containing PY DNA sequences. The 4.6×10^6 dalton piece is clearly seen in Fig. 2, lane 6′; the 0.1×10^6 dalton fragment would have run off the gel under the electrophoresis conditions employed. These results indicate that the PY DNA insert in pPR21 is oriented as described in model A. Digestion by Hind III of a PY-pBR322 recombinant plasmid oriented as shown in model B should yield three Hind III fragments, each containing PY DNA sequences. In addition to a band comigrating with the Hind III PY DNA marker (1.55×10^6 daltons) (Fig. 2, lanes 4 and 4′), two other DNA fragments with mobilities corresponding to 2.0×10^6 and 2.6×10^6 dal-

Table 1. Hybridization of ^{3}H-labeled RNA from minicells to pBR322, PY, or *E. coli* DNA immobilized on nitrocellulose filters. Minicells were isolated from *E. coli* χ1776 containing various plasmids under P4 conditions essentially as described (*20*). Differential sedimentation was repeated three times, after which the minicell band was centrifuged and the pellet was suspended to an A_{620} of 0.2 in uracil-free M9 medium supplemented with diaminopimelic acid (100 μg/ml), threonine (40 μg/ml), methionine (10 μg/ml), thiamine (4 μg/ml), biotin (1 μg/ml), and thymidine (50 μg/ml). RNA was labeled with tritiated uridine (New England Nuclear, 50 μCi/ml) and the labeled RNA was isolated essentially as described (*21*). The ^{3}H-labeled RNA from each minicell preparation was hybridized with three nitrocellulose filters each containing approximately 1 μg of either *E. coli*, pBR322, or PY DNA in a reaction mixture (0.3 ml) containing 1*M* NaCl, 0.04*M* TES (N{tris[hydroxymethyl]methyl-2 amino}ethanesulfonic acid), *p*H 7.4, 0.1 percent sodium dodecyl sulfate, and 50 percent formamide. After incubation at 37°C for 36 hours, the filters were rinsed three times with 1X SSC (1X SSC is 0.15*M* NaCl and 15 m*M* sodium citrate), incubated at 20°C for 1 hour in 1X SSC containing 10 μg/ml of ribonuclease A, washed again in 1X SSC, dried at 60°C, and counted.

Origin of minicells	Input (count/min)	^{3}H-labeled RNA hybridized to (count/min)		
		pBR322 DNA	Polyoma DNA	*E. coli* DNA
χ1776	2,380	2		8
χ1776 (pBR322)	13,700	655	0	2
χ1776 (pPB5)	36,000	2350	2020	12
χ1776 (pPB6)	34,400	2140	1400	5
χ1776 (pPR18)	183,000	2610	2730	0
χ1776 (pPR21)	4,520	252	252	8

