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Interrupting the early region of polyoma virus DNA enhances tumorigenicity

(papovavirus/T antigen/tumorigenesis/cell transformation/restriction enzyme)

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ABSTRACT **The tumorigenicity of DNA from polyoma virus after cleavage with a variety of restriction enzymes was evaluated in suckling hamsters. Cleavage with enzymes that interrupt the region of the genome coding for the large tumor (T) antigen of polyoma virus markedly enhanced the tumorigenicity above that observed with DNA I of the virus. Cell lines established *in vitro* from tumors induced by polyoma virions, polyoma virus DNA I, or polyoma virus DNA that had been cleaved with restriction endonucleases in the early region all contain the polyoma virus middle and small T antigens but not the large T antigen. These findings indicate that the large T antigen of polyoma virus is not required for maintenance of the transformed state and probably not for initiation of tumorigenesis by viral DNA.**

Serological, biochemical, and genetic studies all indicate that gene products specified by the early region of papovavirus genomes play a key role in the oncogenic process (1). The viral tumor antigen(s) (T Ag), a hallmark of cells transformed by papovaviruses, is encoded by the region of the viral genome transcribed early in lytic infection (2); in contrast, proteins from the late region of the viral genome are rarely detectable in transformed cells. Also, conditional lethal mutants defective in the transformation process have lesions that map in the early region of the viral genome (3, 4).

Recent studies of proteins immunoprecipitated with hamster antiserum against T Ag from cells infected or transformed by simian virus 40 or polyoma virus have shown that T Ag is not a single protein but consists of several related polypeptides—at least two for simian virus 40 (2) and at least three for polyoma virus (5, 6). The polyoma virus-encoded T Ags have molecular weights of approximately 105,000 (large T Ag), 56,000–63,000 [middle T Ag(s)], and 20,000 (small T Ag), as estimated by acrylamide gel electrophoresis (6). Genetic studies and tryptic peptide analyses of these proteins indicate that the small and middle T Ags are specified by nucleotide sequences located in the proximal portion of the early region (4–6). The large species of T Ag is specified by a portion of the early gene sequences encoding the small and middle T Ags as well as sequences located in the distal part of the early region (4–6) (Fig. 1). The exact role of these antigens in papovavirus-induced tumorigenesis and cellular transformation is currently a research area of great interest.

Two groups of "early" polyoma virus mutants have been isolated. Polyoma tsa mutants, which map in the distal portion of the early gene region (3), fail to replicate their DNA in mouse cells at the nonpermissive temperature (7, 8). Although tsa mutants transform cells normally at the permissive temperature, they fail to transform cells at the restrictive temperature (9, 10). The results of recent experiments conducted by Cuzin and his colleagues (11, 12) indicate that, under certain circumstances, the viral gene product that is altered in tsa mutants is required continuously for the expression of the transformed phenotype in polyoma tsa-transformed rat fibroblasts. The second type of early mutant (hr-t) maps in the proximal portion of the early region (4), does not transform rat or hamster cells, and cannot replicate in certain mouse cell lines (13). The hr-t and tsa mutants can complement one another in transformation assays (14, 15).

Recently we observed that polyoma viral DNA can induce tumors when injected into newborn hamsters (16). This finding suggested the possibility that specifically altered viral DNA molecules could be used to evaluate the role of polyoma virus gene products in mediating virus-induced tumorigenesis. In this study, we have examined the tumorigenicity in suckling hamsters of polyoma virus DNA molecules cleaved with restriction endonucleases within or around the early gene region.

METHODS

Virus and Viral DNA. A large-plaque variant of polyoma virus, originally isolated by Vogt and Dulbecco, was used (17). Polyoma virus DNA I was isolated by differential salt precipitation (18) from infected 3T6 cells as described (16).

