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.

MARK A. ISRAEL
HARDY W. CHAN
DNA Recombinant Research Unit, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014
WALLACE P. ROWE
Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases
MALCOLM A. MARTIN
DNA Recombinant Research Unit

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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-

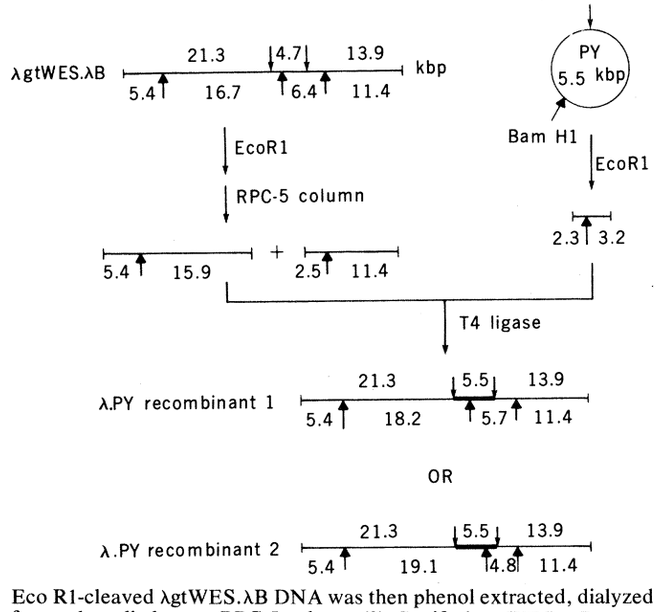

Fig. 1. Construction of λ-PY recombinant DNA. Arrows pointing downward and upward denote Eco R1 and Bam H1 sites, respectively. The sizes of the restriction enzyme fragments are given in kilobase pairs. λgtWES.λB DNA and PY DNA of desired amounts were cleaved with sufficient Eco R1 enzyme in buffer containing 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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mately 10^4 plaques per microgram of DNA.

Twenty plaques were selected, and the phage was propagated in *E. coli* strain DP50 SupF (*4*). To ascertain whether the putative recombinants contained PY DNA, phage DNA was prepared, digested with Eco R1, and the resulting fragments were analyzed by electrophoresis in 0.7 percent agarose gels. In all cases, we observed a DNA fragment that comigrated with a full-length linear PY DNA marker [5.5 kbp or 3.4×10^6 daltons (*5*)] and that contained PY nucleotide sequences as monitored by DNA-DNA hybridization (*6*).

The PY genome can be inserted into the lambda vector DNA in either of two orientations at the Eco R1 site, and cleavage of the recombinant DNA preparations with Bam H1 permitted the un-

