Tumorigenic activity of cloned polyoma virus DNA in newborn rats[1]

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Summary. The proximal portion of the polyoma virus early region as well as the complete viral genome were cloned in pBR322. Recombinant plasmids induced tumors in newborn rats but only after linearization of the DNA by various restriction endonucleases.

Recent studies on the transforming capacity of Simian virus 40 (SV40) and its related polyoma virus (Py) indicate that gene products specified by the early region of papovavirus genomes play a key role in the oncogenic process[2]. The early region codes for several related polypeptides: at least 2 for SV40[3], designated small and large tumor antigens (T antigens), and at least 3 for Py[4,5] designated small, middle, and large T antigens. Although transformation by SV40 seems to implicate large T, there is strong evidence which indicates that Py large T is not essential either for the initiation or the maintenance of transformation[6,7,8]. It has also been reported that Py DNA which has been cleaved in the distal portion of the early region by restriction endonucleases has an enhanced tumorigenic activity compared with uncleaved DNA[9]. A possible explanation for this finding is that Py large T interferes with tumor formation. To define better the biological activity of Py DNA in animals, we have studied the ability of recombinant plasmids containing fragments of Py DNA to induce tumors in newborn rats.

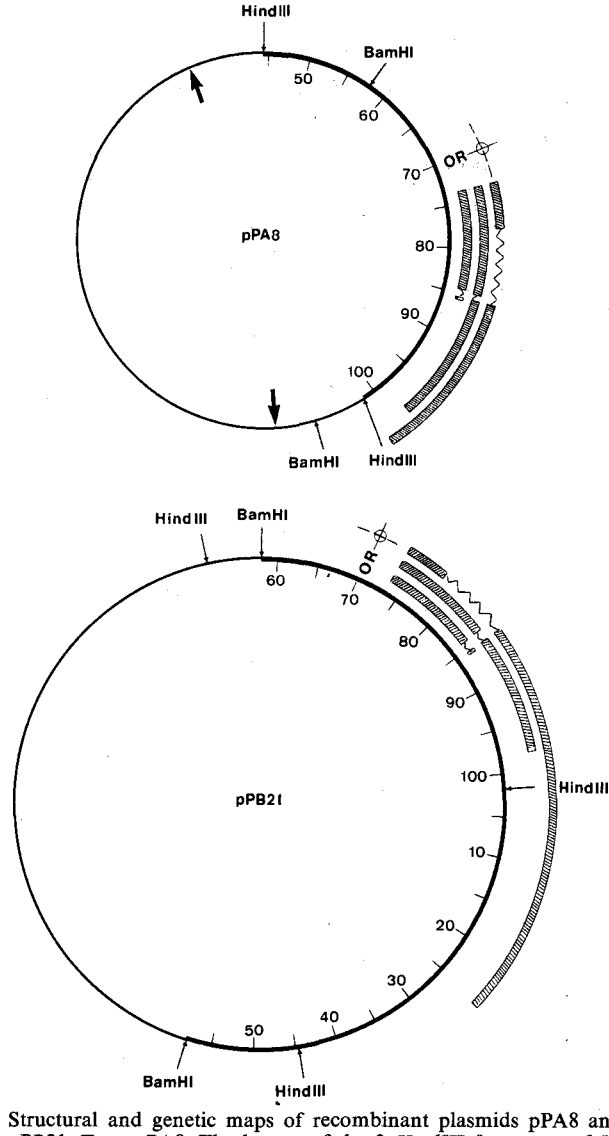
Plasmids containing the entire viral genome were constructed by cleavage of Py DNA with *Bam*HI which produces a single cut in the late region. The resulting linear molecule was then joined, using T4 ligase, to pBR322 DNA previously digested with *Bam*HI (pPB21, fig.). Plasmids containing the proximal portion of the Py early region were obtained by cloning the largest of the 2 *Hin*dIII fragments of Py DNA into the *Hin*dIII site of pBR322 (pPA8, fig.). This fragment extends clockwise from 46 to 1.8 units on the Py map[2]. It contains the origin of viral DNA synthesis as well as sequences encoding both small and middle T antigens, but lacks the sequences coding for the C-terminal segment of large T antigen. The plasmids were used to transform *E. coli* HB101 to ampicillin resistance, a selectable marker carried by the plasmid, and clones containing insertions of Py DNA were identified by screening for a tetracycline sensitive phenotype as previously described[10].

Characterization of the recombinant plasmids by restriction endonuclease mapping showed that the insertion of Py DNA in both *Bam*HI and *Hin*dIII sites of pBR322 occurred in the 2 possible orientations. The infectivity of the cloned viral DNA was determined after releasing the Py DNA from the plasmid by digestion with the same restriction endonuclease used in the plasmid construction. The assay with pPA8 was performed in the presence of *Hin*dIII-

Tumorigenesis by recombinant Py DNAs

Plasmid	DNA configuration	Incidence of tumors in newborn rats Number	%
pPA8	Supercoiled	0/31	0
pPA8	Cleaved by *Hin*dIII	33/55	60.0
pPA8	Cleaved by *Hin*dIII + *Bam*HI	24/56	42.8
pPA8	Cleaved by *Hin*cII	4/24	16.6
pPB21	Supercoiled	0/17	0
pPB21	Cleaved by *Hin*dIII	4/11	36.3
pPB21	Cleaved by *Hin*dIII + *Bam*HI	11/17	64.7
pPB21	Cleaved by *Bam*HI	3/9	33.3
pBR322	Cleaved by *Hin*dIII	0/10	0

1-day-old Fischer rats were inoculated in the neck with 2 µg of DNA s.c. (in 50 µl of phosphate buffer saline) in the configuration indicated.



