Endonuclease Activity Associated with Purified Simian Virus 40 Virions

JOAN C. KAPLAN, SHARON M. WILBERT, AND PAUL H. BLACK

Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Purified simian virus 40 has associated with it an endonuclease activity which converts form I (double-stranded, circular) simian virus 40 deoxyribonucleic acid to a nicked form that sediments as a homogeneous peak in alkaline sucrose gradients. The enzyme is dependent on magnesium ions for activity and is completely inhibited by ethylenediaminetetraacetic acid (0.02 M) or heat (80 C for 10 min). In tris(hydroxymethyl)aminomethane-hydrochloride buffer it exhibits optimal activity between pH 6.7 and 7.1 at 37 C. Gel electrophoretic analysis of purified, disrupted virus indicates the absence of detectable host cell protein contamination.

Simian virus 40 (SV40) is an oncogenic virus which contains double-stranded, closed, circular deoxyribonucleic acid (DNA) with a molecular weight of 3.2×10^6 daltons (4) and six structural polypeptides (5). It has been shown in certain species of cells transformed by SV40 that the viral DNA is covalently integrated into host DNA (6, 15), but infectious virus is not synthesized. However, infectious virus can be induced from these cells by co-cultivation techniques and, from certain hamster lines, by physical and chemical agents as well (2, 3, 7, 14). Although the precise molecular events associated with integration and induction have not been delineated, the most likely model would involve a process of genetic recombination for integration of the viral DNA and a reversal of this process for excision of the viral genome. Such mechanisms are operative in lysogenic bacteria and are dependent on nucleases which are specified by the viral genome (16).

Nucleases have been found to be associated with the virions of several animal viruses. Vaccinia virus contains an endonuclease which is optimally active on single-stranded DNA at pH 7.8 (11). In addition, purified pentons of adenovirus type 2 make double-strand scissions in native DNA (1a). It has also been suggested by Mizutani et al. that Rous sarcoma virus (RSV) contains an endonuclease which hydrolyzes T7 DNA (9). However, other workers have been unable to detect significant endonuclease activity associated with similar strains of RSV (12). Exonuclease activities have also been found in vaccinia virus (11) and RSV (10, 12) preparations. Therefore, we have examined purified preparations of SV40 virus for nuclease

activity. We report here that an endonuclease activity is associated with purified virions which converts form I (double-stranded, closed, circular, supercoiled) DNA into a nicked form. (It is not known, at present whether the endonuclease makes single- or double-strand scissions in native SV40 DNA. In this report nicked form will indicate either or both reaction products because both forms have similar sedimentation velocities under the conditions of our alkaline sucrose gradients.)

MATERIALS AND METHODS

Purified SV40 virus served as the enzyme source. Lysates of SV40 virus-infected MA-134 monkey kidney cells were purified by two different procedures. In the first, virus was banded twice in CsCl density gradients following preliminary purification by the method of Black et al. (1). The second method involved a preliminary banding to equilibrium in saturated KBr solutions prior to banding in CsCl density gradients (13). Each purified virus preparation had a titer of approximately 10^9 plaque-forming units per ml and a protein concentration of 450 μg/ml. Concentrations of SV40 were estimated by using the relationship established by Koch (8), i.e., 3.85 × absorbance at 260 nm = 1.0 mg of virus. It was estimated that the viral protein equals 87% of the absolute amount of virus. Purified virus was stored at −70 C and was utilized after one to four cycles of freezing and thawing.

The substrate for the endonuclease assay was tritium-labeled form I SV40 DNA. Virus was grown in MA-134 cells in medium containing tritiated thymidine (New England Nuclear Corp., NET-027X) with a specific activity of 20 Ci/mmole) at a concentration of 2.0 μCi/ml. Viral harvests were purified (1) and the DNA was extracted by exposure

to 1.0% sodium dodecyl sulfate (SDS) and heated at 50 C for 30 min (17). Form I DNA was isolated following sedimentation in a neutral sucrose gradient (15 to 30%, w/v) for 19 hr at 23,000 rev/min at 10 C. Fractions of the form I DNA peak, sedimenting at 21S, were pooled and dialyzed against 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0 (1.0 × SSC). This DNA preparation had a final specific activity of 7.0 × 10⁴ counts per min per μg.

