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Polyoma large tumor antigen is not required for tumorigenesis mediated by viral DNA

(polyoma virus/restriction endonucleases/polyoma virus–plasmid recombinant DNA/Southern blotting technique)

JANET L. MOORE*†, KAMAL CHOWDHURY‡, MALCOLM A. MARTIN‡, AND MARK A. ISRAEL‡

*Laboratory of Viral Diseases and ‡DNA Recombinant Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT **The arrangement of viral DNA sequences in a hamster cell line derived from a tumor induced by a recombinant plasmid DNA preparation containing the entire polyoma virus genome was examined. In the recombinant plasmid employed, viral DNA sequences specifying the large species of polyoma tumor antigen but not the small and middle tumor antigens were interrupted by the insertion of plasmid DNA at the *Eco*RI restriction endonuclease site. Blot-hybridization analyses of tumor cell DNA indicated that the "joints" linking viral and plasmid DNAs in the original recombinant plasmid used in animal inoculation had been preserved. Integration into the hamster cell genome had apparently occurred within plasmid DNA sequences. These results indicate that polyoma large tumor antigen is not required for tumorigenesis mediated by viral DNA.**

It is now well established that the polyoma (PY) virus genome encodes at least three early proteins: the small (20,000 daltons), middle (56,000–63,000 daltons), and large (105,000 daltons) tumor (T) antigens (Ags) (1–5). The function of each of these proteins in mediating PY virus-induced cellular transformation has been examined by using a variety of techniques. Three types of "early" PY virus mutants have been isolated. PY virus *tsa* mutants (6–8), which map in the distal part of the early region (9), are unable to synthesize functional PY large T Ag or transform cells at the nonpermissive temperature. Cells transformed by PY virus *tsa* mutants at the permissive temperature usually maintain their transformed phenotype at the restrictive temperature (6–8). However, Cuzin and his colleagues have isolated *tsa*-transformed rat cells that express the transformed phenotype only at the permissive temperature (10, 11). A second type of early mutant, the *hr-t* mutants (12), map in the proximal portion of the early region (13) and are defective in their ability to direct the synthesis of small and middle T Ags (14). Large T Ag can be detected in mouse cells infected with *hr-t* mutants of PY virus (14). The *hr-t* mutants are incapable of transforming rat or hamster cells in culture or inducing tumors after injection into newborn hamsters (12). PY virus *hr-t* and *tsa* mutants can complement one another in transformation assays (15, 16). Another type of early PY virus mutant has recently been isolated that has deletions in the region of the viral genome that encodes both PY middle and large T Ags (17). In addition to specifying truncated forms of the large and middle PY T Ags (17), these viruses have altered transforming activities (17).

Although PY virus mutants have proven to be invaluable tools for defining early gene products, they have not been successfully utilized to unambiguously delineate the functional role of the three virus-encoded T Ags in virus-mediated oncogenesis. Due to the complex physical organization of the early region of PY virus DNA, which seems to specify three different polypeptides in two different reading frames (1–5), a single mutation, depending on its location, could affect the synthesis of one, two, or three of the early proteins. Recently, we reported that hamster cell lines established in tissue culture from tumors induced by PY virus contain only the small and middle T Ags (18), and that the integrated PY virus DNA in such cell lines has deletions located in the distal part of the early gene region that preclude the synthesis of PY large T Ag.§ These data indicate clearly that the PY large T Ag is not required for maintenance of the transformed state and confirm genetic studies that suggested this possibility (6–8).

