JOURNAL OF VIROLOGY, Nov. 1980, p. 566-574
0022-538X/80/11-0566/09$02.00/0

Vol. 36, No. 2

A Cloned Polyoma DNA Fragment Representing the 5′ Half of the Early Gene Region Is Oncogenic

KAMAL CHOWDHURY,[1]* SUSAN E. LIGHT,[1] CLAUDE F. GARON,[2] YOSHIAKI ITO,[1] AND MARK A. ISRAEL[1]

DNA Recombinant Research Unit, Office of the Scientific Director,[1] and Macromolecular Biology Section, Laboratory of Biology of Viruses,[2] National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

The two polyoma DNA fragments generated by cleavage with *Bam*HI and *Eco*RI were cloned in pBR322, and their oncogenic potential was tested in vivo and in vitro. Only recombinant plasmid DNA containing a polyoma DNA fragment which extends clockwise from 58 to 0 map units and includes approximately the 5′-proximal half of the early gene region produced tumors in newborn hamsters and transformed rat embryo cells in tissue culture. Southern blotting analysis indicated that the entire 2.2-kilobase polyoma *Bam*HI-*Eco*RI fragment was intact in both a tumor cell line and a cell line transformed in culture which we examined. The presence of polyoma middle and small T antigen in these lines was demonstrated by immunoprecipitation and tryptic peptide mapping. DNA from a recombinant plasmid containing a polyoma genome deleted between 90 and 4 map units failed to induce tumors or transform cells.

Polyoma (Py) virus is capable of inducing tumors in rodents and transforming susceptible cells in tissue culture (37). Cell lines established from Py-induced tumors or from cells transformed in vitro with Py virus contain Py tumor antigens (T Ag's). These proteins are encoded in approximately half of the Py genome and are expressed early in the lytic cycle and in transformed cells. They have been designated the Py large T Ag (81×10^3 to 108×10^3 daltons [81-108K]), middle T Ag (55 to 63K), and small T Ag (22K) (14, 20, 21, 31, 32) (Fig. 1). Usually only the Py small and middle T Ag's are found in cell lines established from tumors induced by Py virus or DNA (17a, 18). Viral DNA sequences from the distal portion of the Py early region, which encode the unique portion of large T Ag, are often absent in these tumor cell lines (19). Some cell lines transformed in vitro by Py also lack these DNA sequences and Py large T Ag (14, 22, 25, 32).

The tumorigenicity of a recombinant plasmid consisting of Py DNA inserted into pBR322 at the *Eco*RI site has been previously reported (15, 28). In such a recombinant plasmid the early gene region of Py was interrupted by pBR322 in a region of the viral genome encoding Py large T Ag. The fact that this recombinant plasmid was tumorigenic indicated that Py large T Ag was required for neither the initiation nor the maintenance of oncogenesis induced by Py DNA (28). However, the entire viral genome was present in such a recombinant plasmid; consequently, the possibility that Py DNA sequences from the region of the genome encoding the unique portion of Py large T Ag were in some way required for the induction of tumors could not be ruled out. In this study we have evaluated the biological activity of cloned segments of Py DNA. Whereas a Py DNA fragment extending clockwise from the *Bam*HI site at 58 map units to the *Eco*RI site at 0 map units efficiently transforms cells in culture and induces tumors, a cloned Py DNA preparation deleted in a region of the genome encoding the carboxy terminus of middle T Ag does not transform cells or induce tumors in newborn hamsters.

MATERIALS AND METHODS

Viruses and cells. Pasadena large-plaque strain Py virus (38) was used in these experiments. An established rat embryo cell line, F111 (kindly supplied by M. Fluck), was used for in vitro transformation experiments.

Hamster tumors were established in tissue culture and cloned as previously described (18). Foci of Py-transformed F111 rat cells were trypsinized in stainless steel cloning rings and established in culture. These cell lines were further cloned by the endpoint dilution technique before further analysis.

Viral, cellular, and plasmid DNA. Py (large-plaque) DNA was isolated from infected 3T6 cells by Hirt extraction (13) followed by cesium chloride-ethidium bromide equilibrium density centrifugation. Cellular DNA from tumor and transformed cell lines was isolated as previously described (19). pBR322 (36) and recombinant plasmid DNAs were isolated from *Escherichia coli* HB101 after propagation and chloramphenicol amplification in M9 medium as previously

