Biological Properties of Polyoma DNA Fragments Cloned in Plasmid pBR322

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SUMMARY

The two HindIII fragments of polyoma virus DNA were cloned in the HindIII site of plasmid pBR322, and the biological activity of the recombinant plasmids was tested in tissue culture cells. A mixture of recombinant plasmids containing the HindIII-A and HindIII-B fragments was infectious, but only after cleavage with HindIII. Recombinant plasmids containing the HindIII-A fragment, but not those containing the HindIII-B fragment, induced the transformation of Fischer rat 3T3 cells. These findings indicate that about half of the early region of polyoma virus DNA is not essential for the initiation or the maintenance of transformation.

Both polyoma (Py) virus and its DNA are able to induce tumours in animals and transform cells in culture (Tooze, 1973). Only the portion of the Py virus genome which is expressed during the early phase of productive infection and which consists of at least two genes, denoted ts-a and hr-t, seems to play a role in virus-induced transformation (Eckhart, 1977; Fluck et al. 1977). In a recent study, however, Israel et al. (1979a) found that the ability of Py DNA to induce tumours in newborn hamsters was enhanced by cleavage of the virus DNA with EcoRI, a restriction enzyme which cleaves Py DNA once (Griffin et al. 1974) in the middle of the ts-a gene (Miller & Fried, 1976; Feunteun et al. 1976). Cleavage at this site would presumably inactivate the ts-a gene which codes for large tumour antigen (T Ag) (Ito et al. 1977) but preserve the hr-t gene (Feunteun et al. 1976) which encodes both middle T Ag and small T Ag (Friedmann et al. 1978; Schaffhausen et al. 1978; Soeda et al. 1979).

Another restriction enzyme, HindIII, cleaves Py DNA twice, once in the late region and once in the early region close to the EcoRI site (Griffin et al. 1974). The present report deals with the construction and the biological properties of recombinant plasmids containing the two HindIII fragments of Py DNA. We show that recombinant DNA containing the HindIII-A fragment, which includes the hr-t gene but only part of the ts-a gene, induced transformation; recombinant DNA containing the HindIII-B fragment did not.

Mouse 3T6 cells were infected at a multiplicity of 10 p.f.u./cell with the A2 large plaque variant of Py virus (Fried et al. 1974) and supercoiled Py DNA was isolated by the Hirt procedure (1967) followed by equilibrium centrifugation in caesium chloride–ethidium bromide solution (Bauer & Vinograd, 1968). The DNA was digested to completion with restriction endonuclease HindIII, generating two fragments A and B of mol. wt. $2 \times 10^6$ and $1.5 \times 10^6$, respectively (Griffin et al. 1974). The fragments were separated by electrophoresis in a 0.7% agarose slab gel, and recovered by freezing and thawing the gel slices at $-20 ^\circ C$ (Thuring et al. 1975). They were ligated separately to HindIII linearized plasmid pBR322 (Bolivar et al. 1977) and the products of the ligation reaction were used to transform E. coli X1776 under D level (P3) physical containment. Colonies of bacteria displaying ampicillin resistance were screened for the presence of Py DNA by the method of Grunstein & Hogness (1975). About half of the colonies annealed with the Py DNA probe.
Table 1. Biological properties of Py DNA–pBR322 recombinants

<table>
<thead>
<tr>
<th>DNA preparation</th>
<th>Treatment</th>
<th>Specific infectivity*</th>
<th>Specific infectivity*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>P.f.u./μg DNA</td>
<td>P.f.u. per full polyoma sequence</td>
</tr>
<tr>
<td>pPA8 + pPB41</td>
<td>---</td>
<td>&lt; 1</td>
<td>&lt;10⁻¹¹</td>
</tr>
<tr>
<td>pPA8</td>
<td>HindIII</td>
<td>&lt; 1</td>
<td>&lt;10⁻¹¹</td>
</tr>
<tr>
<td>pPB41</td>
<td>HindIII</td>
<td>&lt; 1</td>
<td>&lt;10⁻¹¹</td>
</tr>
<tr>
<td>pPA8 + pPB41</td>
<td>HindIII</td>
<td>1.6 × 10⁶</td>
<td>2.5 × 10⁻⁸</td>
</tr>
<tr>
<td>Py A2†</td>
<td>---</td>
<td>1.1 × 10⁸</td>
<td>6.2 × 10⁻⁸</td>
</tr>
<tr>
<td>Py A2†</td>
<td>HindIII</td>
<td>6.0 × 10⁵</td>
<td>3.3 × 10⁻⁹</td>
</tr>
</tbody>
</table>