We have previously shown that PY viral DNA, which had been specifically cleaved with restriction endonucleases that interrupt the viral genome in a region that encodes the large T Ag, induced tumors in newborn hamsters (18). This result raised the possibility that PY large T Ag played little if any role in PY viral DNA-induced tumorigenesis. In those experiments, however, it was not possible to exclude the possibility that PY large T Ag, specified by the few remaining intact molecules in our restriction endonuclease-treated viral DNA preparation, was responsible for initiating the tumors observed. We recently reported that purified DNA from a recombinant plasmid containing pBR322 DNA inserted into the PY virus genome at the *Eco*RI site [PY (*Eco*RI)-pBR322] (19) (Fig. 1) was tumorigenic in hamsters (20). The insertion of plasmid DNA into the PY virus genome at the *Eco*RI site would absolutely block the synthesis of PY large T Ag unless the viral genome was somehow precisely excised from the recombinant DNA molecule and then recircularized at the *Eco*RI site subsequent to infection. Our previous finding that such recombinant DNA molecules were not infectious in mouse cells (19) indicated that precise excision–recircularization does not occur. In this report, a cell line established in culture from a hamster tumor induced by a PY (*Eco*RI)-pBR322 recombinant plasmid was evaluated by blot-hybridization analysis. We observed that the PY (*Eco*RI)-pBR322 plasmid had been integrated into the hamster genome at a site within the plasmid DNA, leaving the entire PY virus genome, covalently linked at the *Eco*RI site to pBR322 DNA, intact. The retention of plasmid DNA sequences adjacent to the viral *Eco*RI site in the integrated PY DNA indicates that PY large T Ag was never synthesized after the hamster inoculation and that this virus-encoded polypeptide played no role in tumorigenesis mediated by PY virus DNA.

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Abbreviations: PY, polyoma; T, tumor; Ag, antigen.

† Formerly Janet M. Ramseur.

§ Israel, M. A., Vanderryn, D. F., Meltzer, M. L. & Martin, M. A., *J. Biol. Chem.*, in press.

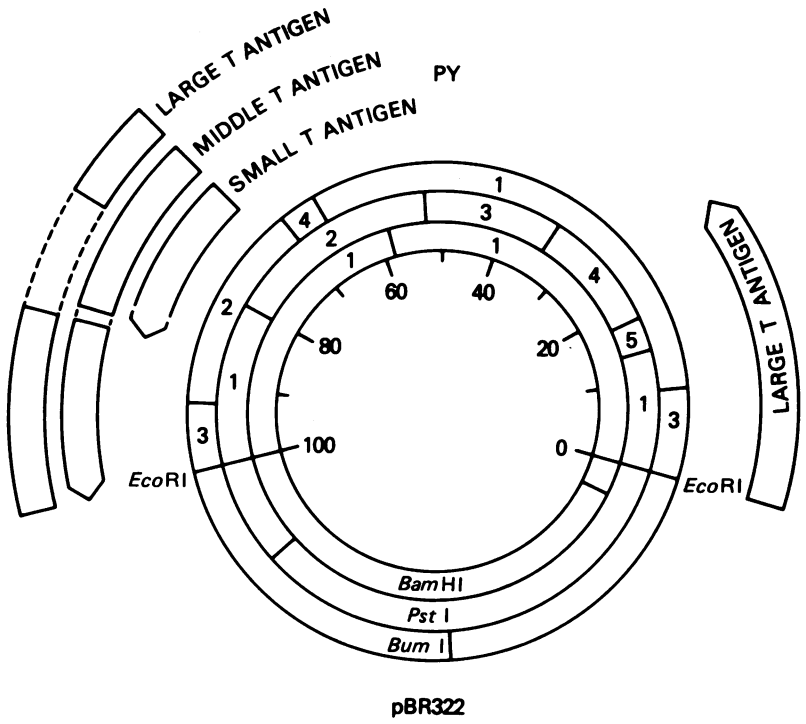

FIG. 1. Structural map of PY (*Eco*RI)-pBR322 DNA. This circular map of recombinant plasmid DNA is shown with the PY viral genome divided in 100 units starting at the *Eco*RI site. The restriction endonuclease cleavage sites for *Eco*RI, *Bam*HI, *Pst* I, and *Bum* I in the PY viral and pBR322 DNA segments are shown. The PY viral DNA fragments produced by cleavage with these various enzymes are numbered according to their size. The arrows outside the circle indicate the putative coding regions for small, middle, and large T Ags (4, 5).

