

# Origin of the Polyoma Virus-Associated Endonuclease<sup>1</sup>

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Endonuclease activity can be found associated with highly purified preparations of polyoma virus. Evidence has been obtained that this enzyme is not an integral part of the virus but is contributed by the fetal calf serum used in maintenance of polyoma-infected cells. This finding is based on: (i) the lack of virion-associated endonuclease activity when virus is produced using serum-free media and (ii) the production of polyoma antibody which neutralizes fetal calf serum endonuclease activity.

Nucleases have been found to be associated with the virions of Rous sarcoma virus (16), vaccinia virus (18), adenovirus (2), frog virus 3 (9), simian virus 40 (10, 11), and polyoma virus (5). The exact function of these enzymes in the viral life cycle is unknown. In the case of papovaviruses, a viral-associated nuclease may have a role in the replication of the viral genome (13, 20) or a possible function in recombinational events occurring between viral and cellular DNA molecules (1, 7, 12, 19, 22, 24, 25). Previous investigations have not determined if the polyoma-associated endonuclease is a viral-coded protein or a host-contributed enzyme. Our early studies demonstrated a very active endonuclease associated with highly purified polyoma virions which converts form I DNA (covalently closed, circular, duplex) into a nicked form, thus confirming the work of others (5). Studies on the origin of this enzyme suggest that it is contributed by the fetal calf serum (FCS) in which the infected cells are maintained.

## MATERIALS AND METHODS

**Virus propagation.** Primary cultures of mouse embryo or baby mouse kidney cells were prepared as described previously (21, 26). Wild-type polyoma (plaque purified) was used to infect cells at a multiplicity of 100. Infected cultures were maintained in Dulbecco modified Eagle medium (26) with 5% FCS or without serum.

**Virus purification.** Infected cells and medium were harvested 72 h after infection and centrifuged. Cell-free supernatant was reserved for concentrating virus. The cell pellet was disrupted in a Sorvall Omnimixer (rheostat setting of 100), and the homogenate was treated with receptor destroying enzyme (Microbiological Associates; 1:20 final dilution) for

3 h at 25 C. Cellular debris was removed by centrifugation at 10,000 rpm for 30 min. The clarified viral supernatant and the above medium were layered over 4 ml of 20% sucrose and centrifuged in a Spinco SW27 rotor at 25,000 rpm for 3 h. Pelleted virus was resuspended in a minimum volume of Tris-hydrochloride buffer (0.01 M, pH 7.4), layered over a preformed CsCl gradient (1.20 to 1.40 g/cm<sup>3</sup>), and centrifuged in an SW50.1 rotor (35,000 rpm, 16 h, 15 C). The two discrete virus bands, consisting of infectious particles (buoyant density, 1.33 g/cm<sup>3</sup>) and empty particles (buoyant density, 1.29 g/cm<sup>3</sup>) were collected separately and dialyzed against Tris-hydrochloride buffer (0.01 M, pH 7.4). The respective virion preparations were layered over a preformed shallow CsCl gradient (1.25 to 1.35 g/cm<sup>3</sup>) and centrifuged in an SW50.1 rotor (35,000 rpm, 2.5 h, 15 C). Fractions were collected, and appropriate fractions of infectious virions, light pseudovirions, and empty virions were pooled, diluted with Tris-hydrochloride buffer (0.01 M, pH 7.4) and centrifuged in an SW50.1 rotor (35,000 rpm, 2 h, 15 C). The pelleted virions were suspended in a minimum volume of Tris-hydrochloride buffer (0.001 M, pH 7.4) and stored at -20 C until used for the experiments described below.

**Polyoma DNA purification.** Polyoma DNA labeled with [<sup>3</sup>H]thymidine (30 μCi/ml of medium) was prepared as described by Chen et al. (3) except that the phenol-extracted and ethanol-precipitated DNA in the Hirt supernatant (8) was centrifuged in a 5 to 20% sucrose gradient (0.01 M Tris, pH 7.6-1 × 10<sup>-4</sup> M EDTA-0.01 M NaCl) in an SW27 rotor at 22,000 rpm and 4 C for 24 h. The polyoma component I DNA was isolated, dialyzed, and stored at -20 C until used.