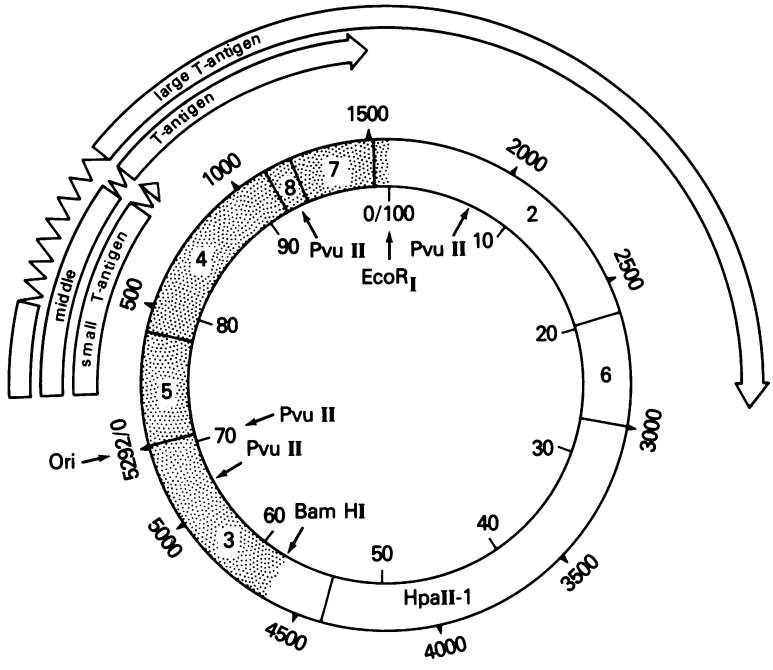

FIG. 1. *Physical map of the Py genome. Several restriction enzyme sites and the regions encoding the Py T Ag's are shown (10, 34). The dotted region shows the 2.2-kb Py BamHI-Eco RI DNA fragment which was cloned and evaluated. Numbers inside the circle indicate map units with the EcoRI site assigned the 0 position; numbers outside the circle indicate base pairs of DNA according to Soeda et al. (34).*

described (16). The supercoiled plasmid DNA was further purified by gel filtration on Sephadex G-100.

Restriction endonucleases, hydrolysis, and agarose gel electrophoresis. All enzymes were purchased either from New England Biolabs (Beverly, Mass.) or Bethesda Research Laboratories (Bethesda, Md.). *Pvu*II, *Sal*I, *Bam*HI, and *Eco*RI digestions were carried out as previously described (19). *Bam*HI and *Eco*RI double digestions were carried out in *Eco*RI buffer. Agarose gel electrophoresis and ethidium bromide staining were performed as previously described (16).

Cloning of Py DNA fragments. Cleavage of Py DNA with *Bam*HI and *Eco*RI yields two DNA fragments of 2.2 and 3.0 kilobases (kb). To clone these fragments in pBR322, we started with recombinant plasmids containing the entire Py genome inserted at the *Bam*HI site in pBR322 (Py-pBR322) in the two possible orientations (pPB5 and pPB6) (16). A Py-pBR322 plasmid, containing the entire Py genome inserted at the *Bam*HI site in an orientation such that the Py early gene region was located adjacent to the plasmid *Sal*I site (pPB5), was cleaved to completion with *Eco*RI, generating 3.38-kb (3.0 kb of Py DNA and 0.38 kb of plasmid DNA) and 6.27-kb cleavage products. The larger fragment, containing the plasmid origin of DNA replication (36), and only the 2.2-kb *Bam*HI-*Eco*RI Py DNA fragment could be circularized and propagated in *E. coli* after bacterial transformation. In a similar fashion, *Eco*RI digestion of Py-pBR322 recombinants containing the entire Py genome in the opposite orientation (pPB6) generated a recombinant plasmid containing the 3.0-kb Py *Bam*HI-*Eco*RI fragment. After cleavage of pPB5 and pPB6 with *Eco*RI, these DNAs were individually circularized by T4 DNA ligase and used for transfection of *E. coli* HB101 (16). Individual ampicillin-resistant colonies were picked. DNA was prepared from cultures of these isolates and then analyzed by gel electrophoresis after cleavage with restriction endonucleases.

To construct a recombinant plasmid containing a Py viral genome deleted in the region of the *Eco*RI site, we treated 2 μg of *Eco*RI-restricted Py DNA with S1 nuclease (Bethesda Research Labs) (30 mM sodium acetate [pH 4.5], 100 mM NaCl, 2 mM $ZnSO_4$, and 2 U of S1 nuclease in 0.07 ml at 25°C for 30 min). This DNA was circularized with T4 DNA ligase and subsequently cloned at the *Bam*HI site in pBR322 as previously described (16). One transformant which contained Py DNA sequences, pOc3-1, was selected and further characterized.

All experiments involving cloning of the Py DNA were performed in accordance with the National Institutes of Health guidelines for handling of recombinant DNA molecules.

Electron microscopy. pOc3-1 DNA was evaluated by electron microscopic heteroduplex mapping. Heteroduplexes were formed by mixing equal amounts (1 to 2 μg) of either *Sal*I-cleaved pPB5 and pOc3-1 DNAs or *Bam*HI-cleaved Py and pOc3-1 DNAs and alkali denaturation and renaturation in 50% formamide, 100 mM Tris-hydrochloride (pH 8.5), and 1 mM EDTA for 2 h at 37°C. Molecules were spread in a solution containing 70% formamide, 0.1 M Tris-hydrochloride

(pH 8.0), 0.25 M NaCl, 0.01 M EDTA, and cytochrome *c* (50 μg/ml) over a distilled water hypophase, picked up on Parlodion-coated grids, stained with uranyl formate, and rotary shadowed with platinum-palladium. Grids were examined in a Siemens Elmiscope 101 at 40 kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at magnifications of 4,000 to 8,000×. Magnification was calibrated with a grating replica (E. F. Fullman; no. 1000), and contour lengths were measured with a Neumonics graphic calculator interfaced to a Wang 2200 computer.

Inoculation of DNA into hamsters. Recombinant DNA preparations were digested with *SalI*, an enzyme that cleaves within pBR322 DNA, and 0.5-μg samples were inoculated into 1-day-old golden Syrian hamsters. Test animals were observed for 6 months for the development of tumors.

In vitro transformation assay. F111 rat cells growing in 50-mm petri dishes were transfected at 30 to 50% confluency with various recombinant plasmid DNAs by using a modification (40) of the calcium precipitation technique (11). Cells were maintained for 3 to 4 weeks; after methanol fixation, the plates were stained with Giemsa. Densely stained foci were scored as transformants.