MATERIALS AND METHODS

Viral and Plasmid DNA. PY viral DNA was prepared from 3T6 mouse cells infected at low multiplicity as described (21). Closed circular pBR322 (22) and PY (*Eco*RI)-pBR322 (19) DNA were purified from chloramphenicol-amplified cultures of *Escherichia coli* RR1 (22) as previously outlined (19). ^{32}P-Labeled hybridization probes were prepared by the *in vitro* labeling (23) of PY viral DNA or pBR322 DNA as previously indicated (24).

Establishment of Hamster Tumor Cell Lines. One-day-old Syrian hamsters were inoculated with PY (*Eco*RI)-pBR322 DNA as described (20). Samples of tumor tissue were sterilely resected, trypsinized, and propagated as tissue cultures in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal calf serum as previously outlined (18).

Cellular DNA and Restriction Endonuclease Cleavage of Cellular DNA. High molecular weight DNA from tissue culture cells was prepared as described.[§] Restriction endonucleases were obtained from either New England BioLabs or Bethesda Research Laboratories. The reaction conditions for *Bst*EII, *Bgl* II, *Eco*RI, *Bum* I, and *Bam*HI have been described.[§] *Pst* I cleavage was carried at 37°C in 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM $(NH_4)_2SO_4$/100 μg of bovine serum albumin per ml. Cleavage of cellular DNA by a combination of *Bam*HI and *Pst* I was carried out in *Pst* I digestion buffer. In each case, complete cleavage of cellular DNA was monitored by gel electrophoresis analysis of the digestion of ^{32}P-labeled simian virus 40 DNA or ^{32}P-labeled bacteriophage λ DNA in aliquots of each individual restriction enzyme reaction mixture.

Detection of PY Viral and pBR322 DNA Sequences in Cellular DNA. Restriction endonuclease-cleaved cellular DNA was transferred from 0.7% agarose gels to nitrocellulose filter sheets as described.[§] Hybridization was carried out in sealed polyethylene bags containing 12 ml of hybridization solution with 10–20 × 10^6 cpm of ^{32}P-labeled PY viral or pBR322 DNA (specific activity 6–10 × 10^7 cpm/μg). After incubation at 60°C for 16 hr, the filters were washed, air dried, and exposed to pre-flashed Kodak XR-2 film with intensifying screens.[§]

Evaluation of Transformed Hamster Cells for T Ag. PY virus-transformed hamster tumor cells were labeled as described (3) with [^{35}S]methionine in Eagle's minimal essential medium lacking methionine. The labeled proteins were immunoprecipitated by either nonimmune hamster serum or hamster anti-PY T serum in the presence of protein A-bearing *Staphylococcus aureus* as described by Ito (25) and electrophoresed in a 10% acrylamide gel (3).

RESULTS

Characterization of PY Viral DNA Sequences in FD1738. In these experiments we mapped the PY (*Eco*RI)-pBR322 DNA sequences that had been integrated into the host cell chromosomal DNA of a hamster tumor cell line, FD1738. Because preservation of the linkage between PY viral and pBR322 DNA sequences at the PY virus *Eco*RI site would indicate that this tumor had been induced by PY viral DNA that had not been excised from the recombinant and arose in the absence of PY large T Ag, our efforts were directed to the detection of fragments that spanned the junctions between pBR322 and PY viral DNAs. The experimental design employed to study the arrangement of the PY DNA in this hamster tumor cell line utilized a modification (26) of the Southern blot-hybridization procedure (27–29). Fig. 2 shows such an analysis of PY viral DNA sequences in FD1738. *Bgl* II and *Bst*EII are restriction endonucleases that do not cleave PY viral DNA. Only a single band was observed after the hybridization of ^{32}P-labeled PY DNA to FD1738 DNA cleaved with either *Bgl* II or *Bst*EII (Fig. 2, lanes B and C), suggesting that viral DNA is integrated at only a single locus in this tumor line. Digestion of PY (*Eco*RI)-pBR322 recombinant DNA with *Eco*RI would be expected to

