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Identification of Small DNA Fragments Synthesized in Herpes Simplex Virus-Infected Cells in the Presence of Acyclovir

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The effect of acyclovir on DNA synthesized in cells infected with herpes simplex virus type 1 was examined. DNA that was synthesized in infected cells in the presence of acyclovir during a short pulse with [^{3}H]thymidine remained near the top of an alkaline sucrose gradient after centrifugation. The sedimentation characteristics of labeled DNA were not changed after chasing in isotope-free medium. The slowly sedimenting DNA was identified as viral in origin by hybridization to purified herpes simplex virus nucleocapsid DNA. When cells were infected with acyclovir-resistant virus containing mutations in the polymerase gene, the viral DNA synthesized in the presence of acyclovir was chased into high-molecular-weight DNA. These findings are consistent with chain termination of herpes simplex virus DNA in virus-infected cells.

The nucleoside analog acyclovir (ACV) is a potent and selective inhibitor of herpes simplex virus (HSV) replication (3). Required for the inhibition of HSV replication is the selective phosphorylation of ACV by virus-specific thymidine kinase (3, 6) and the inhibition of the HSV-induced DNA polymerase activity by the triphosphorylated form of the drug (3, 5). It has recently been reported that the inhibition of HSV DNA polymerization in vitro by ACV triphosphate appears to be due to the incorporation of ACV monophosphate into the primer template DNA, in addition to a strong competitive inhibition of the viral DNA polymerase activity with respect to dGTP (2, 5). It was also shown that the 3′-terminal ACV monophosphate residues incorporated into the DNA template were not removed by HSV type (HSV-1) 1 DNA polymerase-associated exonuclease. In whole cells, ACV was incorporated only as a 3′-terminal residue in TK$^-$ cells transformed to the TK$^+$ phenotype with herpesvirus genetic information (4). Cellular DNA synthesized in transformed cells in the presence of ACV consisted of small fragments that were not chased into high-molecular-weight DNA, whereas the presence of ACV in cells without the HSV thymidine kinase gene did not produce these small fragments. It has yet to be demonstrated with HSV-infected cells that chain termination occurs in the presence of ACV. In this communication, we report that, in HSV-1-infected cells, the viral DNA synthesized in the presence of ACV consisted of small fragments incapable of being chased to high-molecular-weight DNA, as was observed in biochemically transformed cells without HSV. The small fragments of DNA in HSV-infected cells were shown to be of viral origin by DNA-DNA hybridization.

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Baby hamster kidney (BHK) cells were grown in Eagle minimal essential medium containing 10% bovine calf serum, 10% tryptose phosphate broth, 1× minimal essential medium vitamin solution, and 3.5 g of glucose per liter. Once confluent, cells were arrested by incubating in medium containing 0.5% fetal bovine serum, 0.5% tryptose phosphate broth, 0.1× minimal essential medium vitamin solution, and 0.35 g of glucose per liter for 36 to 48 h before infection.

Arrested BHK cells were infected with the KOS strain of HSV-1 at a multiplicity of infection of 10 PFU/cell, and the DNA in these infected cells was prelabeled from 6 to 9 h postinfection with [^{14}C]thymidine (1 μCi/ml, 51 mCi/mmol). At 9 h postinfection, prelabeled cells were washed to remove label and then exposed for 30 min to various concentrations of ACV (see Fig. 1). After the ACV was removed, cells were pulse-labeled for 30 min with [^{3}H]thymidine (10 μCi/ml, 60 Ci/mmol) in the absence of ACV. Before harvesting, cells were chased for 60 min in label-free medium. Cells were harvested as described previously (4). The lysates were gently added to the top of a 12.5-ml alkaline sucrose (0.8 M NaCl, 0.3 M NaOH, 0.001 M EDTA [pH 12.5]) gradient (10 to 20%, wt/vol) and centrifuged at 40,000 rpm for 3 h at 18°C in a Beckman SW41 rotor. Gradients were fractionated (0.3 [see Fig. 1] or 0.5 [see Fig. 2] ml per fraction) and analyzed as described previously (4).