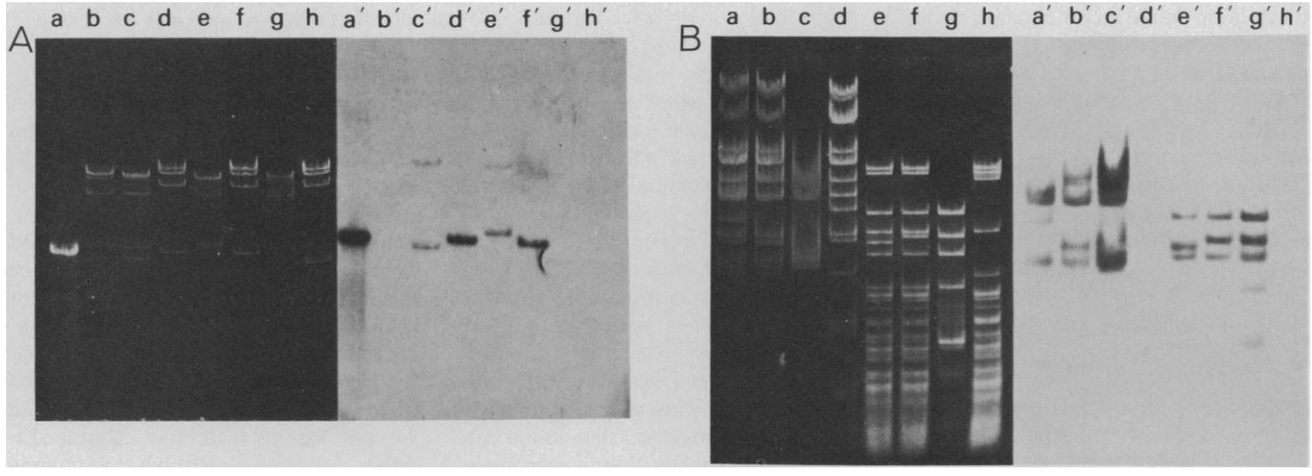

Fig. 2. Restriction enzyme analysis of λ-PY3 and λ-PY63 DNA. (A) Lanes a, d, f, and h are Eco R1 digests of PY, λ-PY63, λ-PY3, and λgtWES.λB DNA's, respectively. Lanes b, c, e, and g are Bam H1 digests of λgtWES.λB, λ-PY63, λ-PY3, and λgtWES.λB DNA's, respectively. Typical reaction mixtures contained 0.3 μg of DNA and sufficient restriction enzyme for complete digestion. Eco R1 buffer is described in the legend to Fig. 1 and Bam H1 buffer contained 20 m*M* tris-HCl, *p*H 7.5, 6.6 m*M* $MgCl_2$, 100 m*M* NaCl, and 2 m*M* β-mercaptoethanol. DNA fragments were analyzed by electrophoresis on a 0.7 percent agarose slab gel (150 V for 3 hours). Lanes a′ to h′ represent the Southern blotting analyses of the gel shown in lanes a to h, by means of a ^{32}P-labeled PY DNA probe. (B) Lanes a to d are Pst I digests of λ-PY3, λ-PY63, PY, and λgtWES.λB DNA's, respectively. Lanes e to h are Hpa II digests of λ-PY3, λ-PY63, PY, and λgtWES.λB DNA's. Reaction mixtures contained 1 μg of DNA and sufficient enzyme for complete digestion. Pst I buffer contained 20 m*M* tris-HCl, *p*H 7.4, 7 m*M* $MgCl_2$, and 1 m*M* dithiothreitol. After incubation at 37°C for 1 hour, DNA fragments were analyzed by electrophoresis on a 1.4 percent agarose gel (60 V for 16 hours). Lanes a′ to h′ represent the Southern blotting analyses of the gel shown in lanes a to h, by means of a ^{32}P-labeled PY DNA probe.