Transformation of FR 3T3 rat cells‡:

<table>
<thead>
<tr>
<th>Number of foci on plastic</th>
<th>Number of colonies in agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>pPA8</td>
<td>---</td>
</tr>
<tr>
<td>pPB41</td>
<td>---</td>
</tr>
<tr>
<td>pBR322</td>
<td>---</td>
</tr>
<tr>
<td>pPA8 + pPB41§</td>
<td>---</td>
</tr>
</tbody>
</table>

* Appropriate dilutions of DNA were assayed in triplicate on mouse 3T6 cells for plaque formation using the DEAE–dextran method (Thorne et al. 1968).
† Isolated from productively infected mouse cells.
‡ Cultures were transfected by the calcium chloride–DMSO method (Stow & Wilkie, 1976) with 1 μg of recombinant DNA.
§ 1 μg of each.

Twenty of the colonies which were found to contain Py DNA sequences were propagated in culture and then used to prepare plasmid DNA by a procedure involving lysis of the cell without detergent (Curtiss et al. 1977). All lysates were found to contain two species of DNA with electrophoretic mobilities somewhat lower than those of the respective form I and form II of pBR322 DNA. Both molecular species were converted into linear molecules with a single-cut restriction endonuclease in order to facilitate their identification by agarose gel electrophoresis. Plasmids pPA8 and pPA4 had a mol. wt. of about 4.8 × 10⁶. Such a value is expected from recombinant plasmids consisting of pBR322 (mol. wt. 2.8 × 10⁶) joined to full-length Py DNA fragment A (mol. wt. 2 × 10⁶) at the HindIII site. Plasmids pPB2 and pPB41 had a mol. wt. close to 4.2 × 10⁶, as expected from the insertion of Py DNA fragment B (mol. wt. 1.4 × 10⁶) into plasmid pBR322. The exact size of the inserted DNA was estimated by digesting the recombinant plasmids with HindIII and determining the electrophoretic mobility of the resulting fragments. All four plasmids yielded a fragment of mol. wt. 2.8 × 10⁶ consisting of pBR322 (Fig. 1 a), as well as another fragment of either 2 × 10⁶ mol. wt. (Py fragment A) or 1.4 × 10⁶ mol. wt. (Py fragment B). When the DNA was transferred to nitrocellulose filters by the technique of Southern (1975), only the smaller HindIII fragments generated from either recombinant annealed with Py DNA labelled with ³²P by nick-translation (Maniatis et al. 1975) (Fig. 1 b). We concluded, therefore, that these four recombinants comprised a full-length Py DNA A or B fragment inserted into the HindIII site of plasmid pBR322.

To ascertain the orientation of the fragments within the recombinants, the latter were digested with various restriction endonucleases. Cleavage with PstI or a mixture of BamHI and HindII yielded distinctly different patterns compatible with the insertion of both fragments in opposite orientations in the recombinants (Fig. 1 c, d).

To verify the fidelity of the cloning procedure and the stability of the foreign sequences
Fig. 1. Restriction enzyme analysis of Py–pBR322 recombinants. The DNA (0.1 to 0.5 μg) was digested with the enzymes indicated and the products were analysed by electrophoresis in 1 % agarose slab gels. Samples were electrophoresed for 5 to 6 h at 80 V per slab in buffer containing 40 mM-tris-HCl (pH 7-9), 10 mM-sodium acetate and 1 mM-EDTA. The DNA was stained by immersing the gel in a solution of ethidium bromide (1 μg/ml), visualized with a u.v. trans-illuminator and photographed with a Polaroid camera. (a) Electrophoresis of a HindIII digest of both polyoma and recombinant plasmid DNAs showing the recovery of fragments A and B after excision with the enzyme. (b) Autoradiogram. The DNA from gel (a) was transferred by blotting to a sheet of nitrocellulose and challenged with 32P-labelled authentic polyoma DNA in a hybridization mixture (Denhardt, 1966). (c, d) Determination of the orientation of the insert by restriction enzyme digestion of recombinant plasmids. The size of the mol. wt. markers (adenovirus type 2 DNA digested with HindIII) × 10⁻⁴ is shown in the margin.
in the bacterial host, the infectivity of cloned Py DNA in mouse 3T6 cell cultures was assayed. The cells were transfected by the DEAE-dextran procedure of Thorne et al. (1968). Intact recombinant plasmid DNA produced less than 1 p.f.u./μg DNA (Table 1). In contrast, a pPA8-pPB41 mixture, cleaved with HindIII, was found to have a specific infectivity of $1.6 \times 10^3$ p.f.u./μg of recombinant DNA ($2.5 \times 10^{-8}$ p.f.u. per HindIII-fragmented Py genome). This is at least as high as HindIII-cleaved Py DNA extracted from productively infected mouse cells. The mixture pPA4 plus pPB2 was also infectious after treatment with HindIII (results not shown). About 10 plaques were isolated independently and used to inoculate mouse 3T6 cell cultures. In all cases the cultures yielded large-plaque Py virus.

