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Transformation of rat embryo fibroblasts by cloned polyoma virus DNA fragments containing only part of the early region

(genetic engineering/DNA transfection/oncogenes)

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ABSTRACT Recombinant plasmids containing either the entire polyoma viral genome or one or the other of the two *Hin*dIII fragments of polyoma virus DNA were constructed and cloned in *Escherichia coli* χ1776, and their DNAs were individually tested for the capacity to transform an established line of rat cells. The recombinant plasmids containing the entire polyoma genome and those containing the *Hin*dIII-1 fragment of polyoma DNA (45–1.4 map units) efficiently transform rat cells, whereas the plasmids containing the *Hin*dIII-2 fragment (1.4–45.0 map units) do not. The properties of many independent transformed cell lines established by infection with the cloned *Hin*dIII-1 fragment were determined. In contrast to the parent cell line, rat cells transformed with the cloned *Hin*dIII-1 fragment grow to high saturation densities, form colonies with high efficiency in dilute agar suspension, produce high levels of plasminogen activator, and display a disorganized arrangement of actin cables. By all criteria examined, these cells transformed by fragments are indistinguishable from cells transformed by whole polyoma viral DNA. Cellular DNA prepared from many *Hin*dIII-1 fragment-transformed cell lines was analyzed for the presence and arrangement of polyoma viral sequences by Southern blot-hybridization. In all cases examined, only those viral sequences contained within the *Hin*dIII-1 fragment of polyoma DNA were detected. These data establish a strong correlation between polyoma DNA sequences mapping within a restricted portion of the early region and the induction and maintenance of the transformed phenotype.

The transforming region of the polyoma viral genome has not been precisely defined, though a wealth of evidence links cell transformation to the early region (70–26 map units, Fig. 1). Mutants containing lesions that map within the early region either fail to transform cells completely [the host-range transformation-defective mutants (1, 2)] or are temperature sensitive for the establishment of transformation and in some cases its maintenance [the tsa mutants (3–6)]. Moreover, RNA complementary to only the "E" strand of the early region of the viral DNA is always detected in transformed cells (7–10), and these cells inevitably contain tumor antigens (T antigens), proteins encoded by the early region of the virus (11–13).

Whether the entire early region of the viral genome is required to maintain cells in a transformed state is not clear. Several analyses of virus-specific RNA in polyoma-transformed cells revealed that some lines either lack or contain smaller amounts of those RNA sequences that are transcribed from the distal part of the early region (7, 9, 10). Moreover, Israel *et al.* (14) have recently observed that digestion of polyoma DNA with restriction endonucleases that cleave in the distal portion of the early region (0–26 map units, Fig. 1) enhances the tumorigenic capacity of the DNA in newborn hamsters. These observations suggest that not all of the early region may be required for transformation. To determine which segment of polyoma virus DNA is required to transform cells, we have measured the capacity of cloned subgenomic fragments of viral DNA to transform cells in culture. Using this approach, we have been able to localize the transforming gene(s) of polyoma virus to a restricted portion of the viral genome containing only part of the early region.

MATERIALS AND METHODS

Cell Culture and Transformation. The Rat-1 cells used in this work were obtained from C. Basilico and recloned in our laboratory. Rat-1 cells are a subclone of Fisher rat F2408 cells (15). Transfections with viral and plasmid DNAs were performed as described (16), using intact plasmids purified on CsCl gradients. The plasmids appeared as homogeneous monomeric molecules after gel electrophoresis, although the presence of small quantities of multimeric molecules is possible. After transfection with DNA, the cells were incubated for 2–3 weeks, and transformants were scored as dense foci. To isolate transformed cell lines, individual foci from separate Petri dishes were ringed with stainless steel cylinders, detached with trypsin, and replated. When the cultures had become confluent they were recloned by plating at high dilution and individual colonies were isolated as described above. To determine the maximum saturation density, 20 sister cultures were inoculated at 1×10^4 cells per cm^2 in 60-mm dishes. The cultures were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified CO_2 atmosphere. The medium was replenished every 2 days, and the cell number of duplicate cultures was determined daily. The saturation density was defined as that density which was maintained for three consecutive days. Clonal growth in agar was assayed as described (17). The cells were plated in 2.0 ml of 0.34% agar at 10^2, 10^3, and 5×10^3 cells per plate in triplicate. Macroscopic colonies were counted after 1–2 weeks of incubation at 37°C. Plasminogen activator present in live cultures, activator secreted by the cultures, and activator present in cell extracts were all assayed by a method based on the digestion of iodinated fibrin. The details concerning these assays have been described (18). The patterns of actin organization were displayed by indirect immunofluorescence as described by Pollack *et al.* (19). The rabbit anti-actin antibody was the generous gift of Keith Burridge.

