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Suppression of Apoptotic DNA Fragmentation in Herpes Simplex Virus Type 1-Infected Cells

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HEp-2 cells underwent apoptosis when the cells were incubated in medium containing sorbitol. Infection with herpes simplex virus type 1 (HSV-1) prior to the sorbitol treatment suppressed this apoptosis completely, indicating that HSV-1 carries an antiapoptosis gene. In addition, HSV-1 multiplication was restricted in these apoptotic HEp-2 cells.

Apoptosis or programmed cell death (PCD) is one type of animal cell death in which cells die by an active cellular process under the genetic control of the cells ("death program") (16). Although many animal viruses are known to induce an apoptotic response in infected cells (5, 23, 25), it has not been known whether herpes simplex virus type 1 (HSV-1) could induce apoptosis. Cells infected with wild-type HSV-1 do not show the typical features of apoptosis, e.g., fragmentation of chromosomal DNA into nucleosomal oligomers and characteristic changes in the morphology of cell nuclei (16). However, the lack of apoptosis in HSV-1-infected cells has been considered to be the result of a viral antiapoptosis gene which inhibits some step in the cellular response toward apoptosis. This idea is supported by the finding that mutant viruses which lack the γ34.5 gene induce PCD in infected human neuroblastoma cells (1) or human foreskin cells (2). Although this mutant virus could grow normally and does not induce PCD in Vero cells or in HEp-2 cells (1), this result has been interpreted as the γ34.5 gene product suppressing HSV-1-induced PCD in a cell type-specific manner (1, 2). Later, this PCD by γ34.5-deficient mutant virus was found to be achieved by the phosphorylation of eukaryotic translation initiation factor eIF-2α and premature shutoff of protein synthesis (3).

The HEp-2 cell line was established from epidermoid carcinoma tissue from the larynx of a male Caucasian (21). One of us found that HEp-2 cells undergo apoptosis when the cells are incubated in medium containing 1 M sorbitol (20a). To examine whether HSV-1 carries, other than the γ34.5 gene, an antiapoptosis gene which can suppress the apoptotic response in infected cells, we characterized the effect of HSV-1 infection on the induction of apoptotic DNA fragmentation in HEp-2 cells.

HEp-2 cells were grown in minimal essential medium with Earle's salts supplemented with 10% newborn bovine serum. HSV-1 strain HF was used throughout the experiments except those with γ34.5 mutant viruses of strain F. HSV-1 strain F and its derivatives [R3616, R4009, and HSV-1(F)R] were kindly provided by B. Roizman (University of Chicago, Chicago, Ill.). Virus infection was carried out by essentially the same procedure as that described previously (18).

To determine the degree of DNA fragmentation, approximately 3×10^6 cells were incubated in medium containing 1 M sorbitol for 1 h at 37°C and then maintained in sorbitol-free medium for 3 h. The medium was removed, and attached cells were resuspended with 0.05% trypsin and added back to the removed medium. Cells were pelleted at $2{,}000 \times g$ for 20 s and resuspended in 100 μl of phosphate-buffered saline. Fragmented DNA was extracted by the Hirt method (13), with minor modifications. Briefly, the cell suspension was lysed by adding 400 μl of TE buffer (10 mM Tris HCl [pH 7.4]—10 mM EDTA) containing 0.6% sodium lauryl sulfate. The cell lysate was gently mixed with 125 μl of 5 M NaCl and kept at 4°C overnight. The mixture was centrifuged at $14{,}000 \times g$ for 30 min, and the chromatin pellet was then removed. After treatment with RNase and proteinase K, DNA in the supernatant was precipitated with ethyl alcohol and resuspended in TE buffer. Samples were analyzed for a nucleosomal DNA ladder by electrophoresis on a 1.5% agarose gel. For quantitation of chromosomal DNA extracted in the fragmented DNA fraction, the cellular DNA was labeled with [^{3}H]thymidine prior to sorbitol treatment and the radioactivities in fractions of the total and extracted DNA were determined as described previously (17). In HEp-2 cells, we usually found a considerable amount (10 to 25% of the total DNA, depending on the experiments) of chromosomal DNA extracted in the fragmented DNA fraction regardless of sorbitol treatment.

