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Inhibition by Ganciclovir of Cell Growth and DNA Synthesis of Cells Biochemically Transformed with Herpesvirus Genetic Information

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The ability of LM cells, thymidine kinase-deficient LM cells (LMTK$^-$), and LMTK$^-$ cells transformed to the LMTK$^+$ phenotype by herpes simplex virus type 1 genetic information (LH_7 cells) to anabolize the acyclovir congener ganciclovir was examined. About 50-fold more ganciclovir triphosphate was produced by LH_7 cells than by either LM or LMTK$^-$ cells. Growth inhibition studies indicated that 180 and 120 μM ganciclovir were required to achieve 50% growth inhibition of LM and LMTK$^-$ cells, respectively; only 0.07 μM ganciclovir was necessary to achieve 50% inhibition of LH_7 cells. DNA synthesis in the transformed cells was significantly reduced by ganciclovir treatment, whereas ganciclovir had little effect on DNA synthesis in the nontransformed cells. Alkaline sucrose gradient sedimentation analysis of transformed cellular DNA indicated that LH_7 DNA synthesized in the presence of ganciclovir chased into mature DNA. Both LM and LH_7 DNA synthesized in the presence of ganciclovir exhibited a concentration-dependent reduction in the rate of elongation into mature DNA. Finally, [^{14}C]ganciclovir was incorporated internally into the growing DNA chains of LH_7 cells.

The acyclovir (Zovirax) congener, ganciclovir, also known as BW759U (24), BW B759U (3), BIOLF-62 (23), DHPG {9-[(1,3-dihydroxy-2-propoxy)methyl]guanine} (5, 6), and 2'NDG (2'-nor-2'deoxyguanosine) (1, 7, 10, 13, 25), is a potent in vitro inhibitor of herpes simplex virus (HSV) (1, 6, 7, 10, 24) and human cytomegalovirus (3). Because of the high frequency of cytomegalovirus infection in patients with acquired immunodeficiency syndrome (approximately 90% of whom develop infections caused by cytomegalovirus [20]) and the poor prognosis of these patients with cytomegalovirus infections, ganciclovir has been made available to the medical community on a compassionate plea basis. As with acyclovir (9), ganciclovir is phosphorylated to the triphosphate form in HSV-infected cells (5, 13, 24). Ganciclovir is, in fact, a more efficient substrate than acyclovir for HSV type 1 (HSV-1) thymidine kinase, the enzyme responsible for initial phosphorylation (1, 10). The same situation exists for conversion of the monophosphate to the diphosphate forms; that is, ganciclovir monophosphate is more rapidly converted to the diphosphate derivative by guanylate kinase than is acyclovir monophosphate (4). As a result, nearly 10-fold more ganciclovir triphosphate (GCVTP) than acyclovir triphosphate (ACVTP) exists in equivalently treated HSV-1-infected cells (24). In uninfected cells, the phosphorylation of ganciclovir to GCVTP is limited (3, 5, 13, 24).

GCVTP functions as a competitive inhibitor (with respect to dGTP) of both HSV-encoded and HeLa S-3 α DNA polymerases (24). HSV-1 DNA polymerases are 35- to 50-fold more sensitive to inhibition by GCVTP than is HeLa S-3 α DNA polymerase. The reduced sensitivity of cellular α DNA polymerase to GCVTP, coupled with the inability of uninfected cells to substantially phosphorylate ganciclovir, contributes to the relative lack of cytotoxicity of ganciclovir.

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L cells deficient in thymidine kinase (LMTK$^-$) biochemically transformed to the TK$^+$ phenotype by transfection with HSV DNA fragments (2, 15, 26) show increased sensitivity to growth inhibition by acyclovir over the nontransformed parent cells (8, 12, 17, 18). This increased sensitivity is probably related to the higher levels of ACVTP produced by the transformed cells (12). Acyclovir (Fig. 1A), which cannot structurally accommodate chain elongation, is incorporated into the DNA of the transformed cells as the terminal 3'-nucleotide, resulting in termination of the growing DNA chain. With its two available hydroxyl groups, ganciclovir (Fig. 1B) has the potential of being incorporated internally into the DNA molecule. It was therefore important to investigate the extent to which ganciclovir could be phosphorylated by these cells and the effect that ganciclovir would have on the growth and DNA synthesis of the TK$^+$ transformant compared with the growth and DNA synthesis of the nontransformed LM and LMTK$^-$ cells.