Infectivity of cloned Py DNA after excision at the site of joining has already been reported for recombinant plasmids containing the whole virus genome as insert (Chan et al. 1979; Fried et al. 1979; Israel et al. 1979c). Our infectivity assays demonstrated that Py DNA was replicated with sufficient fidelity in E. coli to preserve all the functions required for productive infection and that, after cleavage to separate virus from plasmid DNA, the cloned virus DNA displayed a specific infectivity comparable to that of HindIII fragments of authentic Py DNA.

Transforming activity of the recombinant plasmids was assayed on cell monolayers from a Fischer rat (FR) 3T3 cell line (Seif & Cuzin, 1977) using the calcium chloride-DMSO method (Stow & Wilkie, 1976). After 10 days of incubation at 39 °C, foci of transformed cells were observed growing over the monolayers transfected with recombinant plasmids containing the Py DNA HindIII-A fragment, but not over those transfected with plasmids containing the Py DNA HindIII-B fragment (Table 1). Cells of six foci were propagated in culture after being cloned three times. All clones displayed several properties of transformed cell lines, namely loss of contact inhibition and growth to high saturation density ($2.4 \times 10^6$ cells/cm²) in medium containing only 2 % serum. After transfection, some of the cells were also plated in soft agar medium as described by MacPherson & Montagnier (1964). Seven to 10 days later, colonies were observed that were similar in size to those produced after exposure to virus. The number of colonies per μg of pPA8 DNA was of the same order of magnitude as the number of foci observed on plastic (Table 1). No colonies were detected in plates seeded with cells that had been transfected with pPB41 DNA. Aliquots of $2 \times 10^8$ cells of one transformed clone selected as a focus on plastic were injected into 11 Fischer rats. Nine of these rats developed tumours within 9 weeks.

The transforming activity of the HindIII-A fragment of Py DNA was not totally unexpected in view of the earlier observation indicating that tumorigenicity of Py DNA was enhanced rather than abolished after cleavage with EcoRI (Israel et al. 1979a), or other restriction enzymes which also interrupt the early region of the DNA (Israel et al. 1979b). As mentioned earlier, the HindIII-A fragment of Py DNA includes only part of the sequences coding for large T Ag (Friedmann et al. 1978; Soeda et al. 1979). These results, therefore, firmly establish that large T Ag is not essential for the establishment or the maintenance of transformation by Py virus. That large T Ag is not required for maintenance was already suggested by its absence from some virus-transformed cells (Hutchinson et al. 1978) and tumours induced by injection of restricted DNA (Israel et al. 1979b), as well as by the earlier observation that part of the early region included in fragment HindIII-B does not seem to be transcribed in some transformed cells (Kamen et al. 1974; Bacheler, 1977). However, a requirement for a truncated form of large T Ag in transformation cannot be excluded on the basis of such results.

Early ts (or ts-a) mutations have been mapped in the portion of the early region which is part of the HindIII-B fragment (Feunteun et al. 1976; Miller & Fried, 1976). Mutants such as ts-a transform with a reduced efficiency at the restrictive temperature (Fried, 1970),
while some cells transformed by these mutants seem to have temperature-dependent growth properties (Seif & Cuzin, 1977). As already suggested by others (Hutchinson et al. 1978), it is thus possible that in transformation by the whole Py genome, the ts-a gene plays a regulatory role in the expression of the hr-t gene.

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