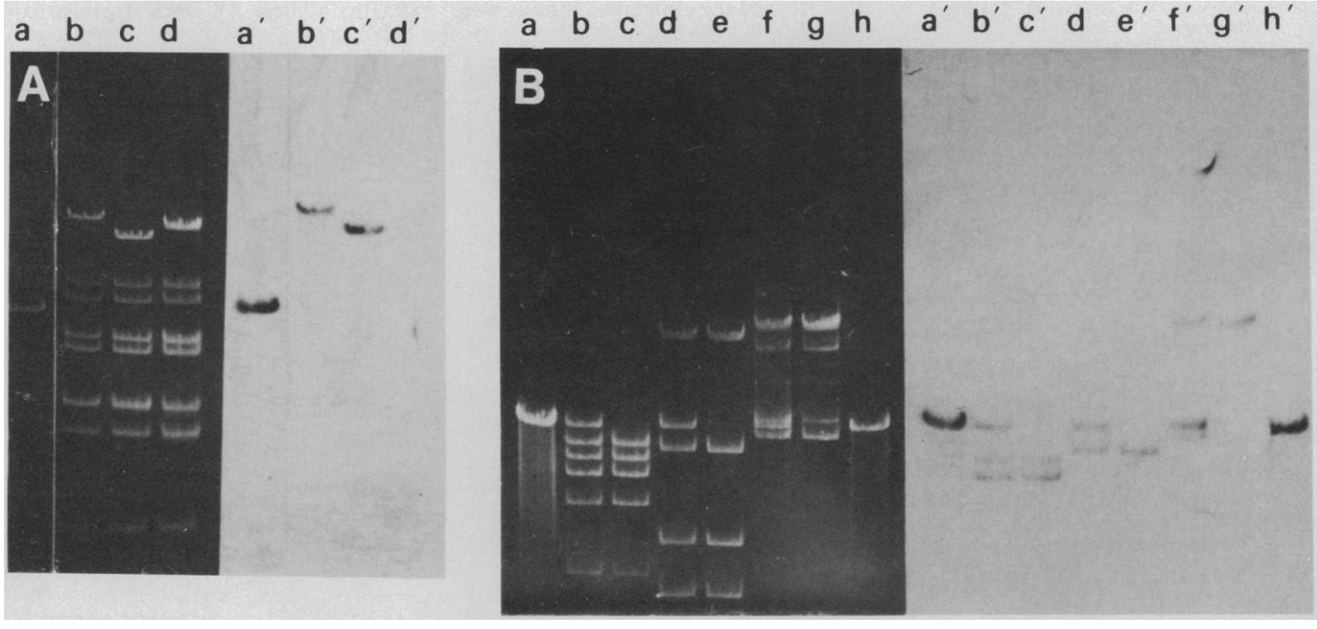

Fig. 3. Restriction enzyme analyses of λ-PY30-5T and λ-PY30-5B DNA's. (A) Lane a, Eco R1 digest of PY DNA; lanes b to d, Hpa I digests of λ-PY30-5B, λ-PY30-5T, and λgtWES.λB DNA's, respectively. Lanes a′ to d′ represent the Southern blotting analyses of the gel shown in lanes a to d, with a ^{32}P-labeled PY DNA probe. (B) Lanes a and h are Eco R1 digests of PY DNA; lanes b, d, and f are Hae II, Bgl 1, and Bam H1 digests of λ-PY30-5B DNA, respectively; lanes c, e, and g are Hae II, Bgl 1, and Bam H1 digests of λ-PY30-5T DNA, respectively. Reaction mixtures contained 1 μg of λ-PY recombinant DNA in Hae II buffer (50 m*M* tris-HCl, *p*H 7.5, 5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol), Bgl 1 buffer (identical to Bam H1 buffer) or Bam H1 buffer (see Fig. 2), and sufficient enzyme for complete digestion. DNA digests were analyzed by electrophoresis on a 1.4 percent agarose gel (120 V for 8 hours). These conditions allowed maximal resolution of DNA fragments in the 5.5-kbp size range, although many of the smaller digestion products have run off the gel. Lanes a′ to h′ represent the Southern blotting analyses of the gel shown in lanes a to h, by means of ^{32}P-labeled PY DNA probe.

ambiguous mapping of the viral DNA inserts (Fig. 1). An example of such an analysis is shown in Fig. 2. Cleavage of phage λ-PY63 DNA with Bam H1 resulted in four DNA fragments, 19.1, 11.4, 5.4, and 4.8 kbp in size (Fig. 2A, lane c). Cleavage of phage λ-PY3 DNA, in contrast, yielded fragments 16.7, 11.4, 6.4, and 5.4 kbp in size (Fig. 2, lane e). DNA-DNA hybridization with a ^{32}P-labeled PY DNA probe indicated that the 19.1- and 4.8-kbp fragments of λ-PY63 (lane c′) and the 16.7- and 6.4-kbp fragments of λ-PY3 (lane e′) contain PY DNA sequences. Thus, these two phages contain monomeric viral DNA inserted in opposite orientations. Digestion with Hpa II and Pst I also confirmed these results (Fig. 2B). Analysis of the 20 recombinant phage DNA's isolated indicated that PY DNA is inserted in one orientation in ten of the preparations and in the other direction in the others.

During the propagation of the recombinant phages, we noticed that, despite being derived from a single plaque, one isolate (λ-PY30) gave rise to progeny particles that banded at two different densities after isopycnic centrifugation in CsCl. The two phage bands (λ-PY30-5T and λ-PY30-5B, corresponding to the top and bottom components, respectively) were extensively purified and their DNA's were reexamined. The enzyme Hpa I, which does not cleave the PY genome, cleaved both of the recombinant DNA preparations into approximately eight fragments (Fig. 3A, lanes b and c). In both cases, the largest fragments contained PY DNA by the Southern (*6*) blotting technique (Fig. 3A, lanes b′ and c′); the fragment derived from λ-PY30-5B was larger than that from λ-PY30-5T. When the two phage DNA preparations were digested with Eco R1, the cleavage patterns were identical (data not shown). These observations suggested the possibility that the λ-PY30-5B recombinant contained more than one copy of PY DNA. Cleavage with restriction enzymes that make a single cut in PY DNA would excise a unit-length PY DNA molecule if head-to-tail oligomeric viral DNA inserts (that is, a tandem repeat in the same orientation) were present, but not with a head-to-head insert. The Bam H1, Bgl 1, and Hae II ("single cut" enzymes for PY DNA) cleavage patterns of λ-PY30-5T and λ-PY30-5B DNA's are shown in Fig. 3B. With the latter DNA, all three enzymes produced a band that comigrated with the full-length PY DNA marker, and hybridization with a ^{32}P-labeled PY DNA probe (Fig. 3B, lanes b′, d′, and f′) confirmed that these bands contained PY DNA sequences. This band was not present in the λ-PY30-5T cleavage products (Fig. 3B, lanes c′, e′, and g′).