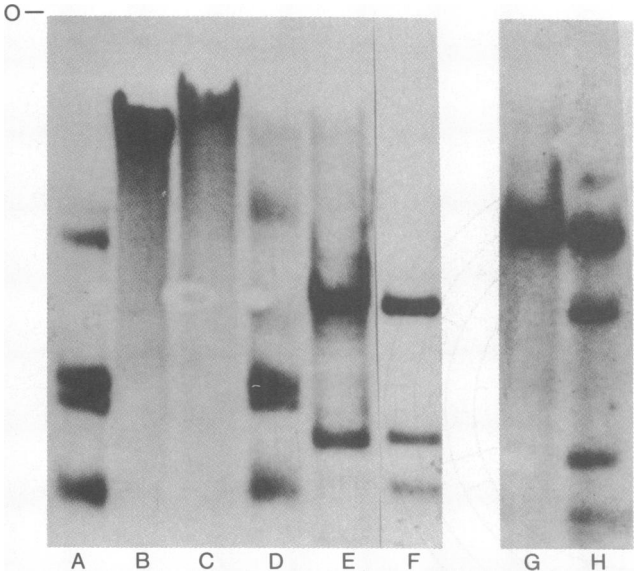

FIG. 2. Hybridization of ^{32}P-labeled PY viral DNA to FD1738 cellular DNA after restriction endonuclease digestion and transfer from agarose gels. Cellular DNA (10 μg per lane) was cleaved with *Bst*EII (lane B), *Bgl* II (lane C), *Pst* I (lane D), *Bum* I (lane E), or *Eco*RI (lane G); electrophoresed in a 0.7% agarose slab gel; denatured; transferred to a sheet of nitrocellulose; and hybridized to ^{32}P-labeled PY viral DNA. Similarly treated PY viral DNA cleaved with *Pst* I (lane A) or *Bum* I (lane F) is included as a marker. Full-length linear PY viral DNA, as well as *Bum* I-cleaved PY DNA, has been electrophoresed in lane H. O indicates the origin of gel electrophoresis.

Table 1. Detectable restriction fragments containing PY viral DNA sequences*

Enzyme	PY viral	PY (*Eco*RI)-pBR322 A	PY (*Eco*RI)-pBR322 B	FD1738
Pst I				4.2
			2.6†	
	1.22			
				1.9
			1.18†	1.18
	0.97	0.97	0.97	0.97
		0.95†		
	0.62	0.62	0.62	0.62
	0.55	0.55	0.55	0.55
		0.28†		
Pst I + *Bam*HI	1.22			
			1.10†	1.10
		0.95†		
		0.90†		
	0.76	0.76	0.76	0.76
			0.70†	0.70
	0.62	0.62	0.62	0.62
	0.55	0.55	0.55	0.55

* Fragment sizes are listed in megadaltons. Under the conditions of electrophoresis employed, restriction fragments smaller than 0.3 megadaltons would not be detected. A and B refer to recombinant PY-pBR322 plasmids containing the PY virus genome in each of the two possible orientations (19).

† These fragments are "joint" fragments containing both pBR322 and PY viral DNA sequences.

yield two fragments that correspond to linear forms of PY viral and pBR322 DNAs. Cleavage of FD1738 DNA with *Eco*RI generated a single band that reacted with ^{32}P-labeled viral DNA (Fig. 2, lane G) and comigrated with a linear PY viral DNA marker (Fig. 2, lane H). The identification of a full-length copy of viral DNA in this tumor cell line suggests that the recombinant plasmid used for animal inoculation had been integrated into the hamster chromosome at a site within the plasmid DNA, leaving the PY viral DNA sequences uninterrupted.