MATERIALS AND METHODS

Cells and media. LM cells, obtained from the American Type Culture Collection, Rockville, Md., were grown in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum (Sterile Systems, Inc., Logan, Utah) (growth medium). LMTK$^-$ cells obtained from R. G. Hughes were propagated in growth medium containing 20 μg of 5-bromodeoxyuridine per ml. LH_7 cells received from S. Silverstein had been prepared by transfecting LMTK$^-$ cells with a 3.4-kilobase doublet of HSV-1 DNA generated by *Bam*HI digestion (26) and were grown in growth medium supplemented with 1 μg of methotrexate (American Cyanamide Co., Pearl River, N.Y.) per ml, 5 μg of thymidine (Sigma Chemical Co., St. Louis, Mo.) per ml, 15 μg of hypoxanthine (Sigma) per ml, and 15 μg of glycine per ml. Transformed and nontransformed cells were subcultured in

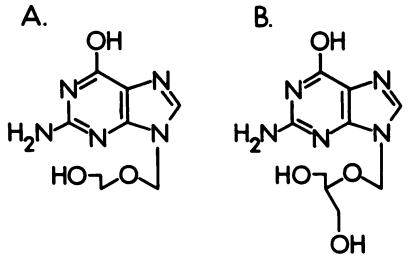

FIG. 1. Structures of (A) acyclovir and (B) ganciclovir.

growth medium before and maintained in medium throughout each experiment, except as indicated.

High-pressure liquid chromatography. LM, LMTK$^-$, and LH_7 cells were exposed to 50 μM ganciclovir or acyclovir for 7 h. The cells were extracted with 3.5% perchloric acid, and the nucleotide content of the extracts was analyzed by high-pressure liquid chromatography. GCVTP and ACVTP were identified as described previously (9, 24).

Determination of cell growth. Tissue culture dishes (60 by 15 mm; Falcon; Becton Dickinson Labware, Oxnard, Calif.) were inoculated with actively growing cells at a density of 2×10^5 per dish. Six to eight hours later, each dish of cells was treated with a different concentration of ganciclovir. After incubation for 20 to 26 h at 37°C, the medium containing the appropriate drug concentrations was replaced. Seventy-six hours after ganciclovir addition, the cells were detached by incubation with 1.5 ml of trypsin (0.25%; GIBCO) for 10 to 15 min at 37°C. The volume was then increased to 5 ml with growth medium. A 1:10 dilution of each cell suspension was made in phosphate-buffered saline (pH 7.4), and the cell number was determined using a model Z_B Coulter Counter.

Estimation of DNA synthesis. The effect of ganciclovir on DNA synthesis of LM, LMTK$^-$, and LH_7 cells was determined by the incorporation of [^{3}H]deoxyadenosine as described previously (12).

Alkaline sucrose gradient sedimentation. LM and LH_7 cells were inoculated in tissue culture dishes (60 by 15 mm; 3×10^5 cells per dish) containing growth medium supplemented with 5% fetal bovine serum and 10% tryptose phosphate broth (GIBCO). The DNA was prelabeled with 0.1 μCi of [^{14}C]thymidine (ICN Chemical and Radioisotope Division, Irvine, Calif.) per ml for 40 h at 37°C. The cells were then washed four times with 5 ml of prewarmed supplemented growth medium and incubated for 2 h in isotope-free supplemented growth medium. The medium was replaced with 1 ml of supplemented growth medium containing the indicated concentrations of ganciclovir, and the cells were allowed to incubate for 30 min. [^{3}H]thymidine (100 μCi/ml; 1 μM; ICN Chemical and Radioisotope Division) was added, and the cells were incubated an additional 30 min. For pulse-chase experiments, the cells were washed four times after completion of deoxynucleoside precursor labeling in supplemented growth medium and incubated for the indicated times in isotope-free, drug-free supplemented growth medium.