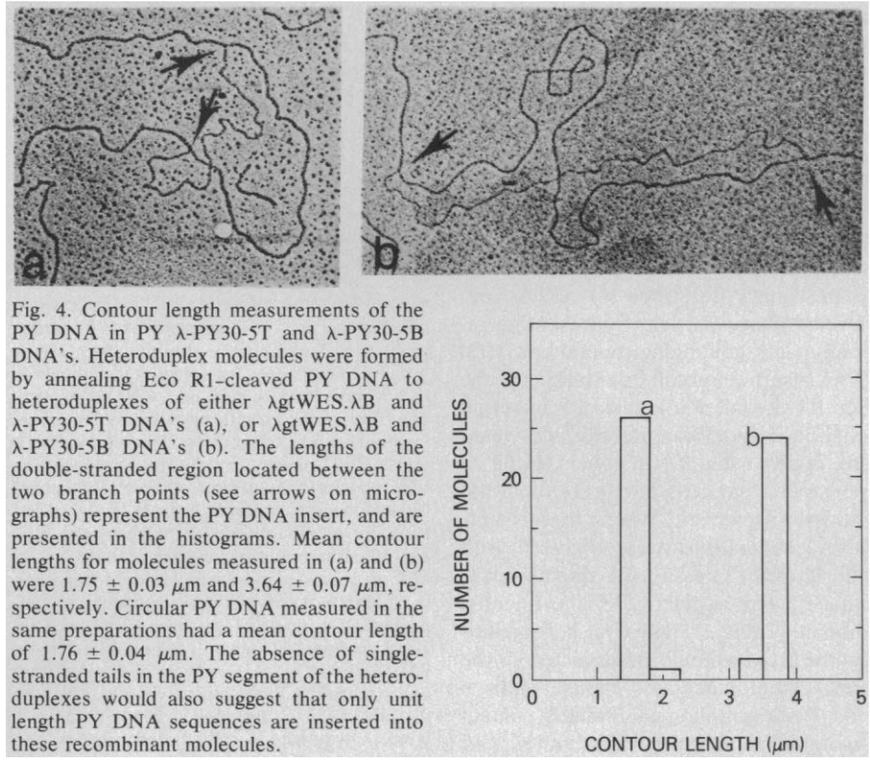

Fig. 4. Contour length measurements of the PY DNA in PY λ-PY30-5T and λ-PY30-5B DNA's. Heteroduplex molecules were formed by annealing Eco R1-cleaved PY DNA to heteroduplexes of either λgtWES.λB and λ-PY30-5T DNA's (a), or λgtWES.λB and λ-PY30-5B DNA's (b). The lengths of the double-stranded region located between the two branch points (see arrows on micrographs) represent the PY DNA insert, and are presented in the histograms. Mean contour lengths for molecules measured in (a) and (b) were 1.75 ± 0.03 μm and 3.64 ± 0.07 μm, respectively. Circular PY DNA measured in the same preparations had a mean contour length of 1.76 ± 0.04 μm. The absence of single-stranded tails in the PY segment of the heteroduplexes would also suggest that only unit length PY DNA sequences are inserted into these recombinant molecules.

The number of tandem copies of PY DNA in the λ-PY30-5B molecule was analyzed by electron microscopy. Heteroduplex molecules formed between DNA's of λgtWES.λB and the λ-PY recombinants revealed a single internal region of strand separation or heterology. One strand in that region represents the Eco R1 B fragment of the λgtWES.λB DNA, and the other strand is the PY DNA insert. While the two heterologous single strands differed in length by a ratio of 2.37 (± 0.17) to 1 in heteroduplex molecules formed between λgtWES.λB and λ-PY30-5B, similar measurements on the heteroduplex constructed between λgtWES.λB and λ-PY30-5T yielded a single-stranded arm ratio near unity (1.037 ± 0.02). One of these single-stranded arms of the heterology bubble could be converted to a duplex structure by the addition of linear Eco R1-cleaved PY DNA to the hybridization mixture (Fig. 4). This permits the accurate measurement of the contour lengths of the "PY-specific" regions of the heteroduplex molecules. Comparisons of these data with the contour lengths of known circular PY DNA molecules present in the same samples established that λ-PY30-5B recombinant phages contained a dimeric PY insert, and confirmed that λ-PY30-5T contained a single copy of PY DNA.

To ascertain whether the two phage populations arose from the genomic duplication of the monomeric viral DNA insert or from the excision of a copy of PY DNA from a recombinant molecule containing a dimeric PY DNA insert, the phage in the λ-PY30-5T and λ-PY30-5B bands were independently propagated in *E. coli* DP50 SupF, and their progeny were analyzed by isopycnic centrifugation in CsCl. Passage of λ-PY30-5T phage uniformly resulted in a single phage band, which contained a single copy of PY DNA as determined by restriction enzyme cleavage analysis (data not shown). However, overnight propagation of λ-PY30-5B again gave rise to both types of phage particles, one of which banded exactly with recombinants containing a single copy of PY DNA. When titrated in *E. coli* DP50 SupF, λ-PY30-5B formed plaques with "single-hit" dose-response kinetics; this indicates that λ-PY30-5B phage is not defective and does not require the helper function of λ-PY30-5T phage. Furthermore, λ-PY30-5T and λ-PY30-5B were comparably infectious for *E. coli*, whether measured with virus particles or free DNA (data not shown).

In serial, low-multiplicity [0.01 plaque-forming units (PFU) per cell] passages of the mixed phage population derived from λ-PY30-5B, progeny phage containing monomeric PY DNA eventually predominated. These experiments indicate that the tandem insertion of more than

one copy of DNA into the λgtWES.λB vector results in unstable recombinants that shed extra copies of the inserts at a high frequency. This phenomenon has been noted by Perricaudet *et al.* (*7*), who examined recombinants containing a segment of the adenovirus genome.