Digestion of DNA with Restriction Enzymes and S1 Nuclease. Polyoma virus DNA I was cleaved with *Eco*RI or *Bam*HI as described (16). The DNA I (50 μg/ml) was cleaved with *Hin*cII plus *Hin*dIII for 3 hr at 37°C in a reaction mixture containing 10 mM Tris·HCl (pH 7.8), 60 mM NaCl, 7 mM $MgCl_2$, and 1 mM dithiothreitol or with *Xba* I for 3 hr at 37°C in a reaction mixture containing 10 mM Tris·HCl (pH 7.4), 100 mM NaCl, and 7 mM $MgCl_2$. In each case, the amount of enzyme added was determined in preliminary experiments to yield complete cleavage of viral DNA incubated under similar conditions. Enzymatic reactions were terminated either by the addition of 1/20 vol of 0.5 M EDTA (pH 8.0) or by incubation of the reaction mixture at 68°C for 6 min. The completeness of each restriction endonuclease digestion was confirmed by slab gel electrophoresis, as described (16). Cleaved viral DNA was digested with S1 nuclease (Miles) at 25°C for 30 min in a reaction mixture (1 ml) containing 50 mM NaOAc (pH 4.5), 300 mM NaCl, and 2 mM $ZnCl_2$. The completeness of the S1 nuclease digestion was monitored by adding denatured simian virus 40 [^{3}H]DNA to an aliquot of the reaction mixture and testing this aliquot for the presence of trichloroacetic acid-precipitable material at the end of the incubation period. In-

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Abbreviation: T Ag, tumor antigen.

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variably, greater than 95% of the denatured simian virus 40 [^{3}H]DNA was rendered acid soluble by this treatment.

Administration of Polyoma Virus DNA to Hamsters. Newborn or suckling (1-day-old) golden Syrian hamsters were inoculated subcutaneously on the back and observed for 5 months for the development of tumors.

Establishment of Hamster Tumor Cell Lines. 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. After the cells reached confluence, they were cloned in microtiter trays by the end-point dilution technique.

Evaluation of Transformed Hamster Cells for T Ag. Hamster cell lines established from tumors induced by polyoma virus or polyoma virus DNA were labeled as described (19) with [^{35}S]methionine in Eagle's minimal essential medium lacking methionine. The labeled proteins were immunoprecipitated by either nonimmune hamster serum or hamster antiserum against polyoma T Ag in the presence of protein A-bearing *Staphylococcus aureus* and electrophoresed in 10% acrylamide gels as described (6, 19).

RESULTS

Polyoma virus DNA, unrestricted or cleaved with the restriction enzymes listed in Fig. 1, was inoculated subcutaneously into 1-day-old golden Syrian hamsters; the injected animals were observed for tumor development over a 5-month period. Except for *Bam*HI (20), the enzymes tested interrupt the viral genome at sites within the coding region for the large species of polyoma virus T Ag and leave the sequences specifying the small and middle T Ags intact (5, 6) (Fig. 1). *Eco*RI and *Bam*HI linearize the viral DNA by cleaving the genome only once (20, 24), in the early and late regions, respectively (23). Digestion with *Xba* I (22) cleaves the genome twice, the two cuts being close to one another in the large T Ag coding region; digestion with *Hin*cII plus *Hin*dIII (21) interrupts the viral genome at multiple sites (Fig. 1), but leaves intact a segment that includes the origin of replication and the proximal portion of the early region. In some experiments, viral DNA was cleaved with a single-cut restriction enzyme (*Eco*RI or *Bam*HI) and subsequently treated with S1 nuclease. The digestion with *Eco*RI and S1 nuclease was of particular interest because it would produce viral genomes in which the synthesis of large T Ag should be altered.