The DNA was prepared for alkaline sucrose gradient centrifugation using the lysing-deproteinization procedure of Smith et al. (22). The extracts were layered at the top of linear gradients of 5 to 15% (wt/wt) sucose solutions that contained 0.7 M NaCl, 0.3 M NaOH, and 0.01 M EDTA. Gradients were centrifuged at 27,000 rpm for 3 h at 20°C in a Beckman SW41 rotor. Fractions (0.3 ml) were collected by bottom puncture, and 150 μl of each fraction was spotted on GF/A glass-fiber paper strips. After drying, the strips were processed and counted as described previously (12).

Enzymatic digestion of DNA. Purified LH_7 cellular DNA was enzymatically digested to 3′-mononucleotides, and terminal nucleosides were digested with micrococcal nuclease (19,000 U/mg; Worthington Diagnostics, Freehold, N.J.) and spleen phosphodiesterase (Cooper Biomedical, Inc., West Chester, Pa.) by the procedure of Pelling et al. (19). Briefly, 180 μg of DNA labeled with [^{14}C]ganciclovir (100 μM; 56 mCi/mmol) was digested with 35 μg of micrococcal nuclease in 1 mM Tris (pH 9.5) and 5 mM $CaCl_2$ for 2 h at 37°C. The partially digested DNA was subsequently incubated with 0.53 U of spleen phosphodiesterase at 37°C and pH 6.5 by the procedure of Josse et al. (14). Another 0.53 U of the phosphodiesterase was added after 1 h and again after a second hour; total incubation time with spleen phosphodiesterase was 3 h. To inhibit any contaminating phosphatases present in the phosphodiesterase preparation, EDTA was added to a final concentration of 1 mM. The digested DNA was analyzed by chromatography on polyethyleneimine cellulose developed with water.

RESULTS

Inhibition of cellular growth by ganciclovir. LM, LMTK$^-$, and LH_7 cells were grown in the presence of ganciclovir for 76 h. The resulting cell growth curves (Fig. 2) indicated that the transformed cells were significantly more sensitive to inhibition by ganciclovir than were either of the nontransformed cell types. The concentrations of ganciclovir required to achieve 50% inhibition of cell growth were 180 μM for LM cells and 120 μM for LMTK$^-$ cells; only 0.07 μM ganciclovir was necessary to achieve 50% growth inhibition of LH_7 cells.

Formation of GCVTP and ACVTP. High-pressure liquid chromatographic analysis of perchloric acid extracts of LM, LMTK$^-$, and LH_7 cells which had been exposed for 7 h to 50 μM ganciclovir or acyclovir indicated a significant difference between the transformed and nontransformed cells (Table 1). LH_7 cells produced 4-fold more ACVTP and 50-fold more GCVTP than either LM or LMTK$^-$ cells. In addition, 50-fold more GCVTP than ACVTP was produced in LH_7 cells when incubated with equivalent concentrations of the respective drugs.

Inhibition of DNA synthesis. The effect of ganciclovir on the incorporation of [^{3}H]deoxyadenosine into the DNA of

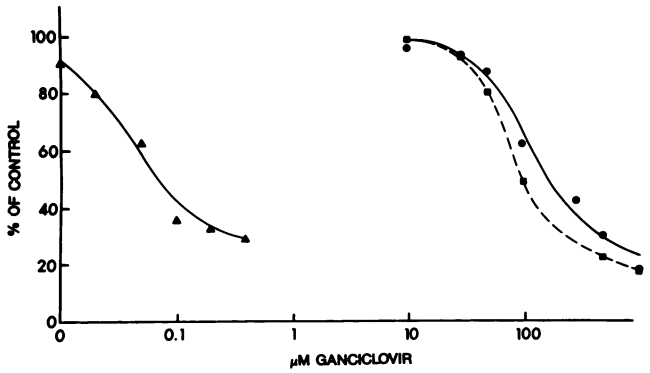

FIG. 2. Effect of ganciclovir on growth of transformed and nontransformed cells. Seventy-six hours after the addition of ganciclovir to actively growing cultures, the cell numbers were determined as described in Materials and Methods. Symbols: ▲, LH_7 cells; ●, LM cells; ■, LMTK$^-$ cells.