**Endonuclease assays.** Endonuclease activity with polyoma DNA as substrate was measured by the nitrocellulose filter disk assay described by Kidwell et al. (11) or by analysis of DNA degradation in alkaline sucrose gradients. In the membrane filter assay, polyoma DNA which has received an endonucleolytic scission is converted to single-stranded DNA by treatment with alkali, followed by reneutralization. The single-stranded DNA is retained by the filter, whereas non-enzyme-treated DNA is

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not. The standard reaction mixture (0.2 to 0.3 ml) contained  $^3\text{H}$ -labeled polyoma DNA ( $10^4$  to  $1.5 \times 10^4$  counts/min),  $6.6 \times 10^{-3}$  M  $\text{MgCl}_2$ , 0.01 M  $\beta$ -mercaptoethanol, 0.05 M Tris-hydrochloride (pH 8.0), and 100  $\mu\text{g}$  of bovine serum albumin. Enzyme, either polyoma virus (5 to 10  $\mu\text{g}$  of protein) or FCS, was added, and incubation was carried out at 37 C. Mercaptoethanol was omitted from the reaction mixture when the polyoma antibody studies were performed.

**Alkaline sucrose gradient analysis.** The endonucleolytic degradation of polyoma DNA was also measured by alkaline sucrose gradient analysis. The endonuclease reaction (described above) was terminated by the addition of EDTA and NaOH to a final concentration of 0.1 M. Samples were layered over 5-ml linear alkaline sucrose gradients (5 to 20% sucrose in 0.3 M NaOH-0.7 M NaCl-0.001 M EDTA) and centrifuged for 3 h in an SW50.1 rotor at 35,000 rpm and 10 C. Fractions were collected directly into aqueous scintillation fluid, and radioactivity was determined.

**Immunological studies.** Antisera used in neutralization studies were prepared by immunization of rabbits with purified polyoma virion preparations that were obtained from infected cells maintained in medium with or without FCS. Rabbits received three footpad injections of purified virions mixed with an equal volume of Freund adjuvant. Hyperimmune sera were collected 2 weeks after the last injection. Polyoma antibody was quantitated by hemagglutination-inhibition and plaque-reduction tests. Endogenous endonuclease activity in the rabbit sera was inactivated by heating at 70 C.

**Other methods.** Infectivity of the virus preparations was determined by the plaque assay (PFU; 4). Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as the standard. Radioactivity was measured in a Beckman LS 233 liquid scintillation counter.

## RESULTS AND DISCUSSION

Polyoma virions obtained from infected cells maintained in medium with FCS contain endonuclease activity (Fig. 1C and 2). With the nitrocellulose membrane filter assay the reaction is linear with increasing protein concentration, requires  $\text{Mg}^{2+}$ , and has a pH optimum of 8.0 (data not shown). Comparable levels of endonuclease activity are also present in purified pseudovirions and empty capsids. These results are in agreement with other workers who have demonstrated the presence of endonuclease activity associated with purified polyoma (5). We have carried out additional studies to determine the origin of the virus-associated enzyme.

We have previously shown that purified polyoma virus obtained from infected cells maintained in medium with FCS and radioactively labeled *in vitro* contains additional proteins on sodium dodecyl sulfate-polyacrylamide gels as