Structural and genetic maps of recombinant plasmids pPA8 and pPB21. Top: pPA8. The largest of the 2 *Hin*dIII fragments of Py DNA (fragment *Hin*dIII-l) is inserted into the *Hin*dIII site of pBR322. The viral region in the plasmid is shown by the thick line and is divided as if the complete viral genome had been divided into 100 units starting at the *Eco*RI site. The cleavage sites for *Hin*dIII and *Bam*HI are indicated. Arrows inside the circle indicate the cleavage sites for *Hin*cII in pBR322. The combed lines outside the circle indicate the putative coding regions for small T, middle T, and the proximal fragment of large T antigen. - Bottom: pPB21. The complete Py genome is inserted into the *Bam*HI site of pBR322.

cleaved pPB41[8], a plasmid carrying the 2nd *Hin*dIII fragment of Py DNA. Both biochemical and biological analyses indicated that the cloned viral sequences had not undergone any detectable modification during the cloning procedure.

To determine the ability of recombinant plasmids to induce tumors in rats, we inoculated s.c. 2 µg of DNA into the neck of 1-day-old Fischer rats and observed the animals for tumor development over a 4-month period. As shown in the table, none of the rats inoculated with either circular pPB21 (complete genome inserted into the *Bam*HI site of pBR322) or circular pPA8 (*Hin*dIII-1 fragment of Py DNA inserted into the *Hin*dIII site of pBR322) developed tumors during the 4-month observation period. The same amount of DNA, however, cleaved by *Hin*dIII or by *Hin*dIII plus *Bam*HI, induced tumors in approximately half of the animals. The tumors appeared within 4–8 weeks following DNA injection and were invariably located near the site of inoculation. The enhanced tumorigenicity observed with the cleaved recombinants was not totally unexpected in view of the earlier observation that tumorigenicity of Py DNA in newborn hamsters was enhanced after cleavage with restriction enzymes that interrupt the distal portion of the early gene region[9]. To determine whether cleavage of the recombinant plasmids outside viral sequences would also stimulate their tumorigenic potential, newborn rats were inoculated with pPA8 digested with *Hin*cII. This enzyme cuts pBR322 twice, 481 and 623 base pairs from its *Hin*dIII site[11], but does not cleave the *Hin*dIII-1 fragment of Py DNA. *Hin*cII-cleaved pPA8 consists, therefore, of linear Py *Hin*dIII-1 DNA flanked on each side by 2 stretches of pBR322 sequences of 481 and 623 base pairs. The table shows that *Hin*cII-cleaved pPA8 induced tumors in 4 out of 24 (16.6%) animals. Although the *Hin*cII fragment is less tumorigenic than its *Hin*dIII or *Hin*dIII plus *Bam*HI counterparts ($p < 0.01$), it appears that cleavage of the recombinant plasmid outside Py sequences enhanced its tumorigenic potential. A possible explanation for the enhanced tumorigenicity is that injection of linear rather than supercoiled DNA molecules may facilitate cellular uptake or integration into the host chromosome. Among other hypotheses that have been entertained is the possibility that interruption of the Py genome in the distal portion of the early region may interfere with the synthesis of virus encoded or induced polypeptides such as the tumor specific transplantation antigen[9] that may play a role in the immunological recognition of tumor cells. Our data do not support such a hypothesis since pPA8, a plasmid lacking the distal portion of the Py early region (1.8–26 units) is not tumorigenic when injected in the circular form. Furthermore, cleavage of pPB21 by *Bam*HI does not interrupt any of the viral early genes, yet stimulates tumorigenicity (table). Therefore, the enhanced tumorigenic potential of cleaved recombinant plasmids cannot be correlated with the inactivation of the tumor specific transplantation antigen gene.

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A cell-marking technique for a cellular slime-mold

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Summary. A simple technique for marking cells of the cellular slime mold *Dictyostelium discoideum* has been developed, using the phagocytic action of the cells. The *D. discoideum* are fed on *E. coli* stained with neutral red and clearly red colored amoebae are obtained.

Dyes such as neutral red, nile blue, and cresyl violet have been used as cell markers for the cellular slime mold *Dictyostelium discoideum* by Bonner and others[1–4]. Farnthworth and Wolpert[5] reported that water soluble dyes were unsatisfactory because they are transferred from cell to cell, so that a mixture of labeled and unlabeled cells gave uniformly labeled cells; on the other hand, acridine orange could be absorbed onto microgranular diethyl amino-ethyl cellulose, and the particles then fed to the amoebae, to obtain colored cells. However, the technique is complicated, so that a simpler and more stable labelling method is necessary in preparation for studies of marked cells.

The amoebae of *D. discoideum* NC-4 (wild type) feed preferentially on bacteria, and multiply by binary fission. *Escherichia coli* B/r is one of the best foods for *D. discoideum* NC-4. In a previous study by Raper[6], *Serratia marcescens (Chrombacterium prodigiosum)* was fed to amoebae, and colored amoebae were obtained. However, Raper's method has not been used as a cell marker for *D. discoideum* NC-4. Using the method described here, marked cells of *D. discoideum* can easily be obtained, and clearly red-colored amoebae persist throughout the developmental period. The amoebae are not immersed in the dye, but take up colored cells by phagocytic action. When this method is used, the dye does not pass from cell to cell.

D. discoideum NC-4 was used throughout the experiments. *E. coli* B/r was grown in liquid nutrient suspension (6.5% Bacto peptone, 2.0% yeast extract, 2.0% glucose, 10 mM