transformed and nontransformed cells was determined. At a ganciclovir concentration of only 1 μM, LH_7 cells were clearly sensitive to inhibition by ganciclovir when compared with LM and $LMTK^-$ cells (Fig. 3). After only 1 h of exposure to 1 μM ganciclovir, LH_7 DNA synthesis was reduced to 40% of that of the control, and after 24 h, DNA synthesis was reduced to 15 to 20% of that of the control. In contrast, 1 μM ganciclovir had no effect on LM cellular DNA synthesis during a 70-h exposure, and $LMTK^-$ cellular DNA synthesis was inhibited only 10% during a 70-h exposure.

In LH_7 cells incubated in 10 μM ganciclovir for 16 h, DNA synthesis was inhibited 90%. Sixteen hours of exposure to 10 μM ganciclovir inhibited DNA synthesis in $LMTK^-$ cells about 30%, while the DNA synthesis of LM cells remained unaffected; however, 70 h of incubation in 10 μM ganciclovir inhibited DNA synthesis of LM and $LMTK^-$ cells 30 and 50%, respectively.

Chain elongation after exposure to ganciclovir. The effect of ganciclovir on the size distribution of DNA from LM and LH_7 cells after exposure to various concentrations of ganciclovir was evaluated by alkaline sucrose gradient sedimentation. The slowly sedimenting, pulsed DNA of untreated LM cells readily chased into mature, prelabeled DNA in 4 h (Fig. 4). The DNA of LM cells treated with 1 μM ganciclovir also chased into mature DNA within 4 h. However, the DNA of LM cells treated with 10 μM ganciclovir was not fully chased into mature DNA after 4 h, but instead it required a 6-h chase. LM cellular DNA synthesized in the presence of 100 μM ganciclovir was not chased into mature DNA after 4 or even 6 h but required an 8-h chase to elongate into fully mature DNA.

The sedimentation profiles of untreated LH_7 cellular DNA were similar to those of LM cells in that the slowly sedimenting, pulsed DNA chased into mature DNA within 4 h (Fig. 5). The DNA of LH_7 cells treated with 1 μM ganciclovir also chased into mature DNA within 4 h, whereas the DNA of LH_7 cells treated with 10 μM ganciclovir required 6 h to chase into mature DNA. At 100 μM, ganciclovir inhibited LH_7 cellular DNA synthesis 100%, and as a result, sedimentation analysis could not be performed at this concentration.

Incorporation of ganciclovir into LH_7 DNA. LH_7 DNA labeled with [^{14}C]ganciclovir was digested to 3′-monophosphates and nucleosides using micrococcal nuclease and spleen phosphodiesterase to determine whether the position of ganciclovir monophosphate in the DNA chain was terminal or internal. Ninety-nine percent of the radioactivity eluted on polyethyleneimine cellulose as the internally incorporated monophosphate (R_f = 0.05), whereas only one percent eluted as the nucleoside with an R_f value of 0.61 (R_f of ganciclovir = 0.65).

TABLE 1. Formation of ACVTP or GCVTP in transformed and nontransformed cells[a]

Cells	Drug (50 μM)	Amt (p mol/10^6 cells)	
		ACVTP	GCVTP
LM	Acyclovir	0.18	
	Ganciclovir		0.83
$LMTK^-$	Acyclovir	0.25	
	Ganciclovir		0.97
LH_7	Acyclovir	1.00	
	Ganciclovir		53.9

[a] Cells were treated for 7 h.