To examine the morphology of cell nuclei, the cells grown under a coverglass were fixed with methyl alcohol-acetic acid (3:1) and stained for 10 min with DNA-binding dye Hoechst 33258 (0.05 μg/ml) by the method of McGarrity (20).

Sorbitol treatment and the induction of apoptosis. When HEp-2 cells were treated with sorbitol at concentrations of more than 0.8 M for 1 h, the cells underwent apoptosis (data not shown). The characteristic fragmentation of chromosomal DNA into nucleosomal oligomers could be detected within 30 min after treatment and became prominent with increased time (Fig. 1). To determine the kinetics of increase in the amount of chromosomal DNA extracted in the fragmented DNA fraction, cellular DNA was labeled with [^{3}H]thymidine prior to the sorbitol treatment and the radioactivities in fractions of the total and extracted DNA were determined. As shown in Fig. 1, the amount of fragmented DNA increased with time and reached the maximum level at approximately 1 h after sorbitol treatment. The time course as well as the extent of DNA fragmentation varied depending on cell physiology (such as cell density).

Apoptosis has been characterized morphologically by cell shrinkage and nuclear fragmentation as well as biochemically by fragmentation of chromosomal DNA into nucleosomal oli-

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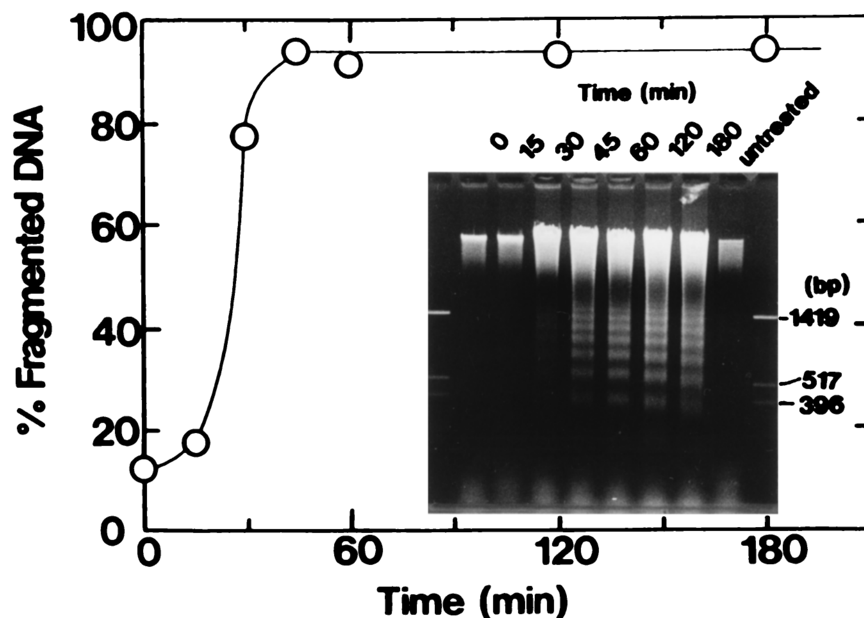

FIG. 1. DNA fragmentation in HEp-2 cells after sorbitol treatment. [^{3}H]thymidine-labeled HEp-2 cells were incubated in medium containing 1 M sorbitol for 1 h. The cells were then incubated in medium without reagent at 37°C. At the indicated intervals, fragmented DNA was extracted, precipitated, and analyzed in a 1.5% agarose gel. For the quantitative analysis, the radioactivities in fractions of total and fragmented DNA were determined separately as described in the text.

gomers. Simultaneously with the progress of this DNA fragmentation, characteristic changes in the morphology of cells and nuclei were observed in the sorbitol-treated cells. At 60 min after treatment, many cells showed a cell shrinkage and an intense perinuclear chromatin condensation (Fig. 2). This sorbitol-induced apoptosis occurred even in the presence of cycloheximide, an inhibitor of protein synthesis, indicating that de novo protein synthesis is not necessary for the induction of apoptosis by sorbitol (18a).