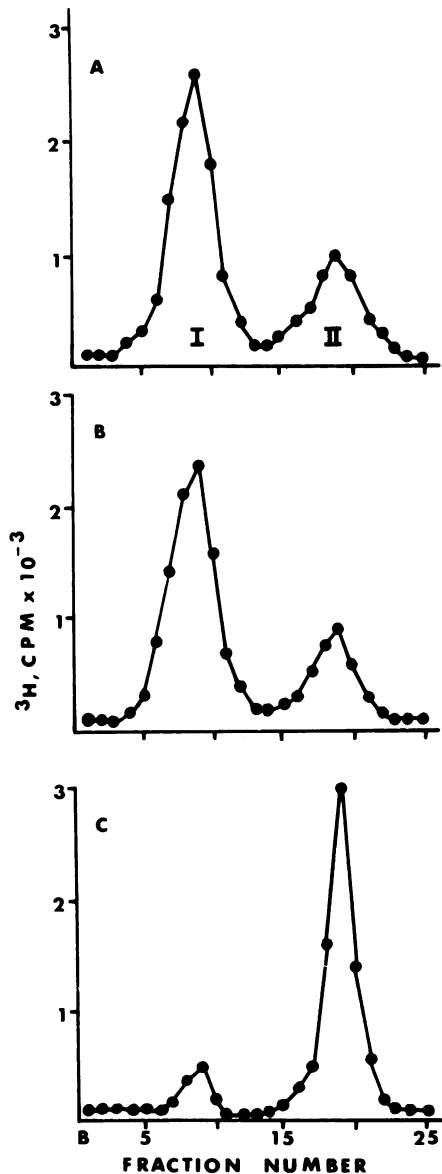


FIG. 1. Degradation of polyoma DNA by polyoma virions as determined by alkaline sucrose gradient analysis. Polyoma virions (9.5  $\mu\text{g}$  of protein) were incubated in the standard reaction mixture for 60 min at 37 C. At the end of the incubation period, polyoma DNA degradation was determined by alkaline sucrose gradient analysis as described. (A)  $^3\text{H}$ -labeled DNA incubated in the absence of polyoma virions; (B)  $^3\text{H}$ -labeled DNA incubated in the presence of purified polyoma virions grown in the absence of FCS; (C)  $^3\text{H}$ -labeled DNA incubated in the presence of purified polyoma virions grown in the presence of FCS.

compared to virus grown in serum-free media (15). It was found that in addition to the seven viral polypeptides (three capsid proteins and

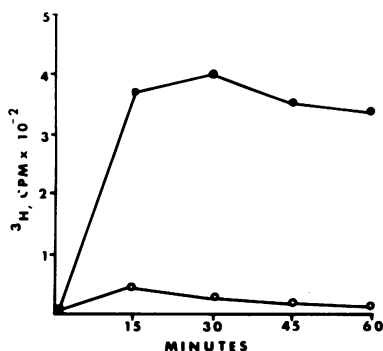


FIG. 2. Polyoma virus-associated endonuclease activity as determined by the nitrocellulose membrane filter assay. Polyoma virions (9.5  $\mu\text{g}$  of protein) were incubated in the standard reaction mixture, and at the times indicated endonuclease activity was determined by the nitrocellulose membrane filter method. Symbols: ●,  $^3\text{H}$ -labeled DNA incubated in the presence of polyoma virions grown in the presence of FCS; ○,  $^3\text{H}$ -labeled DNA incubated in the presence of polyoma virions grown in the absence of FCS.

four host histones), there were five additional peaks of radioactivity. However, *in vitro* labeling of purified virus grown under serum-free conditions resulted in a gel profile which lacked the five extraneous proteins, suggesting that the additional proteins were contributed by the serum. These findings prompted us to determine if the polyoma virus-associated endonuclease was derived from the fetal calf serum used in maintaining the host cells.

As shown in Fig. 1C, polyoma virus obtained from infected cells maintained in serum was capable of degrading polyoma DNA. Virus obtained from cells grown under serum-free conditions was devoid of nuclease activity (Fig. 1B). Similar results were also obtained when endonuclease activity was measured by the membrane filter assay (Fig. 2).

Table 1 presents a tabulation of the results obtained when endonuclease activity was determined with various purified polyoma virus preparations obtained under different growth conditions or after various treatments. Endonuclease activity was determined on alkaline sucrose gradients and is expressed as the percentage of conversion of polyoma component I to component II DNA. When purified virion preparations were obtained from infected cells maintained in medium with FCS, the preparations had an infectious titer of  $4.5 \times 10^{10}$  PFU and were found to have high endonuclease activity. Purified virions obtained from infected cells maintained under serum-free conditions had an infectious titer of  $3.2 \times 10^{11}$  PFU but lacked virion-associated endonuclease activity. Fifteen