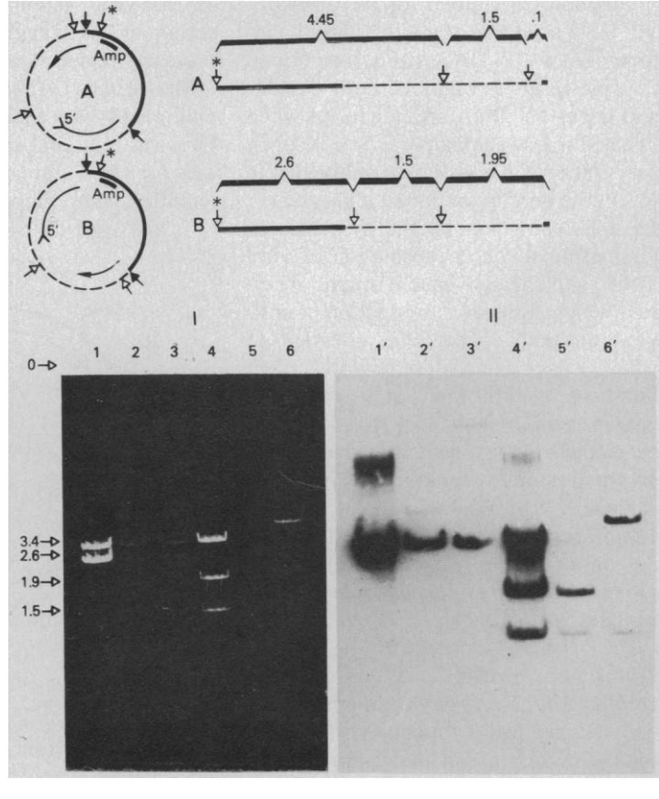

Fig. 2. Orientation of PY genome in PY-pBR322 recombinant plasmids. Insertion of Eco R1 cleaved PY DNA at the Eco R1 site in pBR322 can occur in two possible orientations, A or B. The PY genome is indicated by a dashed line and its orientation indicated by an arrow showing the polarity and extent of early transcription. The pBR322 genome is indicated by a solid line, and its orientation is indicated by the location of the ampicillin resistance gene (*Amp*). To determine the orientation of the PY DNA insertion in these recombinant plasmids, their Hind III cleavage pattern was evaluated by electrophoresis in a 1.4 percent agarose slab gel (*23*) (150 V for 3 hours). Open arrows indicate the location of the Hind III cleavage sites; closed arrows show the Eco R1 cleavage sites. The Hind III fragments of recombinants in the two possible orientations are bracketed in the linearized diagrams; the numbers above each bracket indicate their size ($\times 10^6$ daltons). In panel I, lane 1 contains linearized plasmid and linearized PY DNA markers. The numbers to the left indicate the molecular sizes ($\times 10^6$ daltons) of the various markers. Lanes 2 and 3 contain Eco R1 cleaved pPR18 and pPR21 DNA, respectively. Lane 4 contains the full-lenth linear form of PY DNA as well as the two PY DNA fragments produced by cleavage of PY DNA I with Hind III. Lanes 5 and 6 show the Hind III digestion products of pPR18 and pPR21, respectively. Panel II is a blot analysis (by the method of Southern) of the gel shown in panel I. The DNA in gel 1 was denatured in situ by soaking the gel in a solution of 0.2*N* NaOH and 0.6*M* NaCl for 45 minutes, neutralized for 45 minutes in a solution of 1*M* tris-HC1, *p*H 7.5 and 0.6*M* NaCl, and transferred to a sheet of nitrocellulose, which was then baked at 60°C for 16 hours and soaked in 4X SCC (1X SCC is 0.15*M* NaCl and 15 m*M* sodium citrate) containing 0.02 percent albumin, 0.02 percent polyvinylpyrrolidone, and 0.02 percent Ficoll for 4 hours at 60°C. PY DNA (approximately 1×10^6 count/min) was ^{32}P-labeled in vitro by the nick-translation technique (*24*), was hybridized with the nitrocellulose sheet in a reaction mixture (3 ml) containing 0.02 percent polyvinylpyrrolidone, 0.02 percent Ficoll, 0.02 percent albumin, 0.1 percent sodium dodecyl sulfate and yeast RNA (1 μg/ml) in 4X SCC for 16 hours at 60°C. The nitrocellulose sheet was then washed extensively with 4X SCC, dried, and placed against Kodak XR-2 film to produce the autoradiogram shown in panel II.

tons (Fig. 2, lane 5) were observed. Hybridization of the 2.0×10^6 dalton fragment is seen in Fig. 2, lane 5′. The 2.6×10^6 dalton fragment contains only about 300 nucleotides of PY DNA and was visualized only after very long exposure of the autoradiogram shown in Fig. 2, panel II. The faintly visible band in Fig. 2, lane 5′, corresponding to a 3.4×10^6 dalton DNA segment, is presumably a partial cleavage product. Of the seven clones containing a PY DNA insert at the Eco R1 site, four contained the insertion oriented as described in model A and three contained the insertion in the opposite orientation (model B).