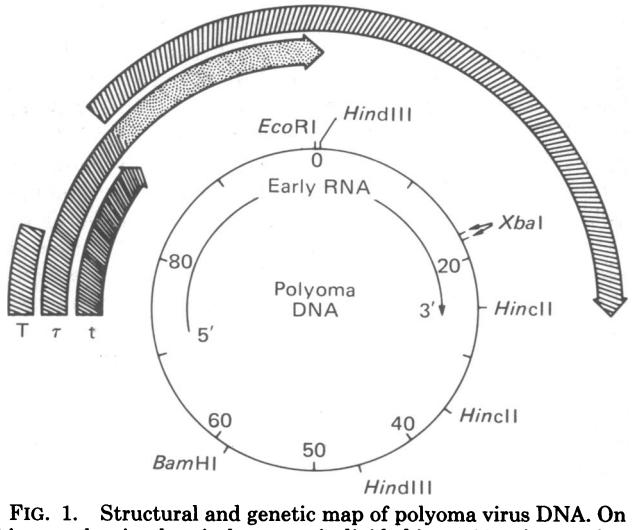

FIG. 1. Structural and genetic map of polyoma virus DNA. On this map the circular viral genome is divided into 100 units starting at the *Eco*RI site (20). The cleavage sites for *Hin*dIII plus *Hin*cII (21), *Xba* I (22), and *Bam*HI (20) are shown. The direction of transcription of early DNA is indicated (23). The large arrows outside the circle indicate the putative coding regions and reading frames (▨ or ▩) for small T (t), middle T (τ), and large T (T) antigens (5, 6).

In some cases, the infectivity of restricted DNA was assayed by parenteral inoculation of newborn or weanling mice, by the mouse antibody production test (16, 25). DNAs cleaved by *Eco*RI, *Eco*RI plus S1, and *Xba* I had approximately 1/5th, 1/100th, and 1/1000th the infectivity, respectively, of polyoma virus DNA I (16, unpublished observations). After cleavage with *Hin*cII plus *Hin*dIII or with *Bam*HI plus S1 nuclease, no residual infectivity could be detected (unpublished observations). Based on the amounts tested (0.3 μg of DNA cleaved by *Hin*cII plus *Hin*dIII and 0.8 μg of DNA cleaved by *Bam*HI plus S1 nuclease) and the known efficiency of infection of mice by full-length supercoiled or linear forms of polyoma virus DNA (16), it is clear that these enzymes cleaved more than 99.9% of the molecules.

As shown in Table 1, 19% of the hamsters inoculated with 0.5 μg of polyoma virus DNA I developed tumors during the 5-month observation period. In a previous study, we found that 100% of animals parenterally injected with 5×10^4 plaque-forming units of polyoma virions (approximately 2×10^{-4} μg of polyoma virus DNA) developed tumors by 4 months (16). Unexpectedly, linearized and fragmented polyoma virus DNAs were not only tumorigenic, but displayed a considerably enhanced tumorigenic potential compared to polyoma virus DNA I. Polyoma virus DNA cleaved by *Eco*RI or *Bam*HI, which were one-fourth to one-sixth as infectious as viral DNA I in the mouse antibody production test (16), induced tumors in 29 of 64 (45%) and 11 of 35 (31%) animals, respectively. Treatment of the cleaved molecules with S1 nuclease in order to decrease their ability to recircularize and simultaneously introduce deletions into the genome led to a further increase in tumorigenicity (Table 1). When polyoma virus DNA digested with *Hin*cII plus *Hin*dIII or *Xba* I was inoculated into hamsters, 85 and 100% of the animals, respectively, developed tumors (Table 1).

An important indicator of papovavirus-mediated tumorigenesis and cellular transformation has been the presence of viral T Ags. Originally detected by complement fixation tests (26, 27), T Ags were later identified in both productively infected and transformed cells by the indirect immunofluorescence technique (28). More recently, immunoprecipitation procedures have been used to analyze polyoma virus polypeptides present in infected or transformed cells (5, 6, 29). We previously reported that a polyoma virus hamster tumor cell line (PYT-54), originally isolated by Takemoto *et al.* (30) and selected for its uniquely high reactivity in immunofluorescence tests, contained all three species of viral T Ag (6). The observation that polyoma virus DNA interrupted in the distal portion

Table 1. Tumorigenesis by polyoma viral DNA

Inoculum	No. tumors/ no. inoculated	% tumors
PY DNA I	14/73	19
PY (*Eco*RI) DNA	29/64	45
PY (*Bam*HI) DNA	11/35	31
PY (*Eco*RI + S1) DNA	17/23	74
PY (*Bam*HI + S1) DNA	12/23	54
PY (*Hin*cII + *Hin*dIII) DNA	17/20	85
PY (*Xba* I) DNA	28/28	100
Saline	0/18	0
pMB9 DNA	0/27	0