Analysis of viral DNA sequences in tumor and transformed cell lines. The arrangement of viral DNA sequences in various transformed cell lines was studied by the Southern blotting technique (35) as previously described (4, 19, 23). However, in these studies, the gels were treated with 0.25 M HCl for 15 min before denaturation and transfer of the DNA to a nitrocellulose filter (39). After hybridization to nick-translated ^{32}P-labeled Py DNA (2×10^7 to 3×10^7 cpm; specific activity, $\cong 10^8$ cpm/μg) (19, 27), the filter was washed at 60°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 5 h and then in 0.5× SSC–0.1% sodium dodecyl sulfate for 30 min, dried, and exposed at −70°C to preflashed Kodak XR-2 film with intensifying screens.

Immunoprecipitation and two-dimensional tryptic peptide fingerprint analysis. Cells were grown to about 80% confluency in 60-mm petri dishes and labeled with [^{35}S]methionine (200 μCi/ml, ca. 1,000 Ci/mmol) for 3 to 4 h at 37°C. Extraction of T antigens, immunoprecipitation using rat anti-Py T serum, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Py T Ag's, and the preparation of dried gels for fluorography were preformed as previously described (20). For peptide mapping, cells from two 100-mm petri dishes were labeled with 5 mCi of [^{35}S]methionine, and T Ag's were isolated (22). Two-dimensional tryptic peptide fingerprint analysis was performed as previously described (20).

RESULTS

Cloning of polyoma DNA fragments in pBR322. When Py DNA was cleaved simultaneously with *Bam*HI and *Eco*RI, two fragments, 3.0 kb and 2.2 kb, are generated (Fig. 1). The smaller fragment contains the origin of Py DNA replication as well as the postulated early and late promoter(s) and coding sequences for Py small and middle T Ag's (Fig. 1) (10, 34). The 3.0-kb fragment contains most of the late region as well as the distal half of the early region. Since the entire Py genome had been previously cloned at the *Bam*HI site in pBR322 in both possible orientations (16), recombinant plasmids (e.g., pPB89 and pPB90) containing the fragments generated by *Bam*HI plus *Eco*RI (*Bam*HI-*Eco*RI) digestion of Py DNA could be easily generated and cloned (see above). Restriction endonuclease examination of DNA from such recombinant plasmids was performed as shown in Fig. 2. Lanes A and B contain the reaction products of *Bam*HI plus *Eco*RI digestion of pPB90 and pPB89, respectively. For comparison, linear Py and pBR322 DNAs were run in the same gel together with the reaction products of Py DNA after cleavage by *Bam*HI and *Eco*RI (lane C). Clearly pPB89 and pPB90 contain only the 3.0- and 2.2-kb Py DNA fragments, respectively.

To study the importance of DNA sequences encoding the carboxyterminal portion of Py mid-

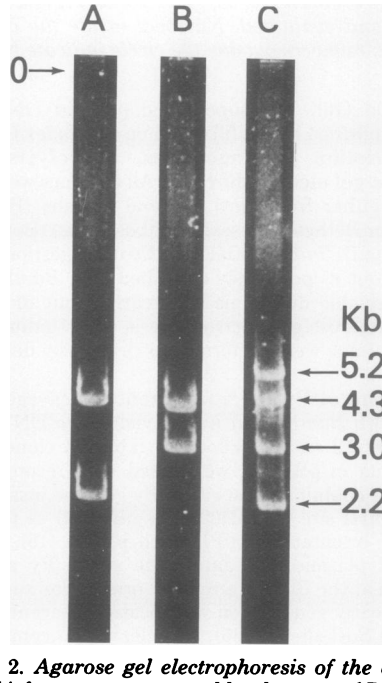

FIG. 2. *Agarose gel electrophoresis of the cloned Py DNA fragments generated by cleavage of Py DNA with BamHI and EcoRI. In lanes A and B 0.5 μg of the recombinant pPB90 and pPB89, respectively, cleaved simultaneously with BamHI and EcoRI are shown. Lane C contains linear Py (5.2 kb) and pBR322 DNA (4.3 kb) and the BamHI and EcoRI cleavage products of Py DNA.*

dle T Ag in transformation, we evaluated the biological activity of a recombinant Py-pBR322 DNA preparation deleted in the region of the Py *Eco*RI site, pOc3-1. The sequence arrangement of pOc3-1 was evaluated by electron microscopy and by restriction endonuclease mapping. Heteroduplex molecules formed between *Sal*I-linearized pOc3-1 DNA and pPB5 (a plasmid containing the entire Py viral genome in a known orientation) DNA (16) revealed the Py DNA to be similarly oriented in pOc3-1 (data not shown) (Fig. 3B). Accurate measurement of the size and map position of the deletion present in pOc3-1 DNA was obtained from heteroduplex molecules formed between *Bam*HI-cleaved Py DNA and pOc3-1 DNA (Fig. 3A). Such an analysis of 48 molecules indicated a contiguous deletion of 732 ± 60 base pairs from a region located between 90.1 and 3.9 map units and represents 13.8% of the Py genome. These values are in close agreement with those obtained by gel analysis of the *Bam*HI-*Hpa*II digestion of pOc3-1 DNA (data not shown) which indicated a deletion of 12.3% of the Py viral genome spanning DNA sequences in the region of the Py *Hpa*II fragments 2, 4, 7, and 8 (Fig. 1).