Plasmid DNA's from the nine ampicillin-resistant, tetracycline-sensitive *E. coli* χ1776 isolates obtained from transformation with the Bam HI pBR322–PY DNA ligation mixture were similarly characterized. All nine contained supercoiled DNA 6×10^6 daltons in size. In each case, cleavage of these DNA preparations with Bam H1 generated a DNA segment that comigrated in agarose gels with full-length linear PY DNA, hybridized efficiently to ^{32}P-labeled PY DNA, and contained the nine Bam H1 plus Hpa II cleavage fragments characteristic of authentic PY DNA (Fig. 3). Digestion with Hind III was also used to determine the orientation of the PY genome in these hybrids. In seven of the clones examined, the PY sequences mapping near the origin of viral DNA replication were located adjacent to the ampicillin resistance determinant of the plasmid DNA, while in the other two isolates, the opposite orientation was found (data not shown).

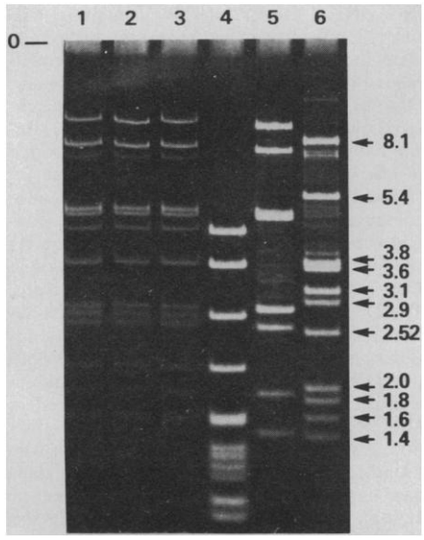

Fig. 3. Composite gel analysis of Bam H1, Hpa II digested DNA. (lane 1) pPB4 DNA; (lane 2) pPB5 DNA; (lane 3) pPB6 DNA; (lane 4) pBR322 DNA; and (lane 5) PY DNA. Each of the recombinant DNA's (2 μg samples) was cleaved with Bam H1 and Hpa II in a reaction mixture (50 μl) containing 20 m*M* tris-HCl, *p*H 7.4, 7 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and gelatin (100 μg/ml) for 3 hours at 37°C. After inactivation of the restriction endonucleases by incubation at 68°C for 6 minutes, portions of the mixtures were subjected to electrophoresis on a 3 percent acrylamide–0.5 percent agarose slab gel (*25*) for 5 hours at 100 V, and stained with ethidium bromide. Lane 6 contains the 11 SV40 DNA fragments digested with Hind II plus III as size markers; their size ($\times 10^5$ daltons) is shown in the margin.

To ascertain whether PY DNA is transcribed in *E. coli*, ^{3}H-labeled RNA was purified from minicells (*20*) of χ1776 containing recombinant plasmids and hybridized to PY, pBR322, or *E. coli* DNA immobilized on nitrocellulose filters. The data shown in Table 1 indicate that viral-specific RNA is synthesized in bacterial minicells containing recombinant PY-pBR322 plasmids. Which regions of the PY genome are transcribed is not known.

The infectivity of PY-plasmid recombinant DNA and bacteria containing such molecules was evaluated in mice by the MAP test (*3*). Animals inoculated with live *E. coli* containing PY-plasmid recombinants were maintained under P4 containment (*6*). Recombinant plasmid DNA's containing full-length PY DNA inserts in both orientations at the Bam H1 or the Eco R1 sites were prepared from chloramphenicol-amplified cultures of *E. coli* χ1776 and were purified as described. When intact recombinant plasmid DNA preparations were inoculated subcutaneously into weanling mice, in no case was there antibody response to PY (Table 2). These results not only indicate that recombinant plasmids are unable to initiate productive PY infections, but they also suggest that no free PY DNA I is present in the bacterial cells, since it would have copurified with the plasmid DNA and would have been infectious in mice.