Molecular Cloning of Polyoma DNA. All research involving cells, viruses, and plasmids was performed in accordance with the Medical Research Council of Canada Guidelines for the Handling of Recombinant DNA Molecules and Animal Viruses and Cells. The details concerning the construction and characterization of the recombinant plasmids will be published elsewhere.

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Abbreviation: T antigen, tumor antigen.

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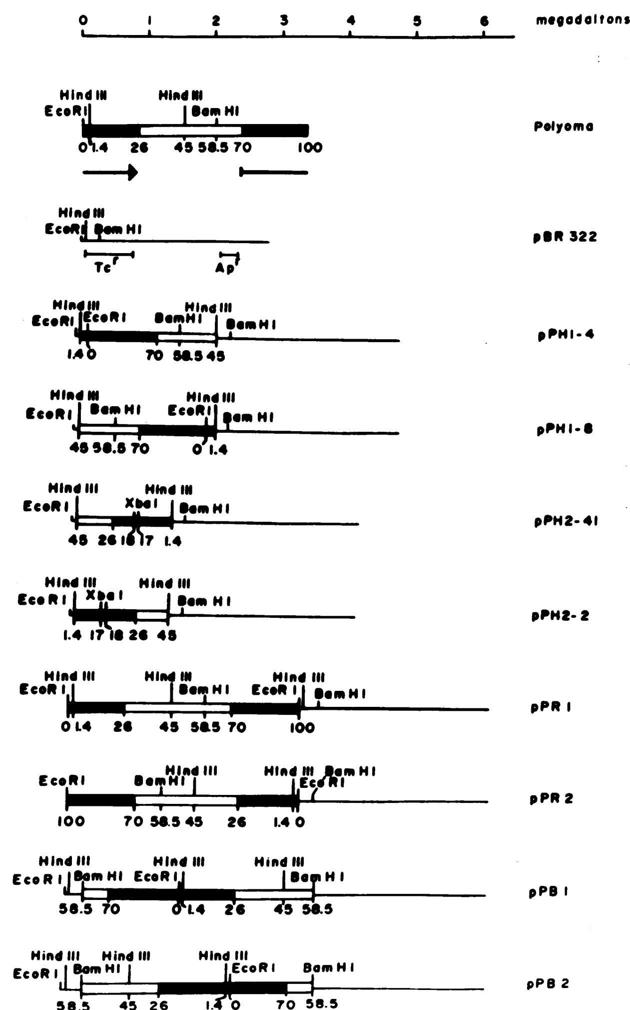

FIG. 1. Physical maps of polyoma and pBR322 DNA and partial restriction endonuclease cleavage maps of polyoma–pBR322 recombinant plasmids. Each circular plasmid has been opened at the *Eco*RI site within pBR322 DNA. The restriction endonuclease cleavage sites for polyoma DNA and the direction of transcription of the early region are indicated. The sites of cleavage of *Bam*HI, *Eco*RI, and *Hin*dIII, and the physical locations of the ampicillin resistance gene (Ap^r) and the tetracycline resistance gene (Tc^r) on pBR322 DNA are shown. The boxed-in areas represent polyoma sequences and the darkened areas represent the early region of polyoma virus. The numbers below each map refer to the restriction endonuclease cleavage sites above and are shown here in polyoma map units. The recombinant plasmid genomes are drawn to scale.