One-day-old Syrian golden hamsters were inoculated subcutaneously with either polyoma virus (PY) DNA I or DNA cleaved with indicated enzymes (0.5 μg in 0.03 ml of phosphate-buffered saline) and observed for 5 months for the development of tumors.

of the early region was tumorigenic in newborn hamsters suggested that the virus-specific proteins detected in these tumors might differ from those found in tumors induced by polyoma virus or the viral DNA I. We therefore examined the [^{35}S]-methionine labeled proteins that could be immunoprecipitated from tumors induced by polyoma virions (two lines), polyoma virus DNA I (two lines), polyoma virus DNA treated with *Eco*RI and S1 nucleases (three lines), the viral DNA treated with *Xba* I (one line), and the viral DNA treated with *Hin*cII plus *Hin*dIII (one line), as well as from PYT-54. Except for PYT-54, the patterns of immunoprecipitable proteins in these lines were identical (Fig. 2). All of the tumor lines (Fig. 2 lanes B–D) contain the small and at least two species of middle T Ag; however, only the PYT-54 line contained the 105,000 M_r form of polyoma virus T Ag (Fig. 2 lanes A). We have previously shown that the 53,000 M_r species of the viral middle T Ag shares methionine tryptic peptides with the small and large forms of viral T Ag, while the 56,000 M_r species, readily detected in transformed cells, is unrelated by tryptic peptide analysis to any of the viral encoded early proteins (6). The 60,000 M_r polypeptide present in this fluorogram is a normal cell constituent (see Fig. 2 lane A-N and figure 1 of ref. 6).

The detection of abundant large T Ag in PYT-54 indicates that our antisera and assay conditions are capable of detecting the 105,000 M_r form of T Ag when present. The absence of large T Ag in cell lines established from tumors induced by cleaved polyoma virus DNA (e.g., *Eco*RI plus S1 nuclease, Fig. 2 lanes D) was not totally unanticipated in view of the location of the restriction enzyme cleavage site (Fig. 1). However, our failure to detect this antigen in any of the four virus- or DNA I-induced lines we established was quite surprising.

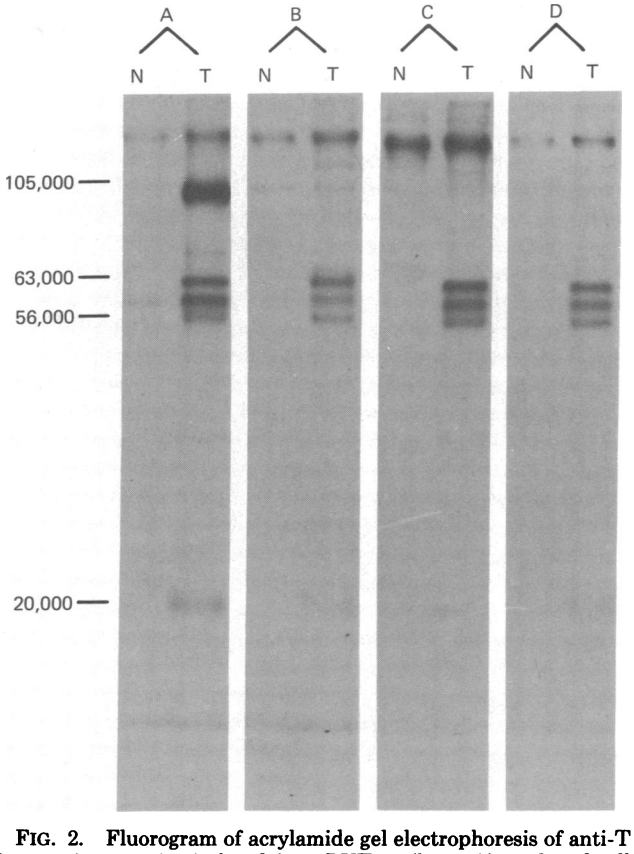

FIG. 2. Fluorogram of acrylamide gel electrophoresis of anti-T Ag reactive proteins isolated from PYT-54 (lanes A) or cloned cell lines established *in vitro* from hamster tumors induced by polyoma virions (lanes B), polyoma virus DNA I (lanes C), or polyoma virus DNA cleaved with *Eco*RI and treated with S1 nuclease (lanes D). Molecular weights are given. N, preimmune serum; T, tumor serum.