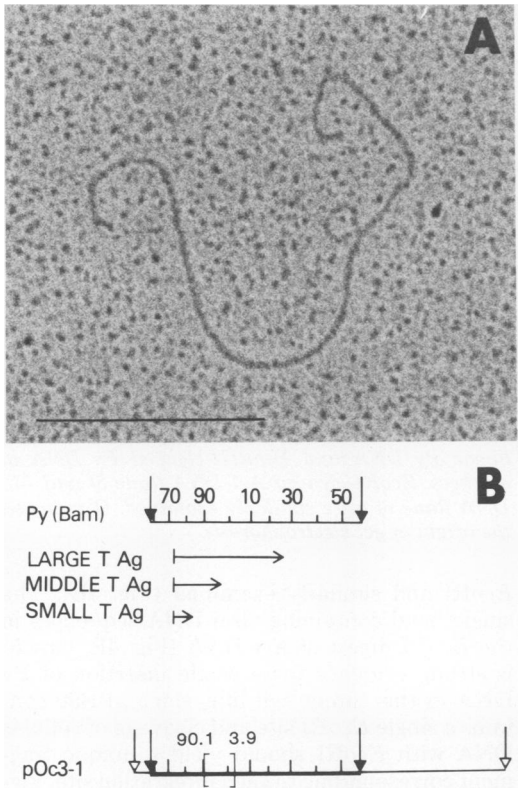

FIG. 3. *Panel A shows a typical heteroduplex molecule formed between BamHI-cleaved pOc3-1 and BamHI-cleaved Py DNA. Each of the 48 heteroduplex molecules measured had a long duplex arm (1.103 ± 0.05 μm), a shorter duplex arm (0.607 ± 0.02 μm), and a single-stranded deletion loop (0.244 ± 0.02 μm). Both single- and double-stranded calibrations were done with Py and pBR322 molecules present in the same field. The bar represents 0.5 μm. Panel B contains a schematic diagram of pOc3-1 in which the Py DNA sequences are represented by a thick line and the pBR322 DNA sequences are represented by a thin line. The map position and extent of the deletion are bracketed and the SalI (▽) and BamHI (▼) restriction endonuclease sites are indicated.*

Oncogenic activity of cloned Py DNA. Rat F111 cells were transfected with purified recombinant plasmid DNAs by using a modified calcium phosphate precipitation technique (11, 40). Transformants were scored as dense foci several weeks after infection. The results of two such experiments are shown in Table 1. The number of transformants observed in plates exposed to the recombinant containing the proximal part of the Py early region (pPB90) (i.e., the 2.2-kb Py *Bam*HI-*Eco*RI DNA fragment) was similar to the number of transformants appearing after transfection with a recombinant plasmid containing the complete Py genome (pPB5). The variation between duplicate samples and the nonlinear response observed in this assay (our unpublished observation) make it impossible to identify with confidence any significant difference in the transforming efficiencies of these molecules. Furthermore, we did not observe any difference in the time of appearance, size, or

TABLE 1. *Transforming activity of recombinant Py DNAs*

Plasmid[a]	Py sequences in recombinant (map units)	In vitro transformation (foci/dish)		Tumorigenesis (no. of tumors/no. inoculated)
		Expt 1	Expt 2	
pPB5	0–100	36,77	16,60	11/20
pPB90	58–100	12,15	47,30	8/17
pPB89	0–58	0,0	0,0	0/22
pOc3-1	4–90	0,0	0,0	0/39
pBR322		0,0	0,0	0/22

[a] pPB5 is a Py-pBR322 recombinant plasmid containing the entire Py genome (16); pPB89, pPB90, and pOc3-1 are recombinant plasmids containing portions of the Py genome. pPB90 contains the Py DNA sequences extending clockwise from 58 to 100 map units; pPB89 contains the viral DNA sequences extending clockwise from 0 to 58 map units. pOc3-1 is described in detail in the text. Component I plasmid DNAs (0.5 μg per dish) were used in in vitro transformation studies, and *Sal*I-linearized recombinant plasmid DNAs (0.5 μg per animal) were used for hamster inoculations.

gross morphology of foci induced by these two substrates. The recombinant plasmid containing Py DNA sequences mapping clockwise from 0 to 58 map units (pPB89) did not induce any transformants.

Several viable mutants of Py virus with mutations located between map units 88.2 and 94.4 have been isolated and found to have altered transformation properties (1, 12, 26). All of these mutations are located in the region of the genome encoding Py large and middle T Ag (1, 12, 26). Analysis of the nucleotide sequence of Py DNA (10, 34) has suggested that the carboxy terminus of Py middle T Ag and a putative polyadenylation signal are present around map unit 99. To study further the importance of this region of the genome to transformation, we evaluated the transforming activity of pOc3-1 DNA, which lacks the DNA sequences extending from 90.1 to 3.9 map units, and found that it failed to transform rat cells (Table 1).

The ability of these same recombinant plasmids to induce tumors in suckling hamsters was also evaluated. Since other experiments had suggested that linearization of viral DNA enhanced its tumorigenic potential (15, 17, 18), the recombinant plasmid DNA preparations were digested with *SalI*, which cleaves the plasmid DNA at a single site. Recombinant plasmids containing either the entire Py genome (pPB5) or only the 5′-proximal half of the early region (pPB90) produced tumors (Table 1) with an efficiency similar to that previously observed for a comparable amount of virus-derived, linearized Py DNA (17, 18). Neither a recombinant plasmid containing Py DNA sequences from the 3′-terminal part of the early region (pPB89) nor pOc3-1 was tumorigenic.