Digestion of Cellular DNA with Restriction Enzymes, Transfer to Nitrocellulose Paper, and Hybridization. Cellular DNA was prepared from dividing cell cultures (20). DNAs were digested with an excess of the restriction endonuclease according to the conditions specified by the manufacturer. DNA fragments obtained by hydrolysis with restriction endonucleases were fractionated through alkaline-agarose gels (21), transferred to nitrocellulose sheets (22), and hybridized with probes labeled *in vitro* by nick translation (23).

RESULTS

The entire polyoma viral genome and restriction endonuclease fragments thereof were cloned with the pBR322–*Escherichia coli* χ1776 plasmid-vector system. Plasmids containing the entire viral genome were constructed by cleavage of polyoma DNA either with *Eco*RI, which cleaves polyoma DNA once in the early region, or with *Bam*HI, which recognizes a single cleavage site in the late region (see ref. 24; Fig. 1). The resulting linear molecules were then joined with phage T4 ligase to pBR322 DNA (previously digested with either *Eco*RI or *Bam*HI, both single-cut enzymes for the plasmid (ref. 25; Fig. 1), and the products were used to transform *E. coli* χ1776 to ampicillin resistance, a selectable marker carried by the plasmid. Clones containing insertions of polyoma DNA were identified by colony hybridization (26). The *Hin*dIII fragments of polyoma DNA were cloned in a fashion similar to that described above, by insertion into the single *Hin*dIII site of pBR322 DNA. The recombinant plasmid DNAs were characterized by hybridization, restriction endonuclease digestion, and measurement of the infectivity of the polyoma DNA released from the plasmids by digestion with the same restriction enzyme used in their construction. The infectivity of the viral DNA contained within the cloned *Hin*dIII fragments was determined after cleavage with *Hin*dIII and ligation with T4 ligase of mixtures of those plasmids containing the *Hin*dIII-1 fragment of polyoma DNA and those containing the *Hin*dIII-2 fragment of polyoma DNA. Both biochemical and biological assays demonstrated that the viral sequences cloned had not undergone any detectable alterations in *E. coli*. The details concerning the construction and characterization of the recombinant plasmids will be described elsewhere. Restriction endonuclease cleavage maps of the various plasmids used in the research reported here are summarized in Fig. 1.

Transformation with Cloned Polyoma DNA. To determine whether the recombinant plasmid DNAs shown in Fig. 1 were capable of transforming cells in culture, we used the modified (16) calcium phosphate coprecipitation technique (27) to transfect Rat-1 cells with DNA from each of the plasmids. Transformants were scored as dense foci appearing in the cultures several weeks after infection. This transfection method typically yields transformation frequencies of 500–2000 transformants per μg of recombinant plasmid DNA per 5×10^5 cells. The results of such a transformation assay are shown in Table 1. All the recombinant plasmids with the exception of those containing the *Hin*dIII-2 fragment of polyoma DNA (pPH2-2 and pPH2-41) are capable of transforming rat cells in culture. Included among these are those plasmids that contain insertions of pBR322 sequences within the early region of polyoma DNA (pPR1 and pPR2) and those that contain only a part of the early region (pPH1-1 and pPH1-8). The orientation of polyoma sequences within the plasmid appears to be unimportant because each pair of recombinant plasmids (pPR1 and pPR2; pPB1 and pPB2; pPH1-4 and pPH1-8) transforms with about the same efficiency. The results shown in Table 1 also reveal that the frequency of transformation obtained with recombinant plasmid DNAs that contain an intact early region (pPB1 and pPB2) is not much different from those that carry an interrupted early region (pPR1 and pPR2). We have de-

Table 1. Transforming activity of various DNAs

DNA source	μg DNA/ dish	Foci/dish	Average foci/ μg DNA
pPB1	0.138	112, 120, 163, 195, 78	968
pPB2	0.218	98, 79, 221, 181, 97	676
pPR1	0.161	103, 91, 128, 153, 105	720
pPR2	0.190	88, 115, 163, 129, 79	604
pPH1-4	0.194	91, 95, 117, 158, 123	602
pPH1-8	0.220	110, 201, 117, 183, 261	792
pPH2-2	0.230	0, 0, 0, 0, 0	0
pPH2-41	0.180	0, 0, 0, 0, 0	0
Polyoma	0.120	210, 251, 163, 221, 181	1710
pBR322	1.84	0, 0, 0, 0, 0	0
Salmon sperm	15.0	0, 0, 0, 0, 0	0

termined that the efficiency of transformation per unit of DNA is essentially linear up to 1 μg of DNA per plate for all plasmid species tested (data not shown).