DISCUSSION

The mechanism of the enhanced tumorigenicity observed with polyoma virus DNA cleaved with restriction enzymes, particularly those that interrupt the distal portion of the early gene region, is unclear. Several possible explanations can be entertained, and more than one mechanism may be involved. The injection of linear rather than supercoiled viral DNA molecules might, in some fashion, facilitate cellular uptake or integration or might ensure that productive, cytolytic infection does not occur. This could explain the progressive enhancement observed with DNA digested with *Bam*HI and *Bam*HI plus S1 nucleases (Table 1). However, the increased tumorigenicity of restricted viral DNA appears to be related chiefly to the site of cleavage rather than to the molecular conformation of the DNA inoculum. Interruption of the polyoma virus genome in the distal portion of the early region may interfere with the synthesis of viral encoded or induced polypeptides that play a role in the immunologic recognition of tumor cells, such as the tumor-specific transplantation antigen. The absence of such antigens might allow transformed cells to escape immunologic surveillance and more rapidly develop tumors. By evaluating the ability of various subgenomic fragments of polyoma virus DNA to transform cells in tissue culture, we hope to examine the transformation event independent of the animal's immunologic reaction. Alternatively, it is conceivable that interruption of early gene sequences by restriction endonuclease digestion prior to animal inoculation may mimic a processing of the viral genome that ordinarily occurs intracellularly. In this regard, biochemical analyses indicate that the integrated viral genomes in cell lines derived from tumors induced by virus and DNA I contain deletions in the distal portion of the early region (unpublished data).

The absence of the 105,000 M_r species of polyoma virus T Ag in hamster tumor cell lines is further evidence for the existence of significant biochemical and functional differences in the early gene regions of simian virus 40 and polyoma virus. Although at least three viral encoded early proteins (large, middle, and small T Ags) have been identified in polyoma virus-infected cells (5, 6), the early region of simian virus 40 appears to encode only two polypeptides (large and small T Ags) (2). The polyoma virus-transformed hamster tumor cell lines we examined contained only the small and middle T Ags; none of the lines we established contained the large species of T Ag. PYT-54, a hamster tumor line selected several years ago for its ability to react strongly in an indirect immunofluorescence assay, contains all three species of polyoma virus T Ag. Simian virus 40-transformed rodent cell lines, on the other hand, invariably contain the large form of T Ag (31, 32). It is tempting to speculate that the large species (97,000 M_r) of simian virus 40 T Ag has at least two functional domains, with a single molecule carrying out the functions that the polyoma virus middle and large T Ags carry out separately. Because one of these antigens is invariably present in tumor cell lines and the other is generally absent, polyoma virus may provide a particularly attractive system for evaluating the role of T Ags in oncogenesis.

The results of both the tumorigenesis and the T Ag studies clearly indicate that large T Ag is not required for maintenance of the transformed state and probably not for initiation of transformation, at least under conditions of *in vivo* tumorigenesis. Our results do not exclude the possibility that polyoma virus large T Ag is transiently synthesized during an early stage of tumor induction after virus inoculation. The production of

this early gene product could be directed by unintegrated input viral genomes or by complete copies of integrated genomes from which certain sequences, mapping between 0 and 0.25 map units, are subsequently deleted. In those experiments involving restricted polyoma virus DNA preparations, we cannot be absolutely certain that a small amount of uncleaved viral DNA played a role in initiating tumorigenesis, but this possibility seems extremely remote. Tests evaluating the tumorigenicity of subgenomic polyoma viral DNA fragments cloned in *Escherichia coli* K-12, and thus consisting only of precisely defined gene segments, should clarify this point.

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