The rate of induction was delayed if the cells were maintained continuously in medium containing sorbitol. The DNA degradation started gradually, increased less steeply, and reached a plateau upon incubation of the cells for 3 h. In the following experiments, we harvested the cells at 3 h posttreatment with sorbitol, although we usually treated the cells with sorbitol for 1 h and then incubated the treated cells in a reagent-free medium.

Suppression of DNA fragmentation by infection with HSV-1. When HEp-2 cells were infected with HSV-1 prior to sorbitol treatment, the fragmentation of chromosomal DNA was suppressed. As shown in Fig. 3A, HEp-2 cells treated with sorbitol immediately after virus infection showed a level of DNA fragmentation similar to that of the uninfected cells treated with sorbitol (control 1), but the degree of chromosomal DNA fragmentation decreased when the cells were treated with sorbitol at later stages of infection and reached a basal level

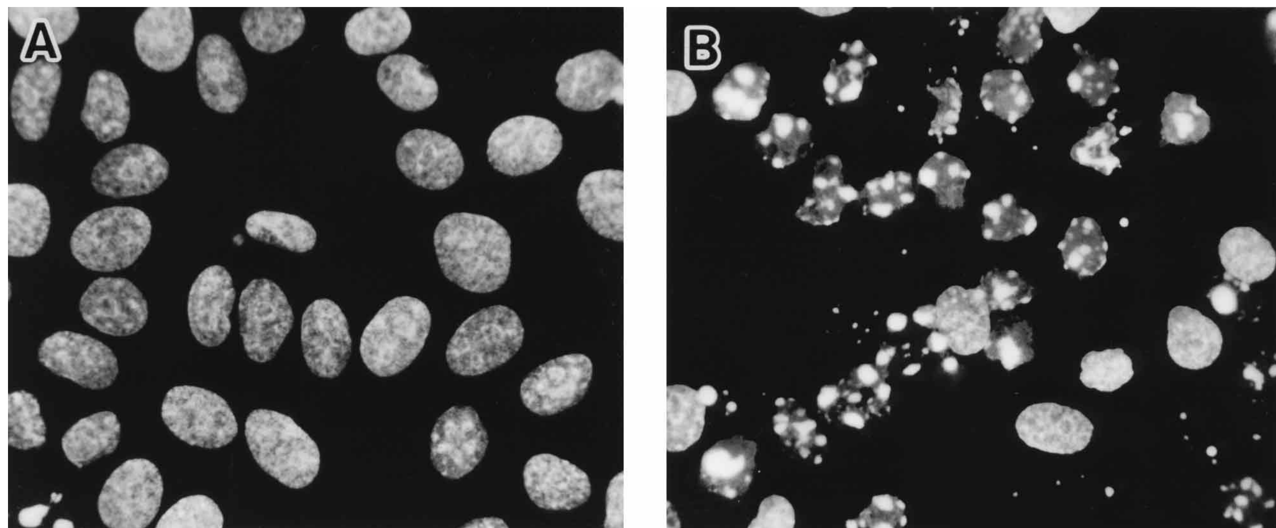

FIG. 2. Morphology of the sorbitol-treated cell nuclei. HEp-2 cells were incubated without sorbitol treatment (A) or were treated with sorbitol as described in the legend to Fig. 1 and incubated for 60 min in medium without reagent (B).