When purified recombinant DNA preparations were first cleaved with the same restriction enzyme as was used in their construction, nearly all of the injected animals developed PY infection (Table 2). This result indicates that PY DNA, when combined with pBR322, is replicated with sufficient fidelity in *E.*

Table 2. Inoculation of mice with cloned PY DNA. Weanling mice from a PY-free colony (Charles River Laboratory) were inoculated subcutaneously with various DNA preparations suspended in phosphate-buffered saline. Serum samples were collected 3 and 6 weeks after the mice were inoculated; the samples were then tested for the presence of hemagglutination inhibiting (HI) antibody to PY as described (*3*). Titers greater than 1:20 were considered indicative of PY infection. Mice infected with PY virions regularly develop HI titers of 1:320 to 1:640 (*3*). Recombinant plasmids pPB5 and pPB6 contain the PY genome that was inserted at the Bam H1 site in pPBR322; the orientation of the viral insert in pPB6 is the reverse of the orientation in pPB5; pPR18 and pPR21 are the Eco R1 recombinant plasmids analyzed in Fig. 2.

Inoculum	Dose (μg/mouse)	No. infected/ No. inoculated
DNA		
pPB5 DNA I	5	0/5
pPB6 DNA I	5	0/5
pPR18 DNA I	5	0/5
pPR21 DNA I	5	0/5
pPB5 DNA cleaved with Bam H1	0.02	5/5
pPB6 DNA cleaved with Bam H1	0.02	5/5
pPR18 DNA cleaved with Eco R1	0.02	5/5
pPR21 DNA cleaved with Eco R1	0.02	5/5
PY DNA I	0.1	9/10
Saline		0/9

Table 3. Evaluation of PY infectivity in mice following administration of live *E. coli* χ1776 containing PY-pBR322 recombinant plasmids. Plasmids pPB1 and pPB3 through pPB6 contain the PY genome inserted at the Bam H1 site. Plasmids pPR18 and pPR21 are the EcoR1 recombinant plasmids in Fig. 2. Weanling mice were inoculated parenterally with approximately 1×10^9 to 3×10^9 bacteria, in the log phase of growth, suspended in phosphate-buffered saline. Other mice were fasted overnight and then fed approximately 1×10^9 to 3×10^9 bacteria in the log phase of growth, suspended in milk and absorbed on bread; the feeding was repeated two to five times at approximately weekly intervals. Serum samples were tested for HI antibodies at 3 and 6 weeks after the initial feeding or injection.

Inoculum	No. infected/No. inoculated		
	Intraperitoneal	Subcutaneous	Oral
χ1776 (pPB1)	0/5		0/4
χ1776 (pPB3)	0/5		0/4
χ1776 (pPB4)	0/5		0/5
χ1776 (pPB5)	0/5	0/5	0/5
χ1776 (pPB6)	0/5	0/5	0/5
χ1776 (pPR18)	0/5		0/5
χ1776 (pPR21)	0/5		0/5

coli to preserve the numerous functions required for productive infection. We have recently shown that, when PY DNA is titrated in weanling mice by subcutaneous inoculation, supercoiled DNA gives an ID_{50} (the dose that infects 50 percent of recipient animals) of 1.3×10^{-4} μg, and Bam HI- or Eco RI-cleaved DNA's give ID_{50} values of about 3×10^{-3} μg, as monitored by the MAP test (*4*). Since 10^{-2} μg of the cleaved PY-plasmid recombinant DNA led to infection in almost all of the animals inoculated, and since only 58 percent of the preparation consisted of PY DNA, it is clear that, after cleavage to separate viral from plasmid DNA, the specific infectivity of the cloned viral DNA is comparable to that of linear forms of authentic PY DNA that had been propagated in mouse cells.

