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Exogenous Thymidine Is Preferentially Incorporated into Human Cytomegalovirus DNA in Infected Human Fibroblasts

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The effect of human cytomegalovirus infection on cellular DNA synthesis in human fibroblasts was measured by fluorometry and by incorporation of radiolabeled thymidine. The results show that although HCMV infection stimulates cellular DNA synthesis in both quiescent and serum-stimulated cells, radiolabeled thymidine is almost exclusively incorporated into viral DNA.

Human cytomegalovirus (HCMV) encodes many viral proteins that modify the transcriptional and metabolic activity of host cells. It has been widely reported that HCMV differs from other herpesviruses in that HCMV infection stimulates the synthesis of cellular DNA (for reviews, see references 1, 12, and 14). The best evidence for HCMV stimulation of cellular DNA synthesis, however, is from experiments with nonpermissive cells (2, 5). HCMV stimulation of cellular DNA synthesis in permissive cells has been reported only when cells are arrested (15, 17). In cultures of dividing, permissive cells, HCMV infection inhibits cellular DNA synthesis (5). An infrequently cited report demonstrates that HCMV infection also inhibits cellular DNA synthesis in serum-stimulated HEL cells (8). The authors of the latter report question the conventional assumption that DNA synthesis in virus-infected cells is adequately measured by [^{3}H]thymidine incorporation but do not test that assumption (8). A recent study with propidium iodide fluorescence-activated cell sorter analysis demonstrated an increase in DNA content in HCMV-infected, serum-stimulated fibroblasts to approximately 3 N after 72 h (10). It is unclear in this case whether HCMV infection stimulates cellular DNA synthesis in serum-stimulated cells or is merely unable to block the cellular DNA synthesis normally seen after serum stimulation (4, 16). It is clear that the opposite conclusions have been drawn from metabolic radiolabeling studies (8) and fluorometric measurement of DNA (10).

The experiments described here examine the effect of HCMV infection on cellular DNA synthesis in growth-arrested human foreskin fibroblasts with metabolic radiolabeling and fluorometric measurement of DNA in parallel. These results indicate that although HCMV infection stimulates cellular DNA synthesis in both quiescent and serum-stimulated cells, radiolabeled thymidine is incorporated almost exclusively into viral DNA.

HCMV AD169 was obtained from the American Type Culture Collection; human foreskin fibroblasts were obtained from T. R. Jones. Both were propagated by standard techniques (11). Fibroblasts were growth arrested in 0.2% fetal bovine serum (FBS) for 48 h prior to infection, at which time the cell density was 60,000 cells per cm^2. They were then either infected at a multiplicity of 8 PFU per cell by dilution of high-titer viral stock 16-fold into the appropriate conditioned medium or mock infected with conditioned medium. The inoculum, with a final concentration of 0.8% conditioned FBS, was withdrawn after 1 h, and the cultures were thereafter maintained in conditioned medium containing 0.2% FBS or fresh medium containing 10% FBS, with or without 45 μg of phosphonoformic acid (PFA) (Sigma P6801) per ml as appropriate. Infected cells were examined by cytoimmunofluorescence spectroscopy 24 h after inoculation to confirm the production of viral protein. Cells were fixed in methanol-acetone and incubated with primary antibody (mouse monoclonal anti-CMV-IE72KD antibody; Dupont NEN 9221) followed by secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse antibody; Southern Biotechnology Associates 1010-02). Cells were then counterstained with diamidinophenylindole (Sigma D9542) and examined with a Zeiss Axioplan fluorescent microscope. Over 90% of the cells infected under these conditions displayed HCMV immediate-early antigen (data not shown).

In order to determine the relative rate of [^{3}H]thymidine incorporation into DNA as well as the total DNA content of cells, they were pulse-labeled with 5 μCi of [^{3}H]thymidine (Amersham TRK 686) per ml for 4 h, washed in phosphate-buffered saline, lysed in 10 mM Tris-HCl (pH 7.5)–10 mM EDTA–50 mM NaCl–0.6% sodium dodecyl sulfate, and incubated with RNAse A at 50 μg/ml for 30 min at 37°C, followed by incubation with Pronase at 1 mg/ml at 37°C overnight. Crude samples were extracted with neutral phenol and chloroform-isoamyl alcohol and then were precipitated from ethyl alcohol thrice and resuspended in distilled water. Aliquots were diluted and counted in Ecolume (ICN Biomedicals, Inc.). Total DNA was measured with Hoechst 33258 reagent (Sigma B-2883) as described previously (7).