Two independent approaches were used to verify that the entire PY virus genome flanked by plasmid DNA sequences was present in FD1738. First, enzymes that cut within the PY virus genome at multiple sites were used to demonstrate the presence of authentic viral DNA fragments. Second, FD1738 DNA was digested with restriction enzymes that generate fragments that span the joint between viral and plasmid DNAs in the original recombinant to ascertain whether this linkage had been preserved in the integrated DNA. Cleavage of FD1738 DNA with *Bum* I yielded two fragments that comigrated with *Bum* I PY viral DNA fragments 1 and 2 (Fig. 2, lanes E and F). The *Bum* I PY viral fragment 3 contains the PY viral *Eco*RI site (Fig. 1) and as such would not be expected to be present in a restriction digest of the original recombinant plasmid DNA. It would also not be detected after cleavage of FD1738 DNA with *Bum* I if the linkage between PY viral and plasmid DNA had been conserved in the tumor cell DNA. As can be seen in Fig. 2, lane E, the *Bum* I PY viral DNA fragment 3 was not detected in FD1738 DNA. A similar restriction analysis of this tumor cell line was carried out with *Pst* I (Fig. 2). The slowest-moving band observed in Fig. 2, lane A, was a partial digestion product. Under the electrophoresis conditions employed, PY viral *Pst* I fragments 1 and 2 (1.22 and 0.97 × 10^6 daltons) ran as a doublet and fragments 3 and 4 (0.62 and 0.55 × 10^6 daltons) comigrated. Fig. 2, lane D, depicts the *Pst* I cleavage of FD1738 DNA and shows fragments that comigrated with *Pst* I PY viral fragments 1–4. Although we anticipated the presence of PY viral *Pst* I fragments 2–4 in FD1738 DNA (Fig. 1, Table 1), the presence of a fragment that appeared to comigrate with PY viral *Pst* I fragment 1 was initially unanticipated. However, *Pst* I cleavage of PY (*Eco*RI)-pBR322 DNA produced a joint fragment of 1.18 × 10^6 daltons, virtually identical in size to the PY viral *Pst* I fragment 1 that spans the *Eco*RI site in the viral DNA (Fig. 1, Table 1). In another experiment, we have detected the hybridization of ^{32}P-labeled pBR322 DNA to this fragment (see below; Fig. 3, panel A), indicating that it also contains plasmid DNA sequences and that it is the authentic *Pst* I PY (*Eco*RI)-pBR322 fragment 1. These experiments suggest that the entire PY virus genome is present in FD1738 and that the joint present in the original recombinant plasmid linking PY viral and pBR322 DNAs is intact in the tumor cell line.

To examine more closely the region of the integrated PY virus genome in the region of the *Eco*RI site, FD1738 DNA was simultaneously digested with *Bam*HI and *Pst* I, which would be expected to generate "joint" fragments containing both PY viral and plasmid DNA sequences after cleavage of the recombinant plasmid (Fig. 1, Table 1). PY viral DNA can be inserted into pBR322 in two possible orientations (ref. 19, Table 1). Cleavage of such preparations with *Pst* I plus *Bam*HI would be expected to yield joint fragments having unique electrophoretic mobilities as well as "internal fragments" comigrating with one another as well as with the *Pst* I plus *Bam*HI fragments of PY viral or pBR322 DNAs (Fig. 1, Table 1). Fig. 3*A* depicts the hybridization of ^{32}P-labeled pBR322 DNA to restriction-endonuclease digested DNAs from the recombinant plasmids in two different orientations (lanes A and B) and FD1738. In addition to the internal 1.94 × 10^6 dalton plasmid DNA fragment, the labeled pBR322 DNA also reacted with additional fragments having sizes that corresponded to predicted joint segments. Bands comigrating with the 1.1 × 10^6 and 0.7 × 10^6 dalton joint fragments present in the recombinant plasmid examined in lane B were also present in the *Pst* I plus *Bam*HI cleavage of FD1738 DNA (Fig. 3*A*). Besides these two joint