The SV40 endonuclease activity was assayed by measuring the percentage of conversion of tritium-labeled form I DNA into a nicked form. The reaction mixture contained, in 0.2 ml, tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.1 at 37 C (2.0 μmoles), magnesium chloride (0.4 μmole), ³H-labeled DNA (0.4 μg, 10 to 30,000 counts/min), and SV40 virus (equivalent to 1 to 20 μg of virus protein). The reaction mixture was incubated at 37 C for 60 min. At the end of the incubation period, 4.0 μmoles of ethylenediaminetetraacetic acid (EDTA) was added to the sample and the tube was chilled in ice. The entire sample was sedimented through a linear alkaline sucrose gradient (10 to 30%, w/v; 3.4 ml) containing NaCl (0.5 M), NaOH (0.3 N), and EDTA (0.01 M) for 2 hr at 55,000 rev/min at 5 C in an SB405 International rotor. The gradient was fractionated into seven-drop fractions. Carrier bovine serum albumin (0.2 mg) was added to the sample which was then precipitated with cold 5% trichloroacetic acid. The precipitate was collected on a glass fiber disc (Reeve Angel Co.) which was washed three times with cold 5% trichloroacetic acid and dried. Radioactivity was determined by counting the disc in toluene scintillation fluid (10 ml) containing Omnifluor, 4 g/liter (New England Nuclear Corp.) in a Packard 3320 liquid scintillation counter. An alternate method involved fractionating the gradient directly into scintillation vials containing 0.2 ml of HCl (0.15 N). Bray's scintillation fluid (10 ml) was added to each vial, and the radioactivity was determined.

RESULTS AND DISCUSSION

Figure 1 illustrates the results of a typical endonuclease assay. Form I DNA was separated from the nicked form by at least three fractions. The conversion product sedimented as a sharp peak, indicating that random breakage did not occur after treatment of form I DNA with the purified virus. In a parallel experiment, in which the fractions were not precipitated with trichloroacetic acid but instead were collected directly into scintillation vials, the sedimentation profiles were identical, indicating that no acid-soluble material was being released from the form I DNA following incubation with purified virus. Conversion of form I DNA to a nicked form was quantitated by measuring the percentage of total DNA which sedimented in the region of the nicked form. The percentage of nicked DNA in the substrate DNA preparations ranged from 10 to 15%, which presumably was due to spon-
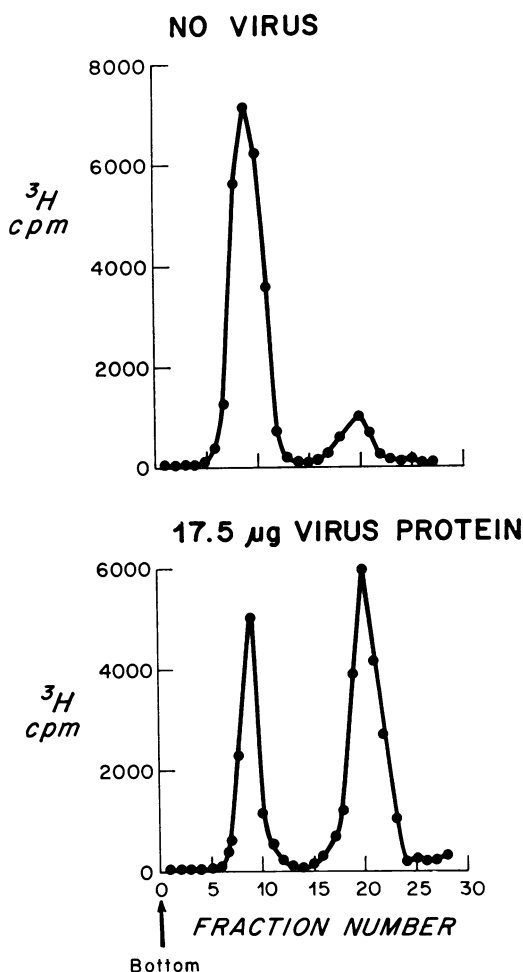

FIG. 1. Cleavage of form 1 simian virus 40 (SV40) ³H-DNA with SV40 endonuclease. SV40 ³H-DNA (form I, specific activity = 7 × 10⁴ counts per min per μg, 0.4 μg) was incubated at 37 C for 60 min in 0.2 ml of reaction mixture either with no enzyme or 20.0 μg of SV40 virus (equivalent to 17.5 μg of virus protein). The reaction mixture also contained 2.0 μmoles of Tris-hydrochloride, pH 7.1 at 37 C, and 0.4 μmoles of MgCl₂. The reaction was terminated by the addition of EDTA (4.0 μmoles), and the samples were sedimented in linear alkaline sucrose gradients as described in the text.

taneous conversion of form I to the nicked form. This percentage did not increase in control incubations at 37 C in which exogenous virus protein was not included.