Arrangement of Py DNA sequences in tumor and in vitro-transformed cell lines. The Py DNA sequences in a cloned cell line established in culture from a hamster tumor induced by the inoculation of pPB90 DNA (cell line HT6279A-1 [A-1]) and a rat cell line transformed in vitro by the same DNA (cell line F1118157915-1 [15-1]) were examined by a modification (39) of the Southern blotting technique (35). When either A-1 or 15-1 cellular DNA was cleaved simultaneously with *Bam*HI and *Eco*RI (Fig. 4A, lanes 1 and 3, respectively) and hybridized to in vitro ^{32}P-labeled Py DNA, only a single fragment which comigrated with the 2.2-kb Py *Bam*HI-*Eco*RI DNA fragment was observed (Fig. 4A, lane 2). This is the same Py DNA segment present in the pPB90 DNA used to establish these transformants.

For further characterization of the viral DNA in these cell lines, cellular DNA preparations from both transformants were cleaved with

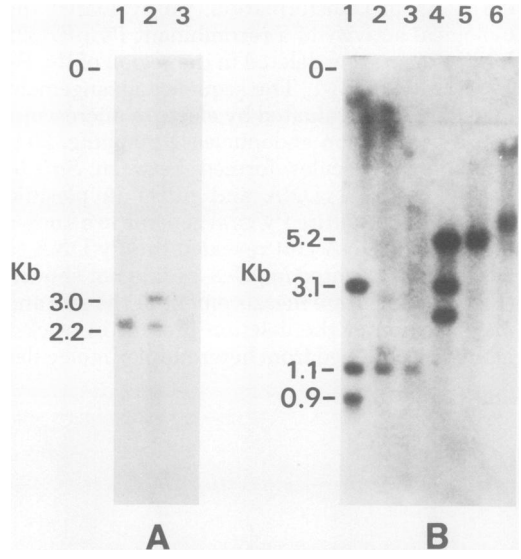

FIG. 4. *Hybridization of ^{32}P-labeled Py DNA to cellular DNAs isolated from tumor (A-1) and in vitro-transformed (15-1) cell lines. Cellular DNAs (5 μg per lane) were cleaved with restriction enzyme(s), electrophoresed in 0.7% agarose gels, denatured, and transferred to nitrocellulose filters for hybridization. In panel A, A-1 (lane 1) and 15-1 (lane 3) cellular DNAs and Py DNA (lane 2) were evaluated after simultaneous cleavage with BamHI and EcoRI. Panel B shows the pattern observed after cleavage with PvuII of Py DNA (lane 1), A-1 DNA (lane 2), and 15-1 DNA (lane 3). Lane 4 contains full-length linear Py DNA and HindIII-cleaved Py DNA as markers. EcoRI-cleaved A-1 DNA (lane 5) and 15-1 DNA (lane 6) were similarly examined. O indicates the origin of gel electrophoresis.*

*Eco*RI and similarly examined (Fig. 4B). The single band containing viral DNA sequences in the *Eco*RI digest of A-1 DNA (Fig. 4B, lane 5) is strong evidence for a single insertion of Py DNA in this tumor cell line, since pPB90 contains a single *Eco*RI site and cleavage of cellular DNA with *Eco*RI should yield a unique fragment corresponding to each integration site. Figure 4B, lane 6 shows the pattern observed when *Eco*RI-cleaved DNA from cell line 15-1 was examined. In this case two bands were observed, suggesting that more than one insertion may be present.

All Py tumor cell lines which have been examined in our laboratory have been found to contain the Py *Pvu*II B fragment which encodes the Py small T Ag and most of the middle T Ag (Fig. 1) (19). Cleavage of Py DNA with *Pvu*II generates four fragments of 3.1, 1.1, 0.9, and 0.13 kb; cleavage of pPB90 DNA with *Pvu*II would generate four fragments of 2.7, 2.2, 1.1, and 0.13 kb. The 1.1- and 0.13-kb pPB90 DNA *Pvu*II

fragments are authentic Py *Pvu*II DNA fragments. The 2.7- and 2.2-kb fragments contain both pBR322 and Py DNA seqeunces. We examined the *Pvu*II digests of A-1 and 15-1 DNA and identified the authentic Py *Pvu*II B fragment (1.1 kb) in both cell lines (Fig. 4B, lanes 2 and 3). In these *Pvu*II digests of A-1 and 15-1 DNA, additional bands, which do not comigrate with other Py *Pvu*II DNA fragments (3.1 and 0.9 kb) or pPB90 *Pvu*II DNA fragments (2.7 and 2.2 kb), can also be observed (Fig. 4B). These cleavage products presumably contain flanking plasmid and cellular DNA sequences in addition to Py viral DNA. Under the electrophoretic conditions used here, the smallest Py *Pvu*II DNA fragment (0.1 kb) has migrated out of the gel.

T antigens in the cells transformed by pPB90. The T antigens present in 15-1 were examined by immunoprecipitation with anti-Py T rat serum and electrophoresis in a polyacrylamide gel (Fig. 5). We observed protein bands that comigrated with the Py middle and small T Ag's isolated from productively infected cells. No Py large T Ag was detectable in the 15-1 cell line. Migrating between the Py middle and small T Ag's are other polypeptides whose nature is unknown at present. A similar pattern consisting of the Py middle and small T Ags, but not the Py large T Ag, was found in the hamster tumor cell line A-1, and a protein kinase activity associated with the 55K Py middle T Ag (7, 29, 33) in both 15-1 and A-1 can be demonstrated (Ito et al., unpublished data).