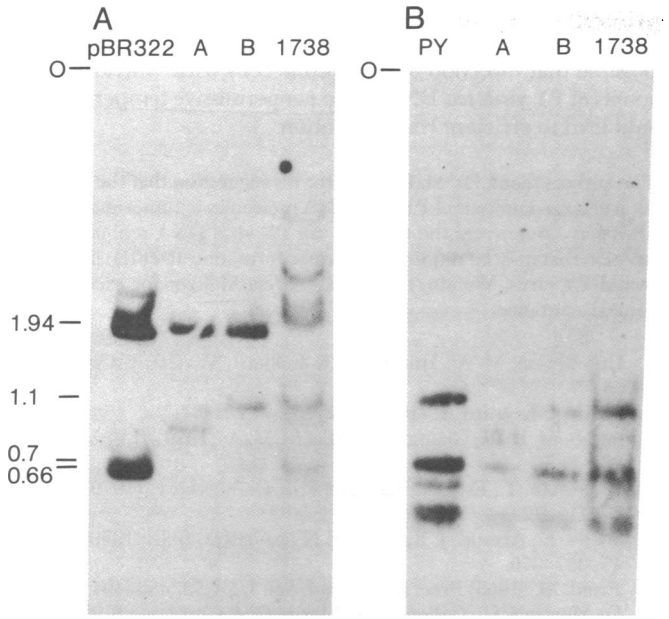

FIG. 3. Hybridization of ^{32}P-labeled plasmid or PY viral DNA to FD1738 cellular DNA. FD1738 DNA cleaved by *Pst* I plus *Bam*HI was electrophoresed, transferred to a sheet of nitrocellulose paper, and hybridized to ^{32}P-labeled pBR322 DNA (*A*) or ^{32}P-labeled PY viral DNA (*B*). Similarly treated PY viral, pBR322, or PY (*Eco*RI)-pBR322 recombinant plasmid DNAs containing the PY viral DNA insert in each of the two possible orientations (A or B) (19) are included as markers. Scale on left is in megadaltons.

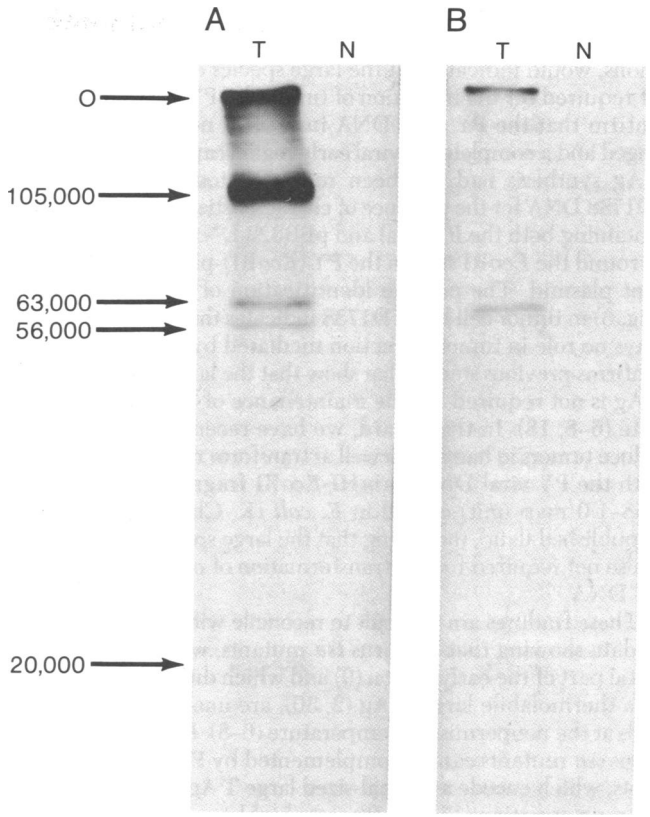

FIG. 4. Acrylamide gel electrophoresis of ^{35}S-labeled polypeptides immunoprecipitated by anti-PY T Ag serum from hamster tumor cell lines. The electrophoretic pattern of proteins immunoprecipitated from PY T-54 (*A*), a PY virus-induced tumor cell line (18) that is known to contain the large, middle, and small T Ags, and FD1738 (*B*) is shown. Samples were immunoprecipitated in the presence of hamster anti-PY T serum (lanes T) or serum from normal hamsters (lanes N).

fragments, other bands containing pBR322 DNA can be seen. While two of these fragments may represent the sites at which the plasmid DNA is linked to the cellular genome, the presence of additional bands suggests that the digestion was incomplete.