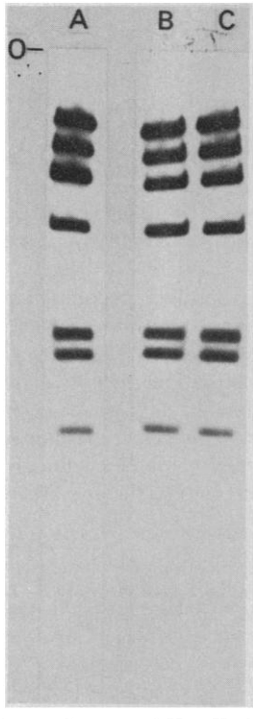

Fig. 4. Autoradiogram of Hpa II cleavage of ^{32}P-labeled viral DNA. The 3T6 cells were inoculated with PY (25 plaque-forming units per cell) (lane A) or 0.1 ml of an inoculum prepared from a clarified supernatant of frozen and thawed primary mouse embryo cells infected with either pPB5 cleaved by Bam HI (lane B) or pPR18 cleaved by Eco RI (lane C). After adsorption of virus, the cells were placed in phosphate-free Eagle's medium containing 10 percent dialyzed fetal calf serum (10 percent) and [^{32}P]orthophosphate (40 μCi/ml) (New England Nuclear). The cells were harvested 65 hours later, and supercoiled DNA was prepared (*4*). A portion of ^{32}P-labeled DNA I (10,000 count/min) was then incubated at 37°C for 3 hours in a reaction mixture containing 20 m*M* tris-HCl (*p*H 7.4), 7 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and Hpa II. The reaction mixture was incubated at 68°C for 6 minutes, and subjected to electrophoresis in a 3.0 percent acrylamide–0.5 percent agarose composite gel for 5 hours at 100 V. The gel was then dried and placed against Kodak XR-2 film.

Table 3 gives the results of exposing mice to viable *E. coli* χ1776 containing recombinant plasmids. Mice were inoculated either intraperitoneally or subcutaneously or were fed concentrated suspensions of bacteria (in the log phase of growth) containing the PY-plasmids. The injected mice received approximately 1×10^9 to 3×10^9 bacteria; if we assume a recombinant plasmid copy number of ten or more per cell, each animal received a minimum of 0.2 μg of PY-plasmid DNA, of which 58 percent is PY DNA. This corresponds to a dose of at least 40 to 150 times the ID_{50} of free linear PY DNA or 130 to 500 times the ID_{50} of PY DNA I. Even when administered as massive doses of bacteria, none of the four *E. coli* χ1776 preparations containing recombinant plasmids resulted in a PY infection after parenteral inoculation, nor did they induce infection when fed repeatedly to animals for total doses of 0.4 to 1.0 μg of PY DNA (Table 3).

These experiments were of necessity carried out with the EK2 host *E. coli* χ1776, an enfeebled bile salt-sensitive derivative of *E. coli* K12 which would not be expected to survive passage through the intestinal tract (*5*). Since the parenterally inoculated bacteria did not induce PY infection, it seems highly unlikely that oral administration of the PY plasmid in strains of *E. coli* capable of surviving in the intestinal tract would have given positive results.

The infectivity of purified recombinant plasmid DNA was also evaluated in cultured primary mouse embryo cells. In none of the seven cases examined was intact PY-plasmid DNA capable of initiating a viral infection (Table 4). When the recombinant DNA molecules were digested with the restriction enzyme used during their original construction, virus production was detected in all cases, confirming the mouse inoculation experiments described in Table 2. While we have not yet determined the titer of the infectivity of PY DNA propagated in *E. coli*, the time of development of the cytopathic effect in mouse cells paralleled closely the development of similar changes in cultures infected with an equivalent amount of authentic viral DNA.

To verify that authentic PY virus was being produced in the cell cultures, we prepared virus stocks from several of the infected cultures (Table 4) and used them to infect 3T6 cells; ^{32}P-labeled supercoiled viral DNA was isolated at 72 hours after infection. The Hpa II cleavage patterns of these preparations and of authentic PY DNA were identical (Fig. 4).