Properties of Rat Cells Transformed with Cloned Polyoma DNA Fragments. Cells transformed by polyoma virus and simian virus 40 acquire a new set of properties that distinguish them from their untransformed counterparts. These include altered morphology, increased saturation density, lowered serum requirement for growth, and the capacity to form colonies in agar suspension (28). The latter property is closely correlated with the capacity of cells to synthesize an activator of serum plasminogen (29, 30), as well as the loss of actin cables (19), and tumorigenicity (31).

Not all the phenotypic markers of transformation are acquired concomitantly (30), and it has been suggested that the different traits of transformed cells may be dependent on the separate activities of the early gene products of the virus (32, 33). We were therefore curious to learn whether rat cells transformed with a segment of polyoma viral DNA capable of encoding only some of these early gene products displayed a partial or complete transformed phenotype. To perform this analysis we chose to study rat cells transformed with the cloned *Hin*dIII-1 fragment of polyoma DNA. For comparative purposes we also isolated a number of rat cell lines transformed by polyoma virus or polyoma viral DNA. The saturation density and the efficiency of colony formation in agar suspension of nine cell lines transformed with pPH1-8 and pPH1-4 DNA (subsequently referred to as fragment-transformed cell lines) and eight cell lines transformed with polyoma virus or viral DNA were measured. Both the fragment transformants and the polyoma DNA transformants grow to high saturation densities compared to Rat-1 cells, the parent cell line from which all the transformants were derived (Table 2). None of the transformed cell lines maintain a truly stable saturation density. Rather, for some lines a density is reached at which the entire cell sheet detaches from the substratum whereas, for the others, individual cells detach and a quasi-stable density is maintained. Examination of the efficiency with which the various transformants form colonies in soft agar revealed that, by contrast to Rat-1 cells, all the transformed lines tested exhibit clonal growth in suspension (Table 2).

Table 2. Properties of transformed cell lines

Cell line	Transforming agent	Saturation density, cells $\times 10^{-4}/cm^2$	EOP* in agar, colonies/100 cells plated
Rat-1	None	14	≤0.001
2-4a	pPH1-8 DNA	≥35	8.1
6-1a	pPH1-8 DNA	≥35	4.5
A8-8a	pPH1-8 DNA	40	4.3
A4-13a	pPH1-4 DNA	≥50	12.5
15-5a	pPH1-4 DNA	61	6.5
2-3a	pPH1-8 DNA	≥17	8.0
A8-10a	pPH1-8 DNA	≥32	7.3
A4-19a	pPH1-4 DNA	≥43	2.7
A8-3a	pPH1-8 DNA	53	10.7
16-2	Polyoma DNA	≥18	8.1
18d2	Polyoma DNA	50	7.0
19a1	Polyoma DNA	≥27	30.0
18a1	Polyoma DNA	26	9.6
33-3a	Polyoma DNA	44	2.1
33-1a	Polyoma DNA	36	3.6
38-1a	Polyoma DNA	51	11.8
PyV1a	Polyoma virus	61	10.0

* The efficiency of plating of cells in agar after 7–14 days of incubation at 37°C.

Table 3. Plasminogen activator production by transformed cell lines

Cell line	Solubilization of iodinated fibrin, %: Live cells*	Cell extract†	Medium‡
Rat-1	4	0	0
2-4a	41	19	16
A4-13a	13	49	36
18d2	15	38	55
19a1	35	20	16

* Fibrinolytic activity is calculated as the release of ^{125}I-labeled peptides from insoluble iodinated fibrin per 10^5 cells per 10^5 available ^{125}I cpm per hr. The results are expressed as a percentage of the cpm of ^{125}I-labeled peptides released by trypsin treatment of insoluble iodinated fibrin (100%).

† Same as above except 1 μg of cellular protein rather than cells was added to fibrin plates.