^{3}H-labeled DNA from infected cells not exposed to ACV migrated to the same position in the gradient as did the high-molecular-weight DNA prelabeled with ^{14}C (Fig. 1). In contrast, [^{3}H]labeled DNA from infected cells exposed to ACV remained near the top of the gradient. There was no change in the position of the ^{3}H-labeled DNA after a 60-min chase in label-free medium, indicating that the DNA was not chased into high-molecular-weight DNA.

For identification of the origin of the ^{3}Hlabeled DNA remaining at the top of the gradient, the DNA was hybridized to purified nucleocapsid (7) and cellular DNA. HSV-1 DNA and BHK cell DNA were prepared from nucleocapsids and uninfected cells by phenol and chloroform-isoamyl alcohol extractions (7). The DNA was then purified by ethidium bromide-cesium chloride gradient centrifugation as described previously (8), except the density of the ethidium bromide-cesium chloride solution was adjusted to 1.576. Gradients were formed in a Sorvall TV-850 vertical rotor by spinning for 16 h at 45,000 rpm. Before hybridizations, the DNA was sheared and then denatured at 100°C for 10 min. The DNA was then attached to nitrocellulose filters with 6×

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TABLE 1. DNA-DNA hybridization[a]

DNA (μg) bound to nitrocellulose filters		Hybridized DNA (cpm)
BHK cells	HSV nucleocapsid	
16		506.8
32		602.2
80		842.4
16	7	3,274.6
16	14	8,443.6
16	28	13,456.6
16	73	18,494.2
16	110	15,138.2
16	147	13,339.0

[a] See text for details of experimental procedure.

SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and hybridized to the slowly sedimenting DNA obtained from the top of the alkaline sucrose gradients. After alkaline sucrose gradient sedimentation, fractions containing labeled DNA corresponding to the slowly sedimenting fragments were pooled, and a 1/3 volume of 7.5 M ammonium acetate was added. Fragments were then precipitated with ethanol. ^{3}Hlabeled fragments were resuspended in TE buffer (10 mM Tris [pH 7.4]–1 mM EDTA). All nitrocellulose filters (24 mm) with various amounts of DNA bound (see Table 1) were incubated in a single vessel in 6× SSC containing ^{3}H-terminated DNA, 100 μg of yeast tRNA per filter, and sodium dodecyl sulfate to 0.1%. Annealing was carried out at 66°C for 20 h. Nonhybridized, single-stranded DNA was removed by washing with 6× SSC, followed by incubation for 45 min at 50°C in 1.0 ml of 100 mM NaCl per filter–37 mM sodium acetate (pH 4.5)–0.1 mM $ZnSO_4$–25 μg of calf thymus DNA per ml containing 500 U of S1 endonuclease (Bethesda Research Laboratories, Inc.) per ml. Filters were then washed in 6× SSC and dried at room temperature. The filters were then assayed for radioactivity by liquid scintillation spectrometry. Minimal hybridization of ^{3}Hlabeled fragments occurred with increasing amounts of filter-bound cellular DNA, whereas significant levels of annealing were observed between labeled fragments and increasing amounts of filter-bound HSV-1 DNA (Table 1).

The sedimentation profiles of viral DNA synthesized in the presence of ACV in cells infected with ACV-resistant mutants PAA^{r}5 and BW^r are shown in Fig. 2. The DNA synthesized in cells infected with these polymerase mutant viruses migrated as high-molecular-weight DNA on alkaline sucrose gradients, whereas the DNA from cells infected with wild-type virus remained near the top of the gradient as slowly sedimenting DNA (Fig. 2). The absence of slowly sedimenting DNA in cells infected with the virus mutants suggests that these viral polymerases cannot utilize ACV triphosphate as a substrate as efficiently as can wild-type virus.