The infectivity of PY DNA, originally propagated as a plasmid recombinant in bacteria, for mouse cells suggests that eukaryotic DNA propagated in a prokaryotic cell is not recognized as "foreign" in a mammalian cell.

The failure of intact recombinant plasmid DNA to initiate synthesis of PY virus in mouse cells was not unexpected, since the monomeric nature of the PY DNA inserts provides no mechanism (terminal redundancy) to facilitate recombination and excision. In this regard, it should be pointed out that several attempts were made to clone PY dimeric DNA by means of this plasmid vector system. Dimers of PY DNA were prepared by ligation in vitro of PY supercoiled DNA cleaved at the Eco R1 or Bam H1 sites; the dimers were preparatively isolated from a 0.7 percent agarose gel, and incubated with suitably restricted plasmid DNA in the presence of DNA ligase. These recombinant DNA molecules were then used to transform *E. coli* χ1776. Despite repeated attempts, we

Table 4. Tests of intact and cleaved recombinant DNA's for PY infectivity in tissue culture. Confluent primary mouse embryo cells were inoculated with 0.17 μg of supercoiled recombinant plasmid DNA or recombinant plasmid DNA first cleaved with the same restriction enzyme used to construct it. Recombinant plasmids pPB1, pPB3 through pPB6, and pPB8 contain the PY genome inserted at the Bam H1 site in pBR322. Recombinant plasmids pPR17 and pPR22 contain the PY genome inserted at the Eco R1 site in the same orientations as indicated in Fig. 2 for pPR18 and pPR21, respectively. The DNA was suspended in tris-buffered saline containing DEAE-dextran (300 μg/ml) (Pharmacia) and was allowed to adsorb onto the cells for 20 minutes at 37°C. The inoculum was removed, and the cells were placed in McCoy's 5A medium. On day 7 after infection, a portion of tissue culture medium was tested for the presence of PY virus by a standard hemagglutination (HA) test (*3*). A titer of 1:20 was considered positive (+), while the absence of any detectable titer was considered negative (–).

Inoculum	Presence of PY HA antigen in culture fluid	
	Intact recombinant DNA	Cleaved recombinant DNA
pPB1	–	+
pPB3	–	+
pPB4		+
pPB5	–	+
pPB6	–	+
pPB8	–	+
pPR17	–	+
pPR18	–	+
pPR21		+
pPR22		+

were unable to identify or isolate recombinant plasmids containing dimeric inserts of viral DNA. We have isolated a phage recombinant containing a head-to-tail dimer insert of PY DNA; this DNA is infectious, although with extremely low efficiency (*1*). Our failure to construct comparable recombinants with pBR322 in χ1776 most likely reflects the existence of an active recombination system that renders any duplicated DNA insert unstable.

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1 December 1978; 12 December 1978

Molecular Cloning of Polyoma Virus DNA in *Escherichia coli*: Lambda Phage Vector System

Abstract. *The biological activity of recombinant phage and recombinant phage DNA containing monomeric or dimeric polyoma DNA inserts was examined in mice and cultured mouse cells. Recombinant preparations containing a single copy of viral DNA were invariably noninfectious; molecules containing a dimeric polyoma DNA insert were at least seven orders of magnitude less infectious than polyoma virions after parenteral inoculation. No infection was detected with any recombinant preparation after oral administration.*

We have described (*1*) the molecular cloning of polyoma virus (PY) DNA in an *Escherichia coli* plasmid vector system and an evaluation of the infectivity of the recombinant plasmids in mouse cells in vivo and in vitro. We now report the extension of such experiments to include recombinants formed between the EK2 lambda phage vector λgtWES.λB (*2*) and PY DNA.

The phage vector λgtWES.λB (*2*) contains three amber mutations, so that it is unable to grow in *E. coli* that do not contain the appropriate suppressor mutation. Furthermore, because of the deletions of the λ elements *int*, *xis*, and *att* required for integration, and *red*, for phage-promoted generalized recombination, λgtWES.λB cannot undergo either lysogeny or illegitimate recombination with the *E. coli* genome. These are the features that qualify it as an EK2 cloning vector (*2*).