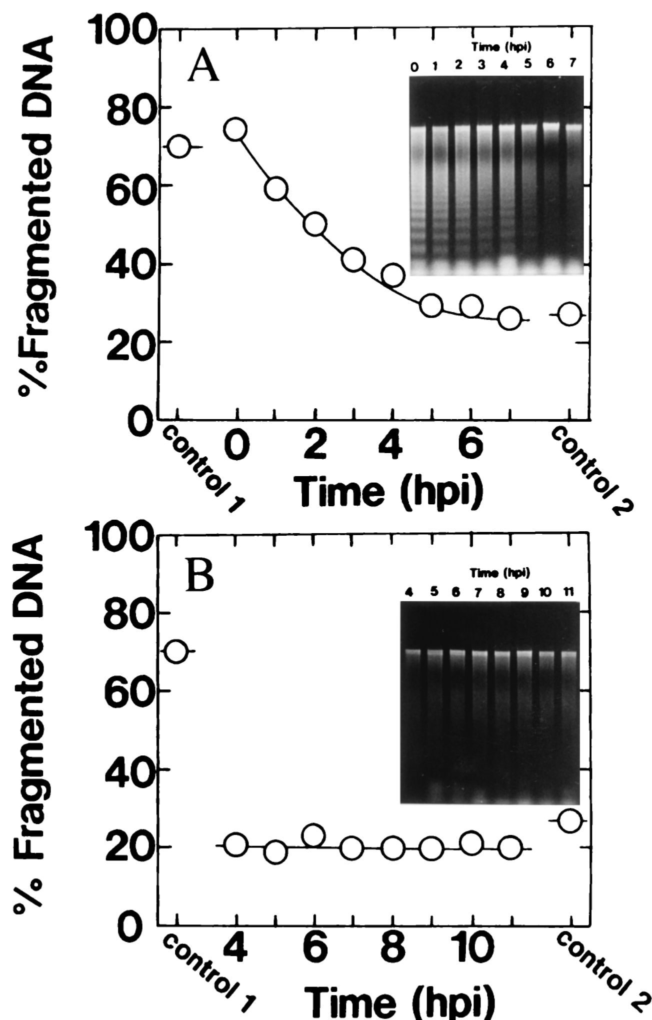

FIG. 3. Effect of HSV-1 infection on DNA fragmentation in sorbitol-treated (A) and untreated (B) HEp-2 cells. [^{3}H]thymidine-labeled HEp-2 cells were infected with HSV-1 HF at a multiplicity of infection of 10. At the indicated times after infection, one group of infected cells was treated with 1 M sorbitol for 1 h, followed by incubation in reagent-free medium for 3 h (A). The other group of cells was kept incubated without sorbitol treatment (B). Cells were harvested, and relative amounts of fragmented DNA to total cellular DNA were determined as described in the text. In parallel, an oligonucleosomal ladder was detected in a 1.5% agarose gel. Control 1, the value of uninfected cells treated with sorbitol; control 2, that without sorbitol treatment.

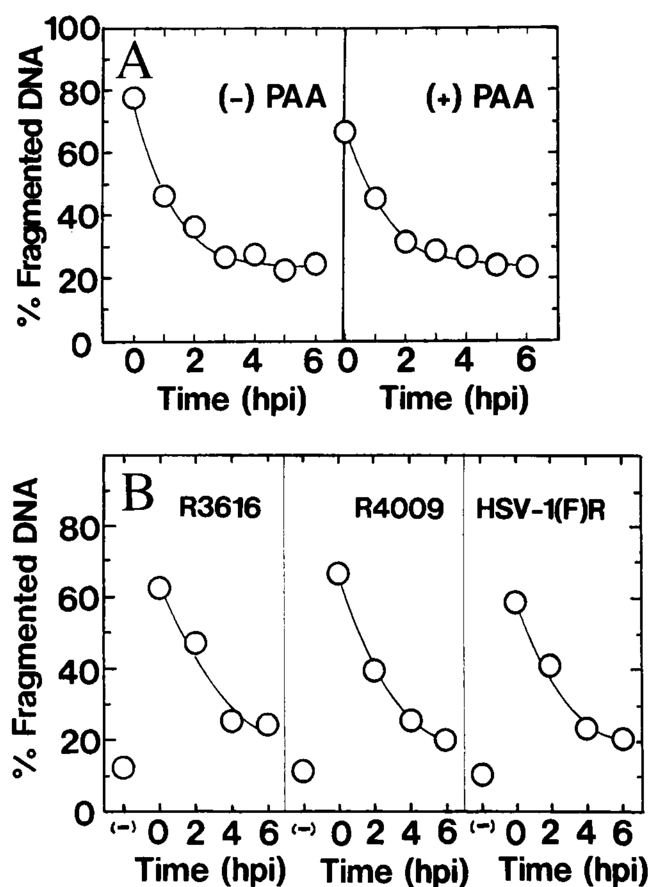

FIG. 4. Effect of PAA (A) or mutation in the γ34.5 gene (B) on sorbitol-induced DNA fragmentation. See the legend to Fig. 3 for details. In the experiment shown in panel A, infection and sorbitol treatment were carried out in the presence (200 μg/ml) or absence of PAA. In the experiment shown in panel B, two γ34.5 mutant viruses (R3616 and R4009) and their repair virus HSV-1(F)R were infected instead of HSV-1 HF. (−), cells without sorbitol treatment.