These data show that small DNA fragments are synthesized in HSV-infected cells in the presence of ACV and that they are viral in origin, as determined by hybridization to purified HSV nucleocapsid DNA. These findings are consistent with those reported previously in transformed cells in which cellular DNA was shown to be chain terminated (4). In studies with Epstein-Barr virus-associated DNA polymerase, it was concluded that a competitive mechanism is the major mode of ACV inhibition of Epstein-Barr virus replication and that chain termination may occur, but only at an extremely low frequency (1). Purified HSV DNA polymerase, chemically synthesized ACV triphosphate, and activated calf thymus DNA as template (1, 4) were used in previous studies, in which it was suggested that chain termination occurs in infected cells treated with ACV; however, this report is the first to demonstrate that small fragments of viral DNA, which cannot be chased to high-molecular-weight DNA, are present in HSV-infected cells after treatment with ACV, suggesting that viral DNA synthesized is chain terminated by the drug.

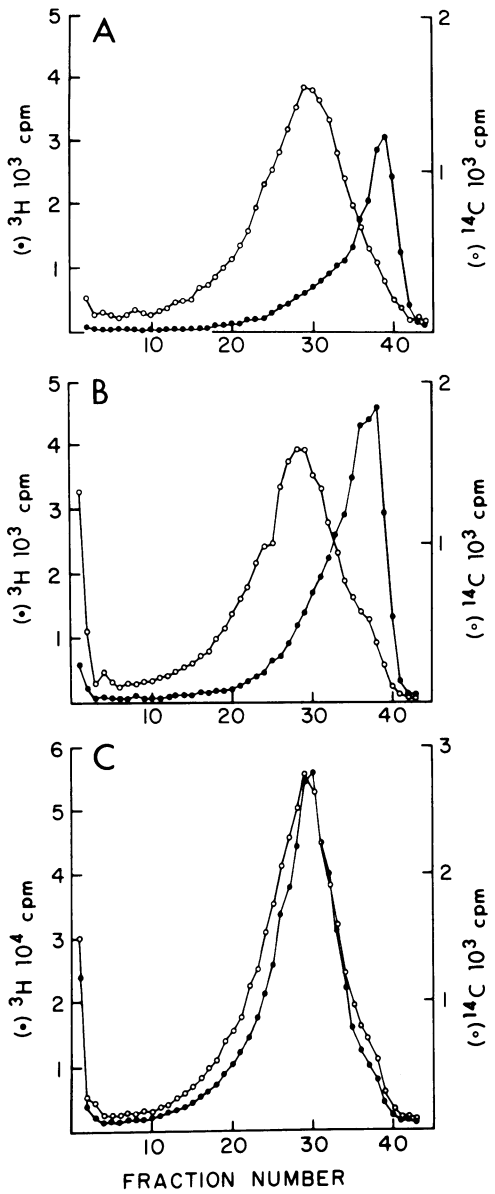

FIG. 1. Alkaline sucrose gradient sedimentation profiles of labeled DNA from HSV-infected cells. Growth-arrested BHK cells were infected as described in the text. Infected cells were prelabeled from 6 to 9 h postinfection with [^{14}C]thymidine (○). Prelabeled cells were then exposed to 50 (A), 25 (B), and 0 (C) μM ACV for 30 min, followed by a 30-min pulse-label of [^{3}H]thymidine (●) in the absence of ACV. The ^{3}H label was then chased for 60 min in label-free medium. When harvested, cell lysates were subjected to alkaline sucrose gradient centrifugation and analyzed. Sedimentation is from right to left.