The infectivity of the recombinant phage particles and their DNA's were assayed in mice or in cultured mouse cells as previously described (*1*). DNA from purified phage particles representing recombinants containing monomeric viral DNA inserted in both orientations at the Eco R1 site failed to induce PY infection when inoculated parenterally into weanling mice (Table 1), or when tested in mouse embryo cells in culture (data not shown). However, when these same DNA preparations were digested with Eco R1 prior to assay, PY infection was induced with regularity in the injected animals (Table 1), and viral hemagglutination activity could be detected in the media from inoculated mouse embryo cells (data not shown). Similarly, intact recombinant phage particles and *E. coli* infected with recombinant phage did not induce PY infection after mouse inoculation (Table 1). These results indicate that while the PY genome is copied faithfully in *E. coli* when it is propagated as a component of a recombinant phage, neither the phage particles containing the monomeric PY DNA insert nor concentrated suspensions of *E. coli* infected with such recombinant phage, nor the DNA itself infected mice. Thus, these results are in agreement with those reported for monomeric PY inserts in a plasmid vector system (*1*).

In contrast to recombinant phage containing a single copy of a viral genome, those containing a head-to-tail dimer insert of PY DNA would, in theory at least, be more likely to be infectious in mouse cells since infectious PY genomes could be generated by intramolecular recombination. PY infection did occur in two of five mice inoculated with 2.8 μg of λ-PY30-5B DNA and in two of five mice injected with 23 μg of DNA prepared from *E. coli* infected with λ-PY30-5B phage (Table 2). Neither DNA (3.6 μg) from λ-PY30-5T, the monomeric recombinant phage that was derived from the same plaque from which the recombinant phage with the dimeric insert was isolated, nor DNA (16.4 μg) isolated from *E. coli* infected with λ-PY30-5T phage was infectious (Table 2), confirming the results shown in Table 1.

PY infection also developed in 6 of 11 mice injected with a crude phage lysate which had been precipitated with polyethylene glycol; this lysate contained 2×10^8 viable PY30-5B phage per inoculum (Table 2). Injection of live *E. coli* DP50 SupF, infected with the λ-PY30-5B phage, failed to induce infection in any of 28 recipient animals (Table 2). Also, feeding of comparable infected bacteria did not induce PY infection in any of the 13 mice tested. (These experiments were done with bacterial cultures infected at a low multiplicity of infection in order to allow multiple cycles of phage infection in the mouse.) We had expected that mice injected with live *E. coli*, infected with λ-PY30-5B, would develop a viral infection since each animal was inoculated with a sufficient number of bacteria to generate amounts of infectious phage (10^8 to 10^9 PFU) known to induce a PY infection (Table 2). The negative results in the inoculated animals may indicate that phage replication is severely inhibited in *E. coli* DP50 SupF when the bacteria are maintained in the subcutaneous environment; for example, the anaerobic conditions in the tissues may be inhibitory since optimal phage production is known to require highly aerobic conditions. Alternatively, if progeny phage are produced, their ability to gain access to mouse cells may somehow be impeded by the presence of bacterial cell components (for example, by phagocytosis of the phage-infected *E. coli* or readsorption of phage particles to cell wall fragments).

Table 1. Inoculation of mice with λ-PY DNA, λ-PY phage, or λ-PY-infected *E. coli*. Weanling mice from a PY-free colony (Charles River Laboratory) were inoculated with preparations of recombinant DNA, recombinant phage, or *E. coli* infected with recombinant phage. At 3 and 6 weeks after the mice were infected, serums were assayed for PY hemagglutination inhibiting antibody (*9*). Titers greater than 1:20 were considered indicative of PY infection. λ-PY3 and λ-PY10 are recombinant phages with monomeric PY DNA inserted in one orientation, whereas λ-PY63 and λ-PY25 phages contain the viral DNA in the opposite orientation (see Fig. 1). Recombinant phage was prepared from bacterial lysates by precipitation with polyethylene glycol (10 percent) in the presence of 0.5*M* NaCl, and was purified by isopycnic centrifugation in CsCl. λ-PY DNA's were prepared by extracting purified phage three times with an equal volume of phenol, equilibrated with 0.05*M* tris-HCl, *p*H 7.5, and dialysis of the extract against 0.01*M* tris-HCl, *p*H 7.5, and 0.1 m*M* EDTA. The λ-PY and PY DNA's were cleaved with Eco R1 as described in Fig. 2. In the experiment involving the inoculation of mice with live bacteria, *E. coli* DP50 SupF (in the log phase of growth) were infected with λ-PY recombinants. After a 15-minute adsorption period at room temperature, the bacteria were grown at 37°C for 20 minutes, then chilled in ice and injected into test animals. An uninoculated control mouse was included in each cage; all remained negative for PY antibody.