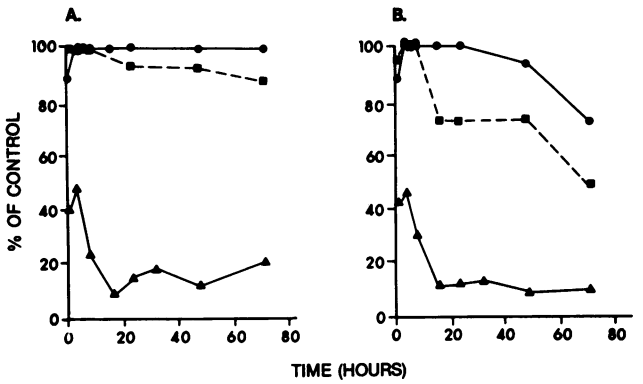

FIG. 3. Effect of ganciclovir on DNA synthesis of transformed and nontransformed cells. DNA synthesis was measured by the incorporation of [^{3}H]deoxyadenosine. At designated times following the addition of (A) 1 μM ganciclovir or (B) 10 μM ganciclovir, the cells were pulse-labeled for 60 min with [^{3}H]deoxyadenosine (15 μCi/ml; ICN Pharmaceuticals Inc., Irvine, Calif.). The cells were lysed, the RNA was digested, and the acid-precipitable material was counted as described previously (9). Symbols: ▲, LH_7 cells; ●, LM cells; ■, $LMTK^-$ cells.

DISCUSSION

$LMTK^-$ cells transformed to the TK^+ phenotype by HSV-1 genetic information anabolized significantly more ganciclovir to the triphosphate form than did either of the nontransformed cell types. This was not unexpected because ganciclovir and its monophosphate derivative are efficient substrates for HSV-1 thymidine kinase and cellular GMP kinase, respectively.

The K_i values obtained for partially purified HeLa S-3 α DNA polymerase of 2.5 μM (24) and for a highy purified α DNA polymerase from peripheral blasts of acute myelogenous leukemia patients of 0.13 μM GCVTP (11) indicate that GCVTP is an inhibitor of cellular DNA synthesis. The difference in inhibition of cell growth and DNA synthesis that exists between the transformed and nontransformed cells is probably a direct result of the higher levels of GCVTP present in the transformed cells. The observed difference in cell growth and DNA synthesis between the two nontransformed cell lines is unexplained. Although the sensitivity of the α DNA polymerases induced by these cells to inhibition by GCVTP has not been determined, it is reasonable to assume that the amounts of GCVTP present in the transformed cells could be sufficient to inhibit their α DNA polymerases. GCVTP concentrations in the nontransformed cells are probably not sufficient for such inhibition.

Results from alkaline sucrose gradient analysis suggest some major differences between the mechanisms of antiviral action of the two guanosine analogs, acyclovir and ganciclovir. In contrast to acyclovir, which causes an accumulation in transformed cells of short nascent DNA chains that do not chase into high-molecular-weight DNA, no accumulation of low-molecular-weight DNA follows ganciclovir treatment. An accumulation of low-molecular-weight DNA was not observed even when transformed cellular DNA was chased in the presence of ganciclovir (unpublished data). In addition, DNA synthesized by both transformed and nontransformed cells in the presence of ganciclovir exhibited a concentration-dependent reduction