‡ Same as above except 25 μl of medium conditioned overnight in the absence of serum was added to fibrin plates rather than cells.

To further compare the phenotypes of the fragment and whole virus transformants, we measured the synthesis of plasminogen activator, and the organization of actin cables in two fragment-derived (2-4a and A4-13a) and two whole virus-derived (18d2 and 19a1) lines. All the transformed lines synthesize substantial amounts of plasminogen activator (Table 3). This is true whether cell extracts, live cells, or released activator (medium) is measured. By contrast, Rat-1 cells produce low levels of plasminogen activator.

The distribution and state of organization of actin are shown in Fig. 2. Both the fragment-transformed cells (2-4a and A4-13a) and the polyoma DNA-transformed cells (19a1) have lost the long and brightly stained actin cables that are characteristic

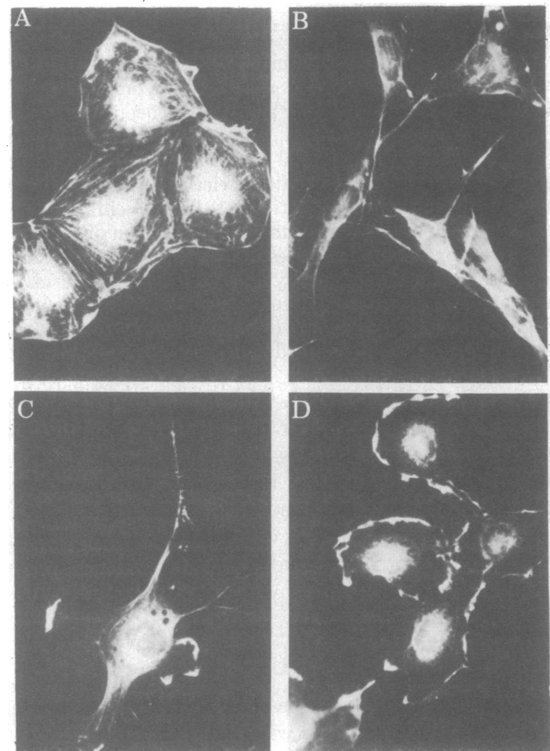

FIG. 2. Organization of actin in Rat-1 cells and their transformed derivatives. (*A*) Rat-1; (*B*) 19a1; (*C*) 2-4a; (*D*) A4-13a. 2-4a and A4-13a were established by transfection with pPH1-8 and pPH1-4 DNA, respectively. 19a1 was obtained by transfection with polyoma viral DNA. (×800.)

of Rat-1 cells. These results show that cells transformed with DNA containing sequences from only the proximal part of the early region express the transformation-specific markers characteristic of maximal transformants.

Viral DNA in Transformed Cells. The observation that only the proximal part of the polyoma early region was required to cause transformation of cells in culture was surprising. To ensure that the fragment-transformed cells contained pPH1-8 or pPH1-4 plasmid DNA and no other polyoma sequences, we screened 16 cell lines for polyoma sequences, using the Southern blot-hybridization technique (22). High molecular weight cellular DNA prepared from the fragment-transformed cell lines was digested with *Hin*dIII, and the resulting fragments were fractionated by electrophoresis through an alkaline-agarose gel (21). The DNA within the gel was then transferred to a nitrocellulose sheet and hybridized to three different probes that had been labeled *in vitro* by nick translation (23). The probes included pBR322 DNA, the *Hin*dIII-1 fragment, and the *Hin*dIII-2 fragment of polyoma virus DNA. These polyoma DNA fragments were obtained by cleavage of the pPH1-8 and pPH2-41 plasmid DNAs with *Hin*dIII and isolation by agarose gel electrophoresis. Those fragments of cellular DNA containing sequences complementary to the probes were identified by autoradiography. The results of this analysis with one representative fragment-transformed cell line (15-5a, see Table 2) are shown in Fig. 3. There are many fragments produced after cleavage of 15-5a cellular DNA with *Hin*dIII that are complementary to the *Hin*dIII-1 fragment probe (Fig. 3*A*). The most prominent band is one that comigrates with the *Hin*dIII-1 marker fragment. When the *Hin*dIII-2 fragment of polyoma virus DNA was used as a probe there was no hybridization detected even after prolonged exposure times (Fig. 3*B*). Hybridization with ^{32}P-labeled pBR322 DNA revealed an array of bands, including an intense species that comigrated with the linear form of pBR322 DNA (Fig. 3*C*). We can readily detect 10^{-7} μg of linear pPH1-8 DNA by using these methods. By analyzing 50 μg of cellular DNA it is possible to detect as little as 0.002 copy of recombinant plasmid DNA per diploid equivalent of cellular DNA. We can therefore calculate that the 15-5a cell line contains less than 0.002 copy of the *Hin*dIII-2 fragment of polyoma DNA per cell, and, in all likelihood, does not contain this fragment at all. We have analyzed 15 other fragment-transformed cell lines. All of these contain sequences complementary to the *Hin*dIII-1 fragment of polyoma DNA and to pBR322 DNA. However, none contain sequences complementary to the *Hin*dIII-2 fragment of polyoma virus DNA (data not shown). These data confirm our earlier result (Table 1) that the *Hin*dIII-1 fragment of polyoma DNA contains all the genetic information necessary for transformation.