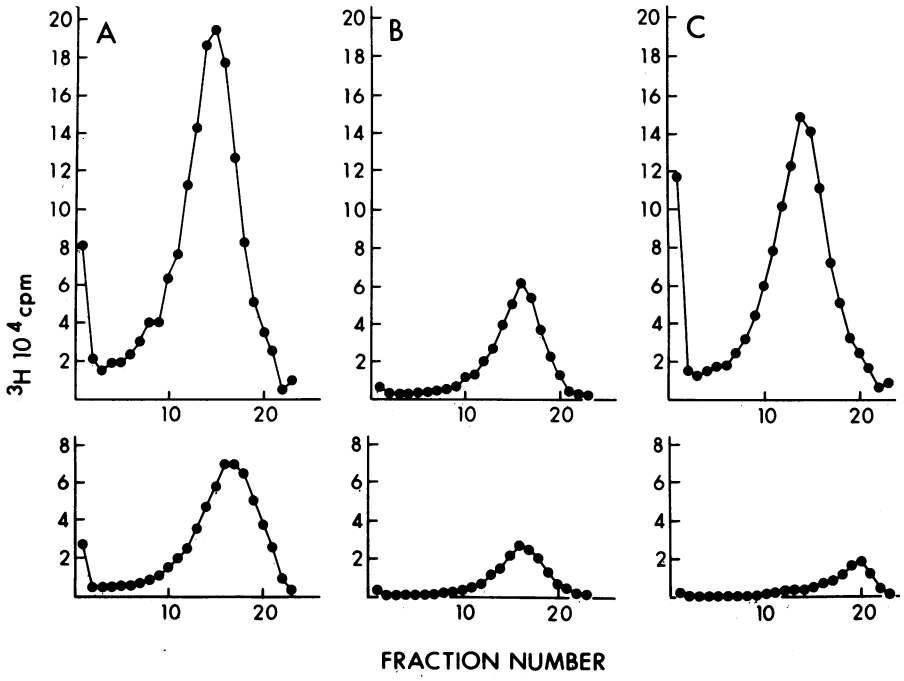

FIG. 2. Alkaline sucrose gradient sedimentation profiles of labeled DNA from cells infected with ACV-resistant mutant HSV and wild-type HSV. Growth-arrested BHK cells were infected as described in the text. Infected cells were exposed to 0 (top row) and 10 (bottom row) μM ACV from 9 to 9.5 h postinfection, followed by a 30-min pulse-label of [^{3}H]thymidine in the absence of ACV. The ^{3}H label was then chased for 60 min in label-free medium. When harvested, cell lysates were prepared and gently added to the top of an alkaline sucrose gradient. Gradients were fractionated and analyzed as described in the text. Sedimentation is from right to left. (A) PAA^{r}5-infected cells; (B) BW^r-infected cells; (C) KOS-infected cells.

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LITERATURE CITED

1. **Datta, A. K., B. M. Colby, J. E. Shaw, and J. S. Pagano.** 1980. Acyclovir inhibition of Epstein-Barr virus replication. Proc. Natl. Acad. Sci. U.S.A. **77**:5163–5166.
2. **Derse, D., Y. C. Cheng, P. A. Furman, M. H. St. Clair, and G. B. Elion.** 1981. Inhibition of purified human and herpes simplex virus-induced DNA polymerase by 9-(2-hydroxyethoxymethyl)guanine triphosphate. J. Biol. Chem. **256**:11447–11451.
3. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. deMiranda, L. Beauchamp, and H. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. U.S.A. **74**:5716–5720.
4. **Furman, P. A., P. V. McGuirt, P. M. Keller, J. A. Fyfe, and G. B. Elion.** 1980. Inhibition by acyclovir of cell growth and DNA synthesis of cells biochemically transformed with herpes virus genetic information. Virology **102**:420–430.
5. **Furman, P. A., M. H. St. Clair, J. A. Fyfe, J. L. Rideout, P. M. Keller, and G. B. Elion.** 1979. Inhibition of herpes simplex virus-induced DNA polymerase activity and viral DNA replication by 9-(2-hydroxyethoxymethyl)guanine and its triphosphate. J. Virol. **32**:72–77.
6. **Fyfe, J. A., P. M. Keller, P. A. Furman, R. L. Miller, and G. B. Elion.** 1978. Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. J. Biol. Chem. **253**:8721–8727.
7. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8**:125–132.
8. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual, p. 93–94. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.