Hybridization of ^{32}P-labeled PY viral DNA to similarly cleaved DNA preparations is shown in Fig. 3*B*. PY viral form I DNA was digested with *Bam*HI plus *Pst* I and four of the expected six cleavage fragments could be identified under these electrophoresis conditions, although bands 3 and 4 comigrated. In Fig. 3*B*, lane PY, the light bands migrating at the top of the lane and between *Pst* I plus *Bam*HI PY viral DNA fragments 2 and 3 are partial digestion products. Digestion of both recombinant plasmid DNA preparations with *Pst* I plus *Bam*HI would be expected to result in appearance of three of the four viral cleavage fragments (Fig. 1, Table 1). The largest PY viral restriction fragment should not be detected because it contains the *Eco*RI restriction site and would be interrupted by the pBR322 insertion (Fig. 1). Hybridization of radiolabeled PY viral DNA to *Pst* I- plus *Bam*HI-digested recombinant plasmid DNAs confirmed these predictions and, in addition, revealed the presence of PY viral DNA in junction fragments, as was most convincingly demonstrated by the detection of the 1.1×10^6 dalton *Eco*RI joint fragment (Fig. 3*B*). The presence of the 0.7×10^6 dalton joint fragment could not be unambiguously shown because it comigrated under these electrophoresis conditions with the PY viral *Pst* I plus *Bam*HI fragment 2 (Table 1). The pattern of ^{32}P-labeled PY viral DNA hybridization to *Pst* I- plus *Bam*HI-cleaved FD1738 DNA was very similar to that observed with one of the recombinant plasmid DNA preparations (Fig. 3*B*). The 1.1×10^6 dalton fragment that links the plasmid to PY viral DNA in the recombinant plasmid was clearly recognizable in the tumor cell DNA preparation and comigrated with a cellular DNA fragment that hybridized with radiolabeled pBR322 DNA (Fig. 3*A*).

Characterization of PY Virus T Ags in FD1738. The blot-hybridization analyses of viral and plasmid DNA present in FD1738 described above provide unambiguous evidence that the early region of the integrated PY virus genome was interrupted by the presence of pBR322 DNA and is, therefore, incapable of directing the synthesis of large T Ag. This expectation was confirmed by examining the [^{35}S]methionine-labeled proteins immunoprecipitated by PY-specific antiserum from FD1738 on sodium dodecyl sulfate/polyacrylamide gels (Fig. 4). Only PY middle (56,000–63,000 dalton) and small (20,000 dalton) T Ags and not PY large T Ag were detected in FD1738 (Fig. 4), a pattern previously reported by us to be present in nearly all hamster tumor lines we have evaluated (18). The presence of full-sized middle T Ag in this cell line indicates that the coding region of PY middle T Ag does not extend beyond the *Eco*RI site.