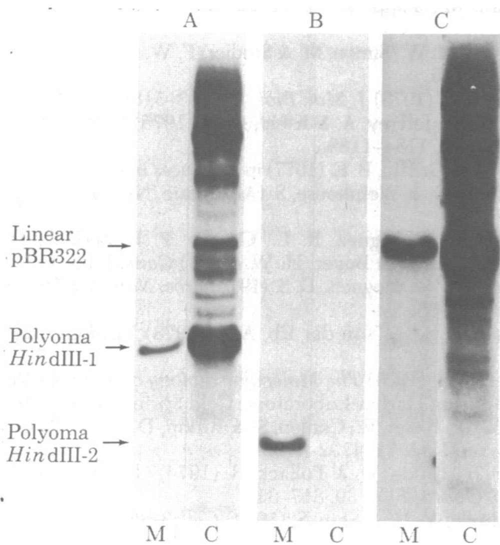

FIG. 3. Detection of DNA fragments that contain polyoma or pBR322 DNA sequences after cleavage of 15-5a cellular DNA with *Hin*dIII. The products of *Hin*dIII digestion of 15-5a cellular DNA were fractionated through 1.0% alkaline-agarose gels in each of three tracks labeled C (50 μg of cellular DNA per well). Markers (labeled M) of pPH1-8 and of pPH2-41 DNA (10 pg per well each) cleaved with *Hin*dIII were electrophoresed in adjacent slots next to the cellular DNA. The fragments were transferred to a nitrocellulose sheet, the sheet was cut into three strips, and each of these was separately hybridized with one of three ^{32}P-labeled probes. (*A*) ^{32}P-Labeled *Hin*dIII-1 fragment of polyoma DNA; (*B*) ^{32}P-labeled *Hin*dIII-2 fragment of polyoma DNA; (*C*) ^{32}P-labeled pBR322 DNA. The *Hin*dIII-1 and *Hin*dIII-2 fragments used as probes were isolated from the pPH1-8 and pPH2-41 plasmids by cleavage with *Hin*dIII followed by preparative agarose gel electrophoresis. The autoradiogram shown in *A* was exposed for 15 hours, that in *B* for 33 hours, and that in *C* for 24 hours. The faint band in track A (marker) comigrating with linear pBR322 is the result of a small amount of pBR322 contaminating our gel-purified *Hin*dIII-1 fragment. The band comigrating with linear pBR322 in the cellular DNA probed with this contaminated preparation of *Hin*dIII-1 (*A*, track C) probably arises from hybridization to this contaminant.

DISCUSSION

The early region of polyoma virus DNA encodes three known proteins: large (molecular weight 100,000), middle (55,000), and small (22,000) T antigens (11–13, 34). These proteins are translated from three related but differentially spliced mRNAs that are derived from the same transcription unit (ref. 35, see also refs. 36 and 37). The coding sequences for small and middle T antigens are located exclusively in the proximal part of the early region (70–100 map units), whereas the sequences encoding large T antigen are located in the proximal and distal part of the early region.