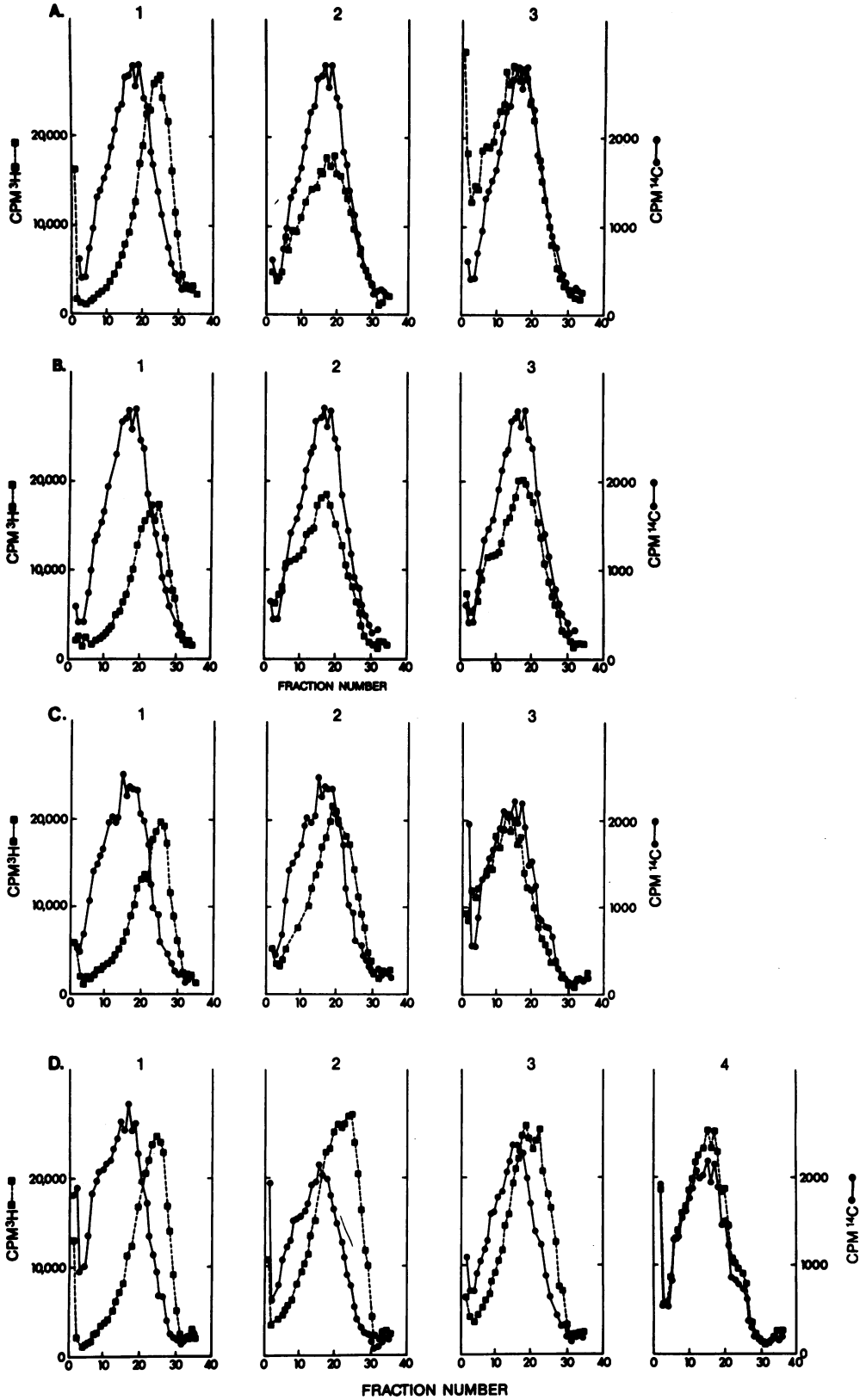

FIG. 4. Alkaline sucrose gradient sedimentation profiles of labeled DNA from LM cells. Cells were prelabeled for 40 h with [^{14}C]thymidine (●) and then pulse-labeled for 30 min with [^{3}H[thymidine (■) in the presence of (A) 0 μM ganciclovir, (B) 1 μM ganciclovir, (C) 10 μM ganciclovir, or (D) 100 μM ganciclovir as described in Materials and Methods. Cells were then chased for (1) 0 h, (2) 4 h, (3) 6 h, or (4) 8h in label-free medium in the absence of ganciclovir.