(control 2) after treatment at 5 h postinfection (p.i.). After 5 h p.i., the sorbitol treatment did not affect the stability of chromosomal DNA.

In agreement with the results in the quantitative analysis, an oligonucleosomal DNA ladder created by agarose-gel electrophoresis disappeared upon infection; the sorbitol treatment immediately after the infection did not affect the observed DNA ladder, but upon sorbitol treatment at increased times after infection, the DNA ladder became less clear and was absent at later stages of the infection (Fig. 3A). These results indicated that HSV-1 has an antiapoptosis gene which suppresses apoptosis induced in sorbitol-treated HEp-2 cells. Although we are not aware of the mechanism of induction of apoptosis in sorbitol-treated HEp-2 cells, this gene likely works as the presumed antiapoptosis gene against virus-induced apoptosis in HEp-2 cells.

In addition, infection with HSV-1 itself did not induce apoptotic DNA fragmentation in HEp-2 cells (Fig. 3B). Without sorbitol treatment, the amount of fragmented DNA in infected cells remained below the level of that of uninfected cells (control 2) and no DNA ladder was observed in the sample at any time after infection. Even at 16 h p.i., a DNA ladder was not observed in HSV-1-infected cells (18a).

Expression of HSV-1 genes is regulated temporally (15), and one class of genes (γ genes) is expressed maximally after the onset of viral DNA replication (14). To characterize the nature of the antiapoptosis gene, we examined the effect of phosphonoacetic acid (PAA) on the expression of this gene. This reagent is known to inhibit viral DNA replication, but as shown in Fig. 4A, the addition of PAA did not affect the virus's ability to suppress DNA fragmentation. The kinetics of expression (Fig. 3A) as well as the fact that expression was not affected by the inhibition of viral DNA replication (Fig. 4A) suggest that this antiapoptosis gene is regulated as an early (α or β) gene.

Suppression by γ34.5 mutant viruses. Previously Chou and Roizman indicated that the γ34.5 gene product plays an essential role in the prevention of programmed cell death in neuroblastoma cells (1) or in human foreskin fibroblasts (2). To test whether the γ34.5 gene is responsible for the observed suppression of sorbitol-induced apoptosis in HEp-2 cells, we examined the ability of two γ34.5-deficient mutant viruses to