Inoculum	Dose* (μg per mouse)	No. infected/No. inoculated
λ-PY3 DNA	1.43	0/4
	1.43×10^{-2}	0/5
λ-PY3 DNA + Eco R1	1.43	5/5
	1.43×10^{-2}	4/5
λ-PY63 DNA	1.43	0/5
	1.43×10^{-2}	0/5
λ-PY63 DNA + Eco R1	1.43	5/5
	1.43×10^{-2}	2/5
λ-PY10 DNA	1.43	0/4
	1.43×10^{-2}	0/5
λ-PY10 DNA + Eco R1	1.43	5/5
	1.43×10^{-2}	4/5
λ-PY25 DNA	1.43	0/5
	1.43×10^{-2}	0/5
λ-PY25 DNA + Eco R1	1.43	5/5
	1.43×10^{-2}	1/5
PY DNA I	1.0×10^{-1}	5/5
	1.0×10^{-3}	3/5
PY DNA I + Eco R1	1.0×10^{-1}	5/5
	1.0×10^{-3}	1/5
λ-PY3-infected *E. coli*	2×10^9 PFU/10^8 *E. coli*	0/5
λ-PY63-infected *E. coli*	2×10^9 PFU/10^8 *E. coli*	0/5
λ-PY3 phage	2×10^9 PFU†	0/5
λ-PY63 phage	2×10^9 PFU†	0/5
Saline	0.1 ml	0/5
	0.1 ml†	0/5

*Unless otherwise stated, the material was administered by intraperitoneal injection. †Injected subcutaneously.

To what extent can these results be extrapolated to the assessment of the possible risks involved in cloning other eukaryotic viral genomes? Polyoma virus is a member of the Papovaviridae, which

The relative infectivity of various forms of PY DNA when administered to mice by injection or feeding is summarized in Table 3. Viral DNA's are far less infectious (10^5-fold or more) when they are free as compared to when they are packaged into virions. We have recently shown that this is true of PY DNA as well (*8*), and that the free PY DNA is noninfectious when fed to mice. When contained in a recombinant molecule, the PY DNA is far less infectious still than the free viral DNA, by about three orders of magnitude for dimer inserts and by at least four orders of magnitude when present as a monomeric insert. Thus, the free recombinant DNA molecules are of the order of 10^9-fold less infectious by injection than is the polyoma virus itself, and the recombinants have been noninfectious when given by mouth, either as DNA or in live *E. coli*. When intact lambda phage particles containing recombinant DNA with a dimeric PY DNA insert were injected into mice, the DNA was infectious at a level comparable to free PY DNA. However, injection or feeding of mice with *E. coli* infected with these phage did not result in PY infection.

also includes SV40, the human papovaviruses JC and BK, the various papilloma viruses, and a number of other viruses of vertebrates. These are relatively simple DNA viruses, having icosahedral symmetry, no envelope, a relatively small genome, and a virion made up of only three virus coded polypeptides. It thus seems clear that a similar reduction in, or lack of, infectivity of cloned genomes can be predicted for other papovaviruses as well as the majority of other DNA viruses, which contain larger and more complex genomes.

One of the main objectives of the polyoma cloning experiments was to gain insight into the extent to which DNA molecules can be transferred from *E. coli* to mammalian cells in potentially functional form. The results with the dimeric recombinant DNA molecules are most relevant to this question, since molecules containing a single copy of viral DNA would initiate infection only if site-specific endonucleases recognizing Bam H1 or Eco R1 sites were present in the mouse cells, or if there were some still unknown mechanism for accurate excision of the PY genome. With the dimers, however, there is no need to postulate a specific excision system or novel replication mechanism in order to generate infectious PY molecules from a linear DNA template, since intramolecular recombination can produce an intact circular PY genome. The rarity of infection by the dimer-containing molecules (Table 3) is thus all the more striking.

The polyoma system can provide another important test of the ability of DNA molecules to move from *E. coli* into host cells, namely, the development of tumors after the injection of bacteria containing PY DNA recombinants into newborn hamsters. PY DNA is capable of inducing tumors in suckling hamsters (*8*), and we have found that restriction enzyme-cleaved DNA preparations can be tumorigenic in the absence of residual infectivity. It is thus conceivable that the recombinant-containing bacteria, although not capable of eliciting productive viral infection, might be able to deliver a tumorigenic dose of PY DNA to the hamster cells. Tests of this hypothesis, which require long-term observation, remain to be completed.

While it is clear from our studies that risk assessment analysis of the cloning of DNA viral genomes should take into consideration whether head-to-tail tandem inserts might be cloned, in most experimental systems they are highly unlikely to occur. It is particularly unlikely that dimeric viral inserts would be formed during the course of a shotgun

Table 2. Inoculation of mice with phage recombinants containing one or two copies of PY DNA. *Escherichia coli* DP50 SupF (in the mid-log phase) were infected with λ-PY30-5B at a multiplicity of 0.04 PFU per cell and allowed to absorb for 15 minutes at room temperature; 0.1-ml samples containing approximately 5×10^7 *E. coli* were then inoculated into test animals. Other mice that had been fasted overnight were fed approximately 1×10^8 bacteria suspended in milk and absorbed on bread. Two other feedings were given during the first week of the test. In another experiment DNA was prepared from *E. coli* DP50 SupF infected with λ-PY30-5T or λ-PY30-5B at a multiplicity of 20. The organisms were grown for 20 minutes at 37°C, chilled in ice in the presence of 0.02*M* sodium azide, and then incubated at 37°C for 1 hour with lysozyme (50 μg/ml). Samples were then digested with Proteinase K (50 μg/ml) in the presence of 1 percent sodium dodecyl sulfate for 1 hour at 37°C, extracted three times with an equal volume of phenol, and dialyzed against 0.01*M* tris-HCl, *p*H 7.5. λ-PY30-5B phage was prepared as described (Table 1), except that isopycnic centrifugation in CsCl was omitted. Phage DNA was purified as described in Table 1.