Unless otherwise indicated, the enzyme source for the following experiments was virus purified by the method of Black et al. (1). Some of the requirements for the endonuclease activity are listed in Table 1. The reaction is dependent on magnesium ions. When EDTA, at a concentra-

tion of 0.02 M, was added to the incubation mixture, a 100% inhibition of the nuclease activity was observed. Preheating purified virus at 80 C for 10 min completely inactivated the endonuclease activity. In addition, when SV40 endonuclease activity was tested in Tris-hydrochloride buffer in the range from pH 6.7 to 8.5 at 37 C, it was found to be active at all pH values. However, the enzyme demonstrated maximum activity between pH 6.7 and 7.1.

The time course of the SV40 endonuclease reaction is shown in Fig. 2. The rate of reaction was linear up to 15 min, at which time 49% of form I DNA had been converted to a nicked form. The conversion occurred rapidly and leveled off at 97% conversion by 120 min. The rate of reaction decreased after 15 min, presumably because of thermal inactivation of the enzyme under conditions of the incubation. The rate curve did not pass through zero because the substrate DNA

TABLE 1. *Requirements for the simian virus 40 (SV40) endonuclease activity reaction system*[a]

Components	Conversion (%)
Complete.........................	86
Minus Mg^{2+} (0.002 M)...............	27
Minus Mg^{2+} plus EDTA (0.02 M)....	15
Heated virus........................	17
Minus virus........................	16

[a] The complete standard reaction mixture (0.2 ml) contained 0.4 µmole of $MgCl_2$, 2.0 µmoles of Tris-hydrochloride buffer (pH 7.1 at 37 C), ³H-labeled form I SV40 DNA (0.4 µg, 28,000 counts/min), and 20.0 µg of SV40 virus (equivalent to 17.5 µg of virus protein). The mixture was incubated at 37 C for 60 min and processed as described in the text.
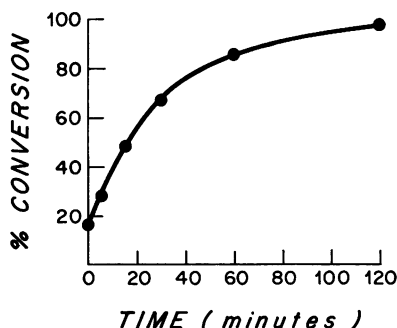
contained 15% of the nicked form before incubation was initiated.

In Fig. 3 the virus concentration dependence of the endonuclease activity is shown. The activity was proportional to the amount of enzyme added in that a linear relationship existed between the concentration of SV40 virus protein included in the assay and the percentage of conversion of form I DNA into the nicked form. Similar results were also obtained utilizing virus which was purified by the KBr method (13).

The data presented here indicate that purified SV40 virus has associated with it an endonuclease which is capable of cleaving double-stranded, closed, circular (form I) DNA. It is possible that host cell protein contamination is responsible for the endonuclease activity described. In an effort to insure against the presence of contaminating host cell proteins, the virus was extensively purified by two different procedures. The KBr method has been shown to be effective, by mixing experiments, in resolving polyoma virus (13) and SV40 virus (R. Roblin, *personal communication*) from contaminating host cell proteins. Virus purified by the KBr method was subjected to acrylamide gel (10%) electrophoresis following disruption at 100 C in 10% SDS as described previously (13). The electrophoretic pattern resembled those previously published for SV40 (5) and indicated no detectable contaminating host cell protein within the limits of resolution of this gel technique.

Since no acid-soluble nucleotides were released from form I DNA by this nuclease preparation, under the conditions of standard assay, it is unlikely that it contains an exonuclease. However, it is not yet known whether the enzyme makes single- or double-strand scissions in form I DNA. The sharpness of the nicked-form peak in the alkaline sucrose gradients suggests that the reac-

FIG. 2. *Time course of simian virus 40 (SV40) endonuclease reaction. SV40 was present at 20.0 µg/reaction mixture (equivalent to 17.5 µg of virus protein). Assay conditions were as described in the text. At the indicated time periods, the samples were removed from incubation and processed as described.*
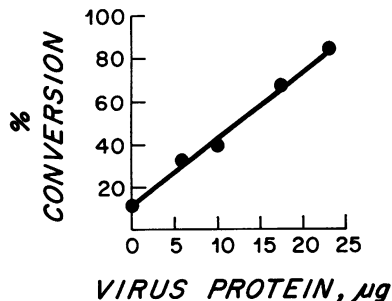

FIG. 3. *Proportionality of endonuclease activity with simian virus 40 (SV40) concentration. SV40 virus concentration ranged from 2.0 to 20.0 µg/reaction mixture (equivalent to 1.75 to 17.5 µg of virus protein). Assay conditions were as described in the text.*

tion product does not result from random cleavage of form I DNA. Studies are being conducted to characterize more definitively the reaction product(s), substrate, and site specificity of this endonuclease. In addition, the location of the enzyme within the SV40 virion is being examined.