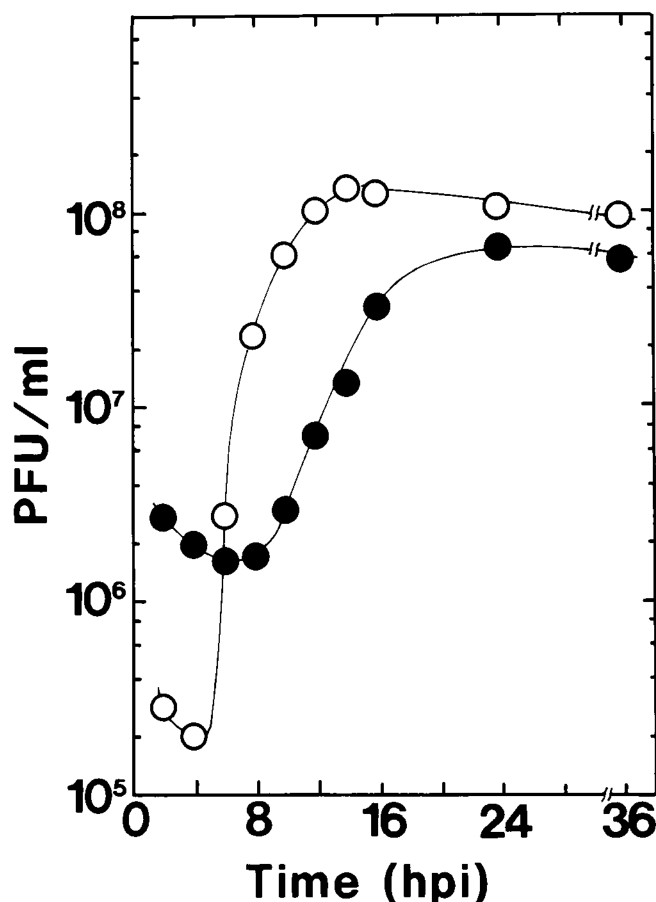

FIG. 5. One-step growth curve of HSV-1 HF in sorbitol-treated cells. HEp-2 cells, preincubated in medium containing 1 M sorbitol for 1 h at 37°C, were infected with HSV-1 HF at a multiplicity of infection of 10. At the indicated intervals, the amounts of progeny virus were determined.

suppress apoptosis. R3616 mutant virus has a deletion of 1 kb of the coding domain of the γ34.5 gene, and R4009 mutant virus has a stop codon inserted at amino acid 28 in the N-terminal region of the γ34.5 open reading frame (1). As shown in Fig. 4B, the γ34.5-deficient virus (R3616 or R4009) could suppress fragmentation as efficiently as the repair virus HSV-1(F)R, indicating that a gene(s) different from the γ34.5 gene is responsible for the observed suppression of the apoptotic response in sorbitol-treated HEp-2 cells.

During the preparation of this article, Leopardi and Roizman (19) reported that the product of the HSV-1 ICP4 gene could suppress apoptosis of Vero cells. This apoptosis was induced by incubating cells at 39.5°C for 30 h and was accompanied by the typical characteristics of apoptosis, like those of the sorbitol-induced apoptosis. It should be noteworthy that the cell death induced by infection of γ34.5-deficient mutant virus is not accompanied by the typical characteristics of apoptosis, suggesting that the mode of action of the γ34.5 gene product is different from that of the ICP4 gene product. Their findings are in good agreement with the results shown here and suggest that the viral antiapoptosis gene described in this communication is the ICP4 gene. In addition, these two independent studies concertedly support the idea that cells of some types induce, by sorbitol or by hyperthermia, apoptotic cell death as a stress response and that HSV-1 carries a gene which can protect infected cells against stress-induced cell death by a mechanism different from that of the γ34.5 gene.

Multiplication of HSV-1 in apoptotic cells. To understand the role of the antiapoptosis gene in the life cycle of the virus, the effect of apoptosis on the multiplication of HSV-1 was examined. When HEp-2 cells were preincubated in medium containing sorbitol before infection, virus yields decreased noticeably with increasing concentrations of the reagent (data not shown). Under these conditions, viral adsorption was not affected as severely as virus yield, because, when the amount of adsorbed virus was compared with that of the control untreated cells, about 70% of the virus adsorbed to the cells treated with 1 M sorbitol (18a).

Figure 5 shows one-step growth curves of the virus in sorbitol-treated and untreated cells. In treated cells, the virus could grow but with a much longer eclipse period and smaller burst size. These results indicate that the multiplication of HSV-1 is sensitive to apoptosis, although not completely abolished.

The results presented in this report, together with those described by Leopardi and Roizman (19), imply that HSV-1 carries a pair of specialized genes (γ34.5 and ICP4) which suppress apoptosis in a cell type-specific manner. The involvement of multiple genes in antiapoptotic activities was apparently observed in common in large DNA viruses (23). Epstein-Barr virus, another member of the human herpesvirus family, is known to have two antiapoptosis genes, LMP1 and BHRF1 (11, 12). Adenovirus also produces multiple viral polypeptides (E1B-19K, E1B-55K, E3-14.7K, and E3-10.4K/14.5K) which are involved in the suppression of apoptosis (6–8, 10, 22, 24, 25). Although the significance of virus-induced apoptosis as well as these viral antiapoptosis genes has not yet been determined, the common presence of multiple antiapoptosis genes in the viral genome strongly suggests the importance of this activity in the viral life cycle and is consistent with the proposed role of the apoptotic response in the host defense mechanism (4, 5, 23). As a mechanism for maintaining viral multiplication, the viruses have evolved the antiapoptosis genes to prevent the induction of apoptosis in infected cells. The observation that multiplication of HSV-1 was suppressed in apoptotic HEp-2 cells (Fig. 5) also agrees with this hypothesis.

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