Fingerprint analysis of T antigens in the cells transformed by pPB90. Two-dimensional fingerprints of methionine-containing tryptic peptides of the proteins of 15-1 cells which comigrate with Py middle and small T Ag are shown in Fig. 6B and C, respectively. For comparison, we prepared a fingerprint of Py middle T Ag from productively infected mouse embryo cells (Fig. 6A). Whereas 15-1 cells are

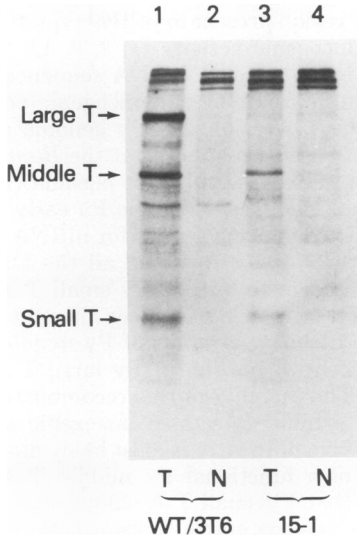

FIG. 5. *Fluorogram of acrylamide gel electrophoresis of [^{35}S]methionine-labeled anti-Py T serum reactive proteins from: 3T6 cells productively infected with wild-type Py virus, anti-Py T serum treated (lane 1); Py-infected 3T6 cells, control serum treated (lane 2); 15-1 cells, anti-Py T serum treated (lane 3); and 15-1 cells, normal serum treated (lane 4).*

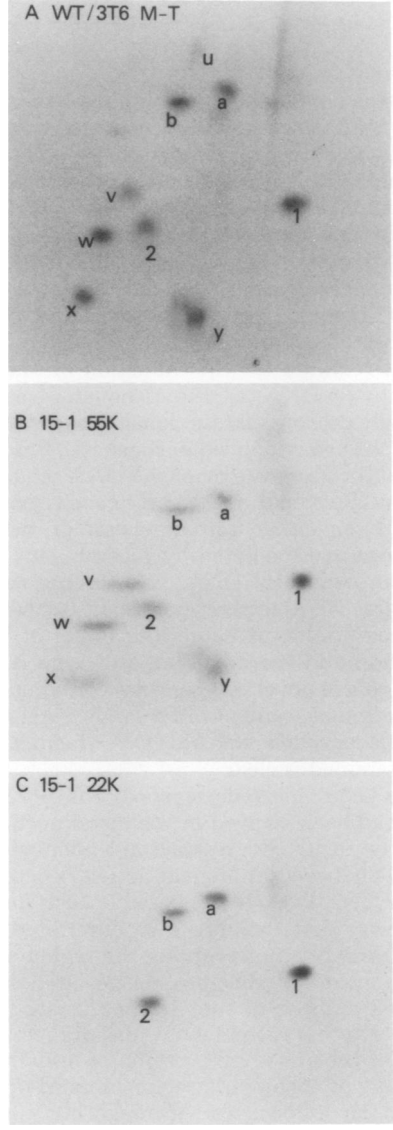

FIG. 6. *Two-dimensional tryptic peptide fingerprints of [^{35}S]methionine-labeled (A) middle T antigen of Py-infected 3T6 cells, (B) 55K protein of 15-1 cells, and (C) 22K protein of 15-1 cells.*

clearly expressing Py middle T Ag (Fig. 6A and B), the peptide marked U in the middle T Ag from productively infected cells (Fig. 6A) does not appear to be present in the fingerprint of Py middle T Ag from 15-1 cells (Fig. 6B).

The methionine peptides of Py small T Ag are a subset of those found in the Py middle T Ag (21, 34). Our fingerprint analysis of the immunoprecipitated protein from 15-1 cells which comigrates with Py small T Ag (Fig. 6C) is indistinguishable from the fingerprint of authentic Py small T Ag from lytically infected cells (20).

DISCUSSION

By using a recombinant plasmid, pPB90, containing Py DNA extending clockwise from the *Bam*HI site at 58 map units to the *Eco*RI site at 0 map units, we have shown that the oncogenic potential of Py virus can be expressed by a DNA fragment encompassing less than 50% of the early region and lacking approximately 60% of the sequences thought to encode Py large T Ag (10, 34). These results complement and extend an earlier observation that the Py large T Ag was not required for the induction of hamster tumors by Py DNA (17, 18). Although we cannot absolutely rule out all functional roles for the Py large T Ag in Py-induced oncogenesis, it is clear that neither this protein nor the DNA sequences encoding the 3′ half of the early gene region are required for either tumorigenesis or cellular transformation mediated by recombinant plasmids containing Py DNA. Neither the role of other viral DNA sequences present between 58 and 0 map units, including the origin of DNA replication and the adjoining late gene region, nor the role of novel polypeptides containing the aminoterminal segment of Py large T Ag which might be encoded by the DNA fragment we have examined is clear.