Inoculum	Dose	No. infected/No. tested	
		Subcutaneous	Oral
E. coli infected with λ-PY30-5B			
Live bacteria	2×10^6 PFU/5×10^7 *E. coli*	0/28	
	4×10^6 PFU/1×10^8 *E. coli*		0/13
DNA	23 μg	2/5	
λ-PY30-5B phage			
Concentrated lysate	2×10^8 PFU	6/11	
DNA	2.8 μg	2/5	
E. coli infected with λ-PY30-5T			
DNA	16.4 μg	0/5	
λ-PY30-5T phage			
DNA	3.6 μg	0/5	
Controls			
PY DNA I	0.01 μg	5/5	
Saline	0.1 ml	0/5	

Table 3. Infectivity for mice of polyoma DNA in various forms. These estimates were calculated by the Poisson distribution from the data in (*1*, *8*, *10*) and in this report. The following assumptions or data were also used. (i) Polyoma virions have a ratio of particle to infectivity of about 3×10^2. (ii) One microgram of PY DNA is 10^{11} molecules. (iii) The PY-plasmids are carried at ten copies per bacterium. (iv) The PY-plasmid is 58 percent PY DNA, the monomer PY-λ DNA is 14 percent, and the dimeric PY-λ DNA is 25 percent PY DNA. (v) At 20 minutes, the time when *E. coli* lytically infected with PY-λ were harvested for the "total *E. coli* DNA" tests, the cells contained ten PY DNA equivalents per cell in the case of the monomeric inserts, and 20 in the case of the dimeric inserts. (vi) These estimates are also assumed for the PY-λ infected *E. coli* administered to mice by injection or feeding; this very low estimate is used because of the inefficiency of the lytic cycle under anaerobic conditions.

Form of DNA	Infectivity		No. of PY DNA molecules in MID*	
	Parenteral	Oral	Parenteral	Oral
Virions	+	+	10^2 to 10^3	10^8 to 10^9
Extracted from virions				
Supercoiled circles	+	−	7×10^7	$> 7 \times 10^{12}$
Relaxed circular	+		10^8	
Eco R1 linears	+		4×10^8	
Bam H1 linears	+		5×10^8	
Cloned in plasmid pBR322 (monomer)				
Live χ1776	−	−	$> 5 \times 10^{11}$	$> 10^{12}$
Plasmid DNA	−		$> 5 \times 10^{12}$	
Plasmid DNA, cleaved	+		$\leq 4 \times 10^8$	
Cloned in λ, monomeric insert				
Live coli in latent period	−		$> 5 \times 10^{10}$	
Total (coli) cellular DNA	−		$> 3 \times 10^{11}$	
Purified phage	−		$> 2 \times 10^{10}$	
DNA from purified phage	−		$> 4 \times 10^{11}$	
DNA from purified phage, cleaved	+		$\leq 5 \times 10^9$	
Cloned in λ, dimer insert				
Live coli in latent period	−	−	$> 3 \times 10^{10}$	$> 8 \times 10^{10}$
Total (coli) cellular DNA	+		3×10^{11}	
Purified phage	+		10^9	
DNA from purified phage	+		10^{11}	

*Mean infectious dose.

experiment with eukaryotic cellular DNA. For example, the phage recombinants we constructed were ligated at a PY DNA concentration of 66 μg/ml, and only one of the 20 clones studied contained a dimeric insert. If, for example, during the course of a shotgun experiment, tissue inadvertently infected with a papovavirus and containing virus or DNA at an average of 100 genome equivalents per cell was used as the source of DNA, the ligation reaction mixture containing 66 μg of cellular DNA would contain approximately 0.006 μg of viral DNA. Not only would the number of recombinants containing single copies of viral DNA be very low (about one clone per 10^4), but the number containing a dimeric insert would be negligible, since dimer formation would be highly concentration-dependent. Furthermore, our results and those of others (*7*) indicate that recombinants containing more than one copy of viral DNA are unstable in the rec^+ bacteria used for their propagation and would eventually disappear from the population.

In view of the widespread interest in the polyoma cloning experiments as a way of evaluating certain postulated potential biohazards of recombinant DNA research, it is important to give our perception of what the studies presented in this and the preceding report (*1*) do and what they do not contribute to the assessment of risk. First, no single experimental system or program can declare recombinant DNA research to be "dangerous" or "safe." Risk assessment of a large and complex area of research is incremental, and our findings should be viewed as a useful addition to, and confirmation of, the immense body of relevant preexisting knowledge of virology and bacteriology that has already been considered in the context of risk assessment.