λgtWES.λB DNA was cleaved with Eco R1, and the right and left arms were separated from the smaller middle DNA fragment by chromatography on an RPC-5 column (*3*). By removal of the middle fragment, the formation of nonrecombinant phage in the subsequent population is eliminated since a ligated molecule consisting of the two arms of λgtWES.λB alone provides insufficient DNA mass for phage packaging (*2*). Linear PY DNA was prepared by digesting superhelical viral DNA, obtained from infected mouse cells as described (*1*), with Eco Rl restriction enzyme. Ligation reaction mixtures containing λgtWES.λB arms alone or arms mixed with the cleaved PY DNA were incubated with bacteriophage T4 polynucleotide ligase and used to transfect *E. coli* strain LE392 under P4 conditions. No plaques appeared after the transfection of bacteria with lambda arms ligated in the absence of PY DNA. However, the transformation frequency of the ligation mixture containing vector arms with linear PY DNA was approxi-

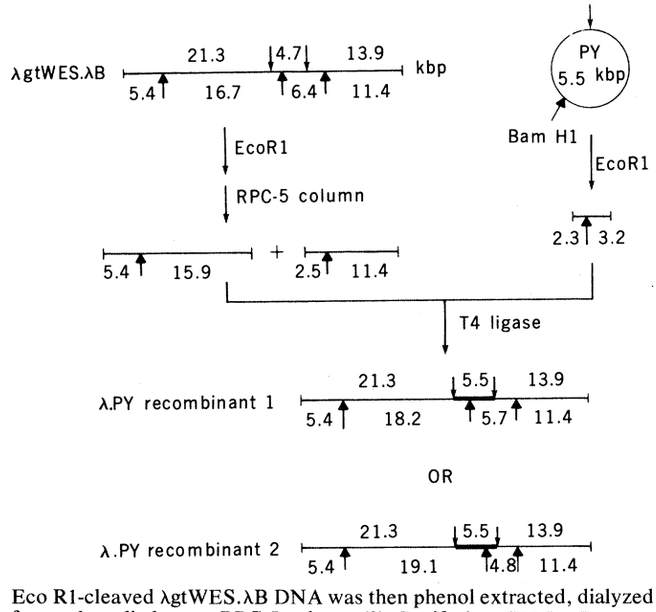

Fig. 1. Construction of λ-PY recombinant DNA. Arrows pointing downward and upward denote Eco R1 and Bam H1 sites, respectively. The sizes of the restriction enzyme fragments are given in kilobase pairs. λgtWES.λB DNA and PY DNA of desired amounts were cleaved with sufficient Eco R1 enzyme in buffer containing 100 m*M* tris-HCl, *p*H 7.9, 50 m*M* NaCl, 0.1 m*M* EDTA, and 12 m*M* $MgCl_2$. Reaction mixtures were incubated at 37° C for 1 hour and were terminated by incubation at 65°C for 7 minutes. The Eco R1-cleaved λgtWES.λB DNA was then phenol extracted, dialyzed against appropriate buffer, and applied to an RPC-5 column (*3*). Purified λgtWES. λB arms (66 μg/ml) and Eco R1-cleaved PY DNA (66 μg/ml) were then ligated with T4 DNA ligase in buffer containing 0.1*M* tris-HCl, *p*H 7.5, 50 m*M* NaCl, 12 m*M* $MgCl_2$, 0.1 m*M* EDTA, 10 m*M* dithiothreitol, 0.1 m*M* adenosine triphosphate, and 50 μg of bovine serum albumin per milliliter. Incubation was at 12°C for 24 hours. Ligated DNA was then used to transfect *E. coli* LE392. Recombinant DNA's were then isolated from the recombinant phages propagated in *E. coli* DP50 SupF.

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