It has been reported previously that Py DNA which had been cleaved in the distal portion of the early region by restriction endonucleases had an enhanced tumorigenic activity compared with Py DNA I (17, 18). A possible basis for this finding was that Py large T Ag interfered with tumor formation; interrupting the region of the genome encoding this protein resulted in an increased number of tumors. In this study we have shown that recombinant plasmids containing either the entire early region or only the 5′-proximal part of the early region induced tumors with similar efficiencies. Although our current experiments do not provide any further explanation for the altered tumorigenicity of restriction enzyme-cleaved viral DNA, they do indicate that the ability to encode a full-sized, functional Py large T Ag, at least in the case of inocula consisting of recombinant Py plasmids, is not a major determinant of oncogenic activity.

A cell line transformed in vitro by the 2.2-kb *Bam*HI-*Eco*RI Py DNA fragment (15-1) contained Py middle and small T Ag's as indicated by our analysis of the tryptic peptide maps of the 55 and 22K proteins immunoprecipitated from this cell line (Fig. 6). The Py middle T Ag of these cells may not be exactly identical, however, to Py middle T Ag from lytically infected cells, since the peptide U in the fingerprint of middle T antigen from 3T6 cells infected with Py virus (Fig. 6A) cannot be unambiguously demonstrated in the fingerprint of the Py middle T Ag of 15-1 cells. The U peptide has been identified in the middle T Ag found in Py-6, a Py-transformed mouse cell line, and is altered in the middle T Ag specified by a Py mutant, dl-8, which contains a deletion in the middle of the region encoding Py middle T Ag (22). Whereas the significance of our inability to demonstrate the U peptide is unclear, we conclude that Py small and middle T Ag's are encoded totally within the 2.2-kb *Bam*HI-*Eco*RI fragment of Py DNA and that it is likely that the carboxy terminus of middle T antigen, as predicted by DNA sequence analysis (10, 34), is encoded proximal (5′ direction of the early RNA) to the *Eco*RI restriction enzyme recognition site at 0 map units.

Py mutants with alterations in that portion of the early region present in pPB90 typically have altered oncogenic activity (1, 2, 8, 12, 26). To further define which Py DNA sequences were critical in determining the oncogenic potential of this virus, we cloned a Py genome deleted between 90 and 4 map units at the *Bam*HI site in pBR322. This recombinant plasmid contains both the 3′ and 5′ ends of the Py early region, the predicted splicing signals for mRNA encoding the three Py T Ags, and all the DNA sequences known to encode Py small T Ag (10, 34). However, DNA sequences thought to encode the carboxy terminus of Py middle T Ag and the central portion of Py large T Ag are deleted. The inability of this recombinant molecule to exhibit detectable oncogenic activity either in vivo or in vitro is most likely due to the absence of a functional Py middle T Ag and suggests that Py small T Ag alone may not be adequate to effect stable transformation.

Our current finding that the 5′-proximal portion of the early region is sufficient to encode the oncogenic activity of Py virus adds to an already substantial body of information suggesting that virus-induced oncogenesis may proceed by more than one mechanism. Py *ts-a* mutants, which encode a thermolabile Py large T Ag (21),

do not transform cells efficiently at the nonpermissive temperature. This finding has led some to propose that the Py large T Ag is required for the initiation of transformation (5, 6, 9). Some Py *ts-a*-transformed cell lines exhibit a temperature-sensitive transformed phenotype (24, 30), indicating that the continued synthesis of Py large T Ag is required for maintenance of the transformed state. Furthermore, cell lines transformed in vitro by Py virus typically, but not invariably, contain full-sized genomic copies of Py DNA (3, 25; our unpublished data) and Py large T Ag (20a, 22). Conversely, large T Ag is not required for tumorigenesis induced by Py DNA (28), and cleavage of Py DNA in the region of the genome encoding large T antigen has been shown to enhance the tumorigenicity of Py DNA (17, 18). Cell lines established from hamster (18, 19) and rat tumors (Israel et al., unpublished data) induced by either Py virus or Py DNA generally do not contain either the 3'-distal portion of the Py early region or Py large T Ag. Our current experiments indicate that the oncogenic activity of Py DNA can be expressed by a 2.2-kb fragment of viral DNA which includes a portion of the early region encompassing only one-half the viral sequences required to encode large T antigen. To clarify these contrasting patterns of Py-induced tumorigenesis and cellular transformation, further experiments to define the physiological activities of Py-encoded proteins in virus-induced oncogenesis are required.

ACKNOWLEDGMENTS

We thank M. Meltzer for her excellent technical assistance, B. Sylvester for her excellent editorial assistance, and P. Howley and M. Martin for their critical reading of this report.

ADDENDUM IN PROOF

Since this paper was submitted, Novak et al. (U. Novak, S. M. Dilworth, and B. E. Griffin, Proc. Natl. Acad. Sci. U.S.A. **77**:3278–3282, 1980) and Hassell et al. (J. A. Hassell, W. C. Topp, D. B. Rifkin, and P. E. Moreau, Proc. Natl. Acad. Sci. U.S.A. **77**:3978–3982, 1980) have described the transformation of rat fibroblasts by a purified fragment of polyoma viral DNA (64.4 to 0 map units) and a cloned polyoma viral DNA fragment (45 to 1.4 map units), respectively.