The results we have presented show that only part of the early region, the 5′-proximal half (70–100 map units) is required to induce transformation of Rat-1 cells. This conclusion is based on the observation that recombinant plasmids containing either the entire polyoma genome interrupted at the viral *Eco*RI site (0/100 map units) or the *Hin*dIII-1 fragment of polyoma DNA (45–1.4 map units) is capable of transforming rat cells. The cloned *Hin*dIII-2 fragment of polyoma viral DNA (1.4–45 map units), which does not include sequences from the proximal portion of the early region, is incapable of inducing the transformed phenotype. Moreover, the frequency of transformation obtained by infection with recombinant plasmid DNAs capable of encoding large T antigen (pPB1, pPB2) and those incapable of doing so (pPR1 and pPR2, pPH1-8 and pPH1-4) are approximately the same. This suggests that large T antigen neither enhances nor inhibits the establishment of DNA-mediated transformation. Our results apparently contradict the observation that mutations located in sequences encoding only large T antigen [between 1 and 26 map units (38)] affect the transforming capacity of polyoma virus at the initiation step (3–6). These mutants, which fall in the *a* complementation group, transform cells at a reduced efficiency at the restrictive compared with the permissive temperature. Perhaps the resolution to this apparent contradiction lies in the fact that we have studied transformation after DNA transfection. In a viral infection, in which most cells are competent to take up virus, each cell is presented with a modest dose of infecting virus. DNA transfection involves quantities of viral DNA similar in magnitude to those added in virions in a viral infection of 10–100 plaque-forming units per cell (assuming a particle-to-plaque-forming unit ratio of 1000); however, far fewer cells are competent to take up the DNA. It is possible that a substantially

increased viral dose per cell in a DNA infection overcomes the need for the *a* gene product to initiate transformation. Whatever the explanation, it is clear that large T antigen is not absolutely required to initiate transformation.

Examination by Southern blot-hybridization of the viral sequences present in many independent *Hin*dIII-1 fragment-transformed cell lines (established by transformation with the pPH1-4 or pPH1-8 plasmid DNA) revealed that these cell lines always and only contain viral sequences that compose the entire *Hin*dIII-1 fragment of polyoma viral DNA or a fraction of it. Invariably the viral sequences are found integrated within cellular DNA and the integrity of the early sequences is maintained. These data establish a strong correlation between the transformed state and the presence of a restricted portion of the polyoma viral genome within transformed cells. Because the fragment-transformed cell lines express only early polyoma DNA sequences as mRNA (unpublished data), it is unlikely that the late sequences present within these cell lines are required to maintain the transformed phenotype.

The results we have obtained are corroborated by several recent observations. Inoculation of newborn hamsters with polyoma virus DNA that was interrupted in the distal half of the early region resulted in the appearance of tumors at the site of injection (39, 40). An analysis of viral mRNAs found in mouse and rat cells transformed by polyoma virus revealed that some cell lines lacked RNA sequences derived from the distal portion of the early region (35). Moreover, large T antigen is not found in rat cells transformed by the cloned *Hin*dIII-1 fragment of polyoma virus DNA (unpublished data), nor is it always present in hamster cells transformed by polyoma virus (13) or in hamster tumor cell lines established by infection with polyoma viral DNA (39, 40). Collectively, these data suggest that large T antigen is not required to maintain the transformed state.

Whether any of the other polyoma early gene products are required to maintain transformation is not clear. There is a strong correlation between the presence and expression of the proximal part of the polyoma early region in transformed cells and the maintenance of the transformed phenotype. This portion of the early region (70–100 map units) encodes small and middle T antigen. These proteins are always detected n polyoma-transformed cells (11, 13, 39, 40), and are also present in rat cells transformed by the cloned *Hin*dIII-1 fragment of polyoma virus DNA (unpublished data). Unless these proteins are fortuitously expressed in polyoma-transformed cells, it seems likely that one of them or both are required to maintain the transformed state. This issue may be settled by the isolation of mutants with lesions in sequences encoding small or middle T antigens or both or by the isolation and characterization of revertants of polyoma-transformed cell lines.

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