different preparations of virus grown in serum-free medium were examined, and all lacked nuclease activity. When the serum-free virions of Table 1, line 2, were reacted for 4 h with commercial bovine DNase (Worthington; 20 units) and then rebanded to equilibrium in a CsCl gradient, the virion preparation contained endonuclease activity, indicating that the commercial nuclease binds to the polyoma proteins (Table 1, line 3). Fifteen different lots of both heat-inactivated (56 C, 30 min) and noninactivated FCS were found to be extremely active in endonuclease activity (Table 1, line 4). When endonuclease-inactive purified polyoma virion preparations were incubated with FCS for 1 h and then rebanded to equilibrium in a CsCl gradient, the previously inactive viral preparation acquired some (11%) endonuclease activity (Table 1, line 5). Polyoma virion proteins appear to have a nonspecific affinity for bovine nuclease. However, this affinity varies with different lots of FCS, since some endonuclease-positive sera did not produce polyoma preparations with endonuclease activity. The reason for this observation is not known at the present time. It was also found that an ATP-dependent endonuclease (6) was not associated with purified preparations of polyoma virions, nor was  $\text{Ca}^{2+}$  required for enzyme activity (17) (data not shown).

Figure 3 demonstrates an experiment in which polyoma antisera, produced against purified polyoma virions that were grown either in the presence or in the absence of FCS (each containing 800 hemagglutination-inhibition units), were used to determine if either could inhibit endogenous endonuclease activity in FCS. Serum endonuclease activity on [ $^3\text{H}$ ]thymidine-labeled polyoma DNA was determined over a 15-min incubation period by the nitrocellulose filter method. As can be seen there

TABLE 1. Virion-associated endonuclease as determined by alkaline sucrose gradients

Sample <sup>a</sup>	Growth conditions	Component II DNA (%) <sup>b</sup>
Virions ( $4.5 \times 10^{10}$ PFU)	+ FCS	55
Virions ( $3.2 \times 10^{11}$ PFU)	- FCS	0
Virions + DNase	- FCS	40
FCS (15 lots)		70
Virions + FCS	- FCS	11

<sup>a</sup> Average of 5 to 15 different preparations.

<sup>b</sup> The percentage of component II DNA after subtracting the percentage of control (not treated with enzyme).

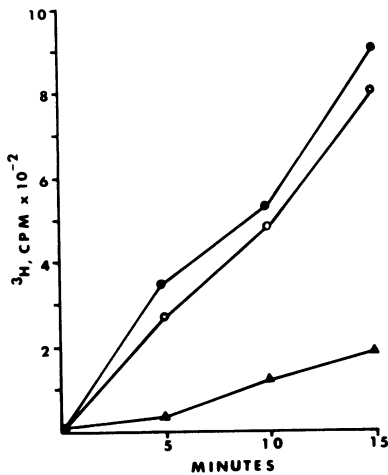


FIG. 3. Neutralization of FCS endonuclease by polyoma antibody. FCS (endonuclease) was incubated with 800 HAI units of polyoma antibody prepared against serum-free or serum-grown virus for 1 h at 37 C and 45 min at 4 C. Reaction mixture containing <sup>3</sup>H-labeled polyoma DNA was added, and incubations were carried out at 37 C. At the times indicated, samples were removed and assayed for endonuclease activity by the nitrocellulose filter method. Symbols: ●, FCS endonuclease; ○, FCS endonuclease incubated with antibody against serum-free polyoma virions; ▲, FCS endonuclease incubated with antibody against polyoma virions grown in the presence of FCS.

was little antibody neutralization of FCS endonuclease when it was reacted with polyoma antibody prepared against serum-free virions. However, when FCS was incubated with the polyoma antibody prepared against virions grown in the presence of serum, there was a marked neutralization of nuclease activity. Essentially identical results have also been obtained by analyzing the degradation of polyoma DNA in alkaline sucrose gradients (data not shown).

The results of these studies therefore suggest that the endonuclease activity associated with highly purified polyoma virus is neither viral coded nor contributed by the host but is rather an enzyme contributed by the FCS used in the maintenance of polyoma-infected cells. Whether the serum endonuclease has a role in the life cycle of polyoma remains to be determined. In the present study we have also shown that exogenous DNase has an affinity for polyoma virion proteins. Tan and McAuslan (23) have also found that highly purified poxvirus can adsorb DNA polymerase from cytoplasmic extracts. These findings demonstrating that purified virions can adsorb exogenous proteins should be considered when

interpreting data which may suggest that virion-associated enzymes are viral coded.

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