DISCUSSION

To determine whether the 105,000-dalton PY large T Ag was necessary for the induction of hamster tumors by PY viral DNA, we examined the PY DNA sequences and PY-specific proteins in a cell line (FD1738) established from a hamster tumor induced by PY (*Eco*RI)-pBR322 recombinant plasmid DNA. This recombinant plasmid was selected because viral DNA sequences specifying the large T Ag were interrupted by the insertion of pBR322 DNA at the *Eco*RI site. This cleavage site is located in the middle of the early region at a position that encodes PY large T Ag but probably outside the region that specifies small and middle T Ags (1–5). The preservation in

FD1738 cells of the joint between viral and pBR322 DNAs, present in the recombinant plasmid used in the animal inoculations, would indicate that the large species of T antigen was not required for the induction of tumors by PY viral DNA. To confirm that the PY viral DNA insert had not become rearranged and a complete PY viral early region capable of PY large T Ag synthesis had not been reconstructed, we examined FD1738 DNA for the presence of characteristic DNA fragments containing both the PY viral and pBR322 DNA sequences that surround the *Eco*RI sites in the PY (*Eco*RI)-pBR322 recombinant plasmid. The positive identification of such fragments (Fig. 3) in tumor cell line FD1738 indicates that PY large T Ag plays no role in tumor induction mediated by viral DNA and confirms previous studies that show that the large species of PY T Ag is not required for the maintenance of the transformed state (6–8, 18). In this regard, we have recently been able to induce tumors in hamster as well as transform rat cells in culture with the PY viral DNA *Bam*HI-*Eco*RI fragment (clockwise, 0.58–1.0 map unit) cloned in *E. coli* (K. Chowdhury *et al.*, unpublished data), indicating that the large species of PY T Ag is also not required for the transformation of cells in culture by PY DNA.

These findings are difficult to reconcile with the large body of data showing that PY virus *tsa* mutants, which map in the distal part of the early region (9) and which direct the synthesis of a thermolabile large T Ag (2, 30), are unable to transform cells at the nonpermissive temperature (6–8). Furthermore, PY virus *tsa* mutants can be complemented by PY virus *hr-t* mutants, which encode a normal-sized large T Ag, for their defect in transformation (15, 16). It seems highly unlikely that all *tsa* mutants are also temperature sensitive for a second early function related to transformation that can be complemented by the *hr-t* mutants. Because all published reports indicating the inability of PY virus *tsa* mutants to transform cells at the nonpermissive temperature have evaluated the efficiency of *in vitro* transformation by mutant virus rather than viral DNA, it is possible that the *a* gene function involved in viral DNA replication could be required for virus-mediated and not PY viral DNA-mediated transformation. In nearly all the tumorigenicity studies we have conducted, newborn hamsters were inoculated with 0.5 μg of PY viral (21) or PY viral-pBR322 DNA (20). This corresponds to approximately $3–5 \times 10^{10}$ DNA molecules, which is equivalent to approximately $1–1.7 \times 10^{8}$ plaque-forming units of PY virus. The subcutaneous tumors that appear after the injection of this amount of viral DNA are invariably located at the site of inoculation. The location of such DNA-induced tumors contrasts sharply with that observed after PY virus inoculation (50% tumor-producing dose [TD_{50}] = 5×10^{3} plaque-forming units [21]). Even after subcutaneous injection of virus, internal as well as subcutaneous tumors appear at multiple sites in the inoculated animals. It is therefore likely the efficiency with which PY virus induces tumors and the multiplicity of tumors observed reflect virus replication in the inoculated animals. Injection of 0.5 μg of viral DNA containing approximately 10^4 to 10^5 times more DNA molecules than are present in one TD_{50} of PY virus may circumvent the necessity for PY viral DNA replication, a function clearly affected by the *tsa* mutation (6–8). Similarly, the *in vitro* transformation observed with the cloned *Bam*HI-*Eco*RI PY viral DNA fragment could represent the delivery of an adequate quantity of viral DNA sequences to sensitive cells, thereby obviating the necessity for the *a* gene replication function. It is possible that infection of susceptible cells with "sufficient" amounts of PY virus *tsa* DNA at the nonpermissive temperature would lead to efficient transformation.

The authors thank Dr. M. Botchan for his suggestion that the inoculation of large numbers of PY viral DNA molecules in tumorigenicity studies may circumvent the necessity for PY viral DNA replication, a function that may be required for tumor formation after the inoculation of PY virus. We also thank Ms. Marcia Meltzer for excellent technical assistance.

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