LITERATURE CITED

1. **Bendig, M. M., T. Thomas, and W. R. Folk.** 1980. Viable deletion mutants in the medium and large T-antigen-coding sequence of the polyoma virus genome. J. Virol. **33**:1215–1220.
2. **Benjamin, T. L.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:394–399.
3. **Birg, F., R. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyoma virus DNA sequences in transformed rat cell lines. J. Virol. **29**:633–648.
4. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
5. **Di Mayorca, G., J. Callender, G. Marin, and R. Giordano.** 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
6. **Eckert, W.** 1975. Properties of temperature sensitive mutants of polyoma virus. Cold Spring Harbor Symp. Quant. Biol. **39**:37–40.
7. **Eckert, W., M. A. Hutchinson, and T. Hunter.** 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell **18**:925–933.
8. **Feuenteun, J., L. Sompayrac, M. Fluck, and T. Benjamin.** 1976. Localization of gene functions in polyoma virus DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:4169–4173.
9. **Fried, M.** 1965. Cell transformation ability of a temperature-sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **53**:486–491.
10. **Friedman, T., A. Esty, P. Laporte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
11. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
12. **Griffin, B. E., and C. Maddock.** 1979. New classes of viable deletion mutants in the early region of polyoma virus. J. Virol. **31**:645–656.
13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cultures. J. Mol. Biol. **26**:365–369.
14. **Hutchinson, M. A., T. Hunter, and W. Eckert.** 1978. Characterization of T antigens in polyoma infected and transformed cells. Cell **15**:65–77.
15. **Israel, M. A., H. W. Chan, M. A. Martin, and W. P. Rowe.** 1979. Molecular cloning of polyoma virus DNA in Escherichia coli: oncogenicity testing in hamsters. Science **205**:1040–1042.
16. **Israel, M. A., H. W. Chan, W. P. Rowe, and M. A. Martin.** 1979. Molecular cloning of polyoma virus DNA in Escherichia coli: plasmid vector system. Science **203**: 883–887.
17. **Israel, M. A., H. W. Chan, W. P. Rowe, and M. A. Martin.** 1979. Biological activity of polyoma virus DNA in mice and hamsters. J. Virol. **29**:990–996.

17a. **Israel, M. A., K. Chowdhury, J. Ramseur, K. Chandrasekron, D. F. Vanderryn, and M. A. Martin.** 1980. Tumorigenicity of polyoma virus in hamsters. Cold Spring Harbor Symp. Quant. Biol. **44**:591–596.

18. **Israel, M. A., D. T. Simmons, S. L. Hourihan, W. P. Rowe, and M. A. Martin.** 1979. Interrupting the early region of polyoma virus DNA enhances tumorigenicity. Proc. Natl. Acad. Sci. U.S.A. **76**:3713–3716.
19. **Israel, M. A., D. F. Vanderryn, M. L. Meltzer, and M. A. Martin.** 1980. Characterization of polyoma viral DNA sequences in polyoma induced hamster tumor cell lines. J. Biol. Chem. **255**:3798–3805.
20. **Ito, Y.** 1979. Polyoma virus specific 55K protein isolated from plasma membrane of productively infected cells is virus coded and important for cell transformation. Virology **98**:261–266.

20a. **Ito, Y., and N. Spurr.** 1980. Polyoma virus T antigens expressed in transformed cells: significance of middle T antigen in transformation. Cold Spring Harbor Symp. Quant. Biol. **44**:149–157.

21. **Ito, Y., N. Spurr, and R. Dulbecco.** 1977. Characterization of polyoma virus T antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:1259–1263.
22. **Ito, Y., N. Spurr, and B. E. Griffin.** 1980. Middle T antigen as primary inducer of full expression of the phenotype of transformation by polyoma virus. J. Virol. **35**:219–232.
23. **Ketner, G., and J. J. Kelly, Jr.** 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1106.
24. **Kimura, G.** 1975. Temperature-sensitive growth of cells transformed by ts-a mutant of polyoma virus. Nature (London) **153**:639–641.

25. **Lania, L., D. Gandini-Attardi, M. Griffiths, B. Cooke, D. DeCicco, and M. Fried.** 1980. The polyoma virus 100 K large T antigen is not required for the maintenance of transformation. Virology **101**:217-232.
26. **Magnusson, G., and P. Berg.** 1979. Construction and analysis of viable deletion mutants of polyoma virus. J. Virol. **32**:523-529.
27. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184-1188.
28. **Moore, J. L., K. Chowdhury, M. A. Martin, and M. A. Israel.** 1980. Polyoma large T antigen is not required for tumorigenesis mediated by viral DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:1336-1340.
29. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigens. Cell **18**:935-946.
30. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cells induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721-728.
31. **Simmons, D. T., C. Chang, and M. A. Martin.** 1979. Multiple forms of polyoma virus tumor antigen from infected and transformed cells. J. Virol. **29**:881-887.
32. **Smart, J. E., and Y. Ito.** 1978. Three species of polyoma virus tumor antigens share common peptides probably near the amino-terminal of the proteins. Cell **15**:1427-1437.
33. **Smith, A. E., R. Smith, B. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma virus middle T antigen in vitro. Cell **18**:915-924.
34. **Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsch, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. Nature (London) **283**:445-453.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.
36. **Sutcliffe, J. G.** 1978. pBR322 restriction map derived from DNA sequences: accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acids Res. **5**: 2721-2728.
37. **Tooze, J.** 1973. The molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Vogt, M., and R. Dulbecco.** 1962. Studies on cells rendered neoplastic by polyoma virus: the problem of the presence of virus-related materials. Virology **16**:41-51.
39. **Wahl, S. M., M. Stein, and S. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextransulfate. Proc. Natl. Acad. Sci. U.S.A. **76**: 3683-3687.
40. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single copy eucaryotic genes using total cellular DNA as donor. Cell **14**:725-731.