ACKNOWLEDGMENTS

ADDENDUM IN PROOF

Since our manuscript was accepted for publication, it has been brought to our attention that a similar endonuclease activity has been found in association with purified polyoma virus: F. Cuzin, D. Blangy, and P. Rouget. 1971. Activité endonucléastique de préparations purifiées du virus du polyome. C.R. Acad. Sci. 273:2650–2653.

LITERATURE CITED

1. Black, P. H., E. M. Crawford, and L. V. Crawford. 1964. The purification of simian virus 40. Virology 24:381–387.

1a. Burlingham, B. T., and W. Doerfler. 1971. Adenovirus endonuclease: association with the penton of adenovirus type 2. J. Mol. Biol. 60:45–64.

2. Burns, W. H., and P. H. Black. 1968. Analysis of simian virus 40-induced transformation of hamster kidney tissue in vitro. V. Variability of virus recovery from cell clones inducible with mitomycin C and cell fusion. J. Virol. 2:606–609.

3. Burns, W. H., and P. H. Black. 1969. Analysis of SV40-induced transformation of hamster kidney tissue in vitro. VI. Characteristics of mitomycin C induction. Virology 39:625–634.

4. Crawford, L. V., and P. H. Black. 1964. The nucleic acid of simian virus 40. Virology 24:388–392.

5. Estes, M. K., E. Huang, and J. S. Pagano. 1971. Structural polypeptides of simian virus 40. J. Virol. 7:635–641.

6. Hirai, K., J. Lehman, and V. Defendi. 1971. Integration of simian virus 40 deoxyribonucleic acid into the deoxyribonucleic acid of primary infected Chinese hamster cells. J. Virol. 8:708–715.

7. Kaplan, J. C., S. M. Wilbert, and P. H. Black. 1972. Analysis of simian virus 40-induced transformation of hamster kidney tissue in vitro. VIII. Induction of infectious simian virus 40 from virogenic transformed hamster cells by amino acid deprivation or cycloheximide treatment. J. Virol. 9:448–453.

8. Koch, M. A., H. J. Eggers, F. A. Anderer, H. D. Schlumberger, and H. Frank. 1967. Structure of simian virus 40. 1. Purification and physical characterization of the virus particle. Virology 32:503–510.

9. Mizutani, S., D. Boettiger, and H. M. Temin. 1970. DNA-dependent DNA polymerase and DNA endonuclease in virions of Rous sarcoma virus. Nature (London) 229:427–428.

10. Mizutani, S., H. M. Temin, M. Kodama, and R. D. Wells. 1971. DNA ligase and exonuclease activities in the virions of Rous sarcoma virus. Nature (London) 230:232–235.

11. Pogo, B. G. T., and S. Dales. 1969. Two deoxyribonuclease activities within purified vaccinia virus. Proc. Nat. Acad. Sci. U.S.A. 63:820–827.

12. Quintrell, N., L. Fanshier, B. Evans, W. Levinson, and J. M. Bishop. 1971. Deoxyribonucleic acid polymerase(s) of Rous sarcoma virus: effects of virion-associated endonuclease on the enzymatic product. J. Virol. 8:17–27.

13. Roblin, R., E. Harle, and R. Dulbecco. 1971. Polyoma virus proteins. 1. Multiple virion components. Virology 45:555–566.

14. Rothschild, H., and P. H. Black. 1970. Analysis of SV40 induced transformation of hamster kidney tissue in vitro. VII. Induction of SV40 virus from transformed hamster cell clones by various agents. Virology 42:251–256.

15. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40-transformed cells. Proc. Nat. Acad. Sci. U.S.A. 60:1288–1295.

16. Shuster, R. C., and A. Weissbach. 1968. Evidence for a new endonuclease synthesized by λ bacteriophage. J. Virol. 2:1096–1101.

17. Thorne, H. V. 1967. Electrophoretic characterization and fractionation of polyoma virus DNA. J. Mol. Biol. 24:203–211.