Our data are most pertinent to the question of cloning viral sequences, but they have some implications for general cloning of eukaryotic DNA in *E. coli* as well. With regard to the former, our findings give no support to the concept, already virtually eliminated by the accumulating knowledge of the complexity of viral gene regulation and synthesis, that bacteria carrying recombinant plasmids or phages containing a eukaryotic viral genome would become producers of virus particles.

In the many discussions with virologists during the planning of these studies, there was a general consensus that the experiments would show *E. coli* carrying PY-λ or PY-plasmids to induce no PY infections when given by mouth, to give some infections when given parenterally, and to be quite infectious by parenteral injection if the insert was an oligomer. The most striking feature of our results, then, is the extremely low or absent infectivity of the recombinant molecules. In no instance was a recombinant molecule with a monomeric insert infectious, and in no instance did oral or parenteral administration of massive doses of live recombinant-containing *E. coli* induce PY infection.

We thus view these results as being highly reassuring with respect to the safety of cloning viral genomes in *E. coli*.

HARDY W. CHAN
MARK A. ISRAEL
DNA Recombinant Research Unit, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014
CLAUDE F. GARON
Laboratory of the Biology of Viruses
WALLACE P. ROWE
Laboratory of Viral Diseases
MALCOLM A. MARTIN
DNA Recombinant Research Unit

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11. The studies described in this and the preceding report (*1*) were carried out under the containment conditions specified by the "NIH Guidelines for Research Involving Recombinant DNA Molecules" [*Fed. Regist.* **41**, 27902 (1976)]. All host vector systems were certified EK2 systems. All laboratory procedures involving viable bacteria or λ phage, including inoculations of mice, were done under P4 physical containment. Mice injected with free recombinant DNA molecules were held under P3 physical containment conditions. We thank Dr. L. Enquist for advice and help; Dr. J. Nutter and S. Nagle for the management of the P4 facility; and R. Boaze, J. Brewer, T. Bryan, J. Byrne, Dr. M. Collins, H. Grove, P. Howten, M. Meltzer, and H. Stull for technical assistance.

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Melting of Io by Tidal Dissipation

Abstract. *The dissipation of tidal energy in Jupiter's satellite Io is likely to have melted a major fraction of the mass. Consequences of a largely molten interior may be evident in pictures of Io's surface returned by Voyager I.*

The free eccentricity of Io's orbit is approximately .00001 (*1*). If this eccentricity accounted for all of the variation in the Jupiter-Io separation, the dissipation of energy from tides raised on Io by Jupiter would be negligibly small (*2*), since Io is synchronously rotating. But the resonant structure of the Galilean satellite leads to forced eccentricities that are considerably larger than the free values. Although still modest by most standards, these forced eccentricities coupled with the enormous tides induced by Jupiter lead to magnitudes of tidal dissipation that are certainly important and may completely dominate the thermal history of the innermost satellite Io. We will first establish values of the forced eccentricities and later substitute these into an expression for the total tidal dissipation.

The Galilean satellites are numbered in the conventional manner with 1 to 4 corresponding respectively to Io, Europa, Ganymede, and Callisto. Let λ_i, n_i $(=d\lambda_i/dt)$, and $\bar{\omega}_i$ denote the mean longitude, mean orbital motion, and longitude of the perijove. The relation $n_1 - 3n_2 + 2n_3 = 0$ is satisfied exactly within observational error such that $\lambda_1 - 3\lambda_2 + 2\lambda_3 = 180°$ with no observed libration amplitude. This is the well-known Laplace relation and is often called simply the libration. In addition, the mean motions of 1 and 2 and, separately, of 2 and 3 are nearly in the ratio 2:1

$$n_1 - 2n_2 = n_2 - 2n_3 = 0.^{\circ}739507/\text{day} \quad (1)$$

compared to $n_2 = 101.^{\circ}375$/day. The nearness of the two sets of mean motions to the 2:1 commensurability suggests that some of the resonance variables $\lambda_1 - 2\lambda_2 + \bar{\omega}_1$, $\lambda_1 - 2\lambda_2 + \bar{\omega}_2$, $\lambda_2 - 2\lambda_3 + \bar{\omega}_2$, and $\lambda_2 - 2\lambda_3 + \bar{\omega}_3$ may be librating about constant values. A periodic solution of the equations of motion with these resonance variables having constant values was used by de Sitter (*3*) as a first approximation in his theory of the Galilean satellites. Sinclair (*4*) has rederived the complete periodic solution and shown that in fact the first three of the four resonance variables are librating with small amplitude—the first and third about 0° and the second about 180°. This means that conjunctions of 1 and 2 occur when 1 is near its perijove and 2 is near its apojove; conjunctions of 2 and 3 oc-

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