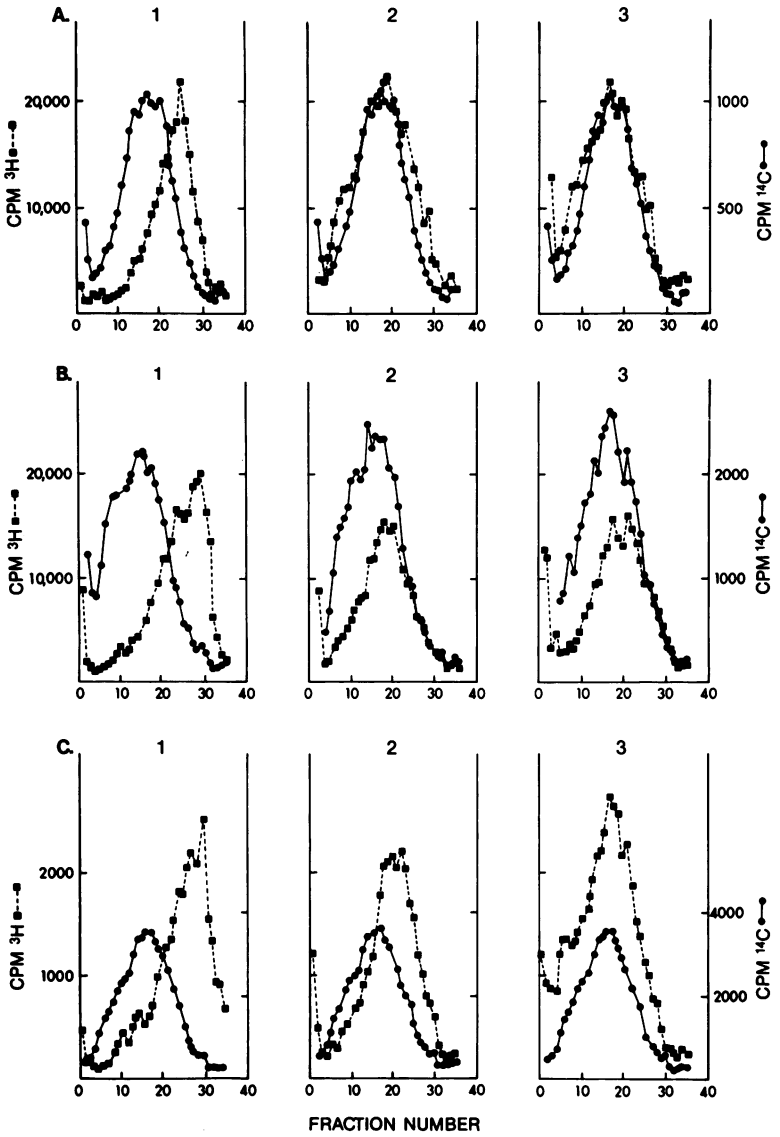

FIG. 5. Alkaline sucrose gradient sedimentation profiles of labeled DNA from LH_7 cells. Cells were prelabeled for 40 h with [^{14}C]thymidine (●) and then pulse-labeled for 30 min with [^{3}H]thymidine (■) in the presence of (A) 0 μM ganciclovir, (B) 1 μM ganciclovir, or (C) 10 μM ganciclovir. Cells were chased for (1) 0 h, (2) 4 h, or (3) 6 h in label-free medium in the absence of ganciclovir.

in the rate of elongation into mature DNA. This observation confirms that of Frank et al. (11), who reported that GCVTP incorporation into a calf thymus template primer slowed the rate of DNA chain elongation catalyzed by a purified HSV-1-induced DNA polymerase relative to unmodified primer. Additionally, Smee et al. (21) observed that 60 to 70% of GCVTP levels persisted in HSV-infected cells 18 h after removal of drug. This persistence of GCVTP may be at least partially responsible for the additional incubation time required for maturation of DNA synthesized in the presence of ganciclovir as compared with DNA from cells not exposed to ganciclovir.

Acyclovir, which lacks the 3'-hydroxyl group, cannot structurally accommodate chain elongation and therefore terminates the DNA molecule (12, 16); ganciclovir, with its two available hydroxyl groups, is incorporated internally into the growing DNA chain. The slowdown of DNA maturation observed for both transformed and nontransformed cells in the presence of ganciclovir is an interesting phenomenon that is under investigation in these laboratories.

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