As expected, growth-arrested fibroblasts showed no significant change in cell number, DNA content per cell, or relative rate of [^{3}H]thymidine incorporation when maintained in conditioned medium with 0.2% FBS for 72 h (Table 1). When stimulated to divide by the addition of fresh medium with 10% FBS, however, the cell number nearly doubled by 72 h, as did total DNA, so that the number of picograms of DNA per cell remained at the 2 N level (13). The serum-stimulated cells showed a 15-fold increase in [^{3}H]thymidine incorporation over quiescent control cells when harvested at 24 h, dropping to a 10-fold increase when harvested at 72 h. As expected, PFA did not affect cell division or [^{3}H]thymidine incorporation into cellular DNA. When quiescent, growth-arrested cells were infected with HCMV, there was no change at 24 h, but by 72 h, the DNA content per cell had increased by 50% to a level of approximately 3 N, accompanied by an 18-fold increase in [^{3}H]thymidine incorporation (Table 1). The increase in DNA per cell was not abolished by PFA, indicating that an early viral

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TABLE 1. Effect of HCMV on DNA content and [^{3}H]thymidine incorporation[a]

Cell culture condition	No. of h post-HCMV infection	No. of cells (10^6)		DNA content				[^{3}H]thymidine incorporation			
				μg		pg/cell		kcpm		kcpm/10^6 cells	
		Mock	HCMV	Mock	HCMV	Mock	HCMV	Mock	HCMV	Mock	HCMV
0.2% FBS	0	1.3		10.7		8.2		10.4		8.0	
	24	1.3	1.3	7.8	9.2	6.0	7.0	35.7	26.3	39.7	25.1
	72	1.2	1.0	10.7	13.1	8.9	13.1	11.4	207.8	16.4	349.2
0.2% FBS + PFA	72	1.1	1.1	7.9	11.2	7.2	10.2	16.5	37.4	23.3	43.6
10% FBS	24	1.4	1.4	9.0	9.2	6.4	6.5	521.3	25.1	586.5	21.6
	72	2.2	1.3	19.0	16.1	8.6	12.4	100.8	210.7	75.0	283.1
10% FBS + PFA	72	2.3	1.2	14.9	13.0	6.5	10.8	180.6	28.6	104.5	31.2

[a] All values are the average of independent duplicate samples.

function is capable of stimulating quiescent cells to synthesize cellular DNA in the absence of cell division. In contrast, the increase in [^{3}H]thymidine incorporation was abolished by PFA, indicating that although growth-arrested cells synthesize both cellular and viral DNA in response to HCMV infection, exogenous [^{3}H]thymidine is preferentially incorporated into viral DNA. Infection of growth-arrested cells with HCMV prior to serum stimulation abolished the 15-fold increase in [^{3}H]thymidine incorporation that is otherwise observed 24 h after serum stimulation (Table 1). Similar results were also obtained when growth-arrested cells were infected with HCMV an hour after serum stimulation (data not shown). The infected serum-stimulated cells did not divide, but they did show a 50% increase in DNA per cell to approximately 3 N, despite the presence of sufficient PFA to effectively block [^{3}H]thymidine incorporation into viral DNA (Table 1). The results from serum-stimulated, HCMV-infected cells are thus consistent with the results from quiescent, HCMV-infected cells. Early viral function(s) stimulate HCMV-infected cells to synthesize cellular DNA and yet alter cellular metabolism sufficiently so that exogenous [^{3}H]thymidine is incorporated into viral but not cellular DNA.

In order to further examine the nature of the radiolabelled DNA synthesized in HCMV-infected cells, samples prepared as described above were separated into viral and cellular fractions by equilibrium centrifugation in CsCl. Aliquots were diluted into a CsCl-Tris-EDTA solution (final density, 1.72 g/ml) and centrifuged in a Beckman Ti 70.1 rotor at 35,000 rpm at 20°C for at least 48 h. Fractions of 0.25 ml were collected with a Buchler Instruments fraction collector, the refractive index was measured with an Abbe refractometer, and aliquots were diluted and counted as described above. The results were identical for both quiescent (Fig. 1) and serum-stimulated (data not shown) HCMV-infected cells. Radiolabelled viral DNA was apparent by 24 h after infection, and the amount of [^{3}H]thymidine incorporated into viral DNA within a 4-h pulse increased in cells harvested 48 and 72 h after infection (Fig. 1). Despite the observed increase in cellular DNA that occurred over the same interval in these cells (Table 1), there was no significant incorporation of [^{3}H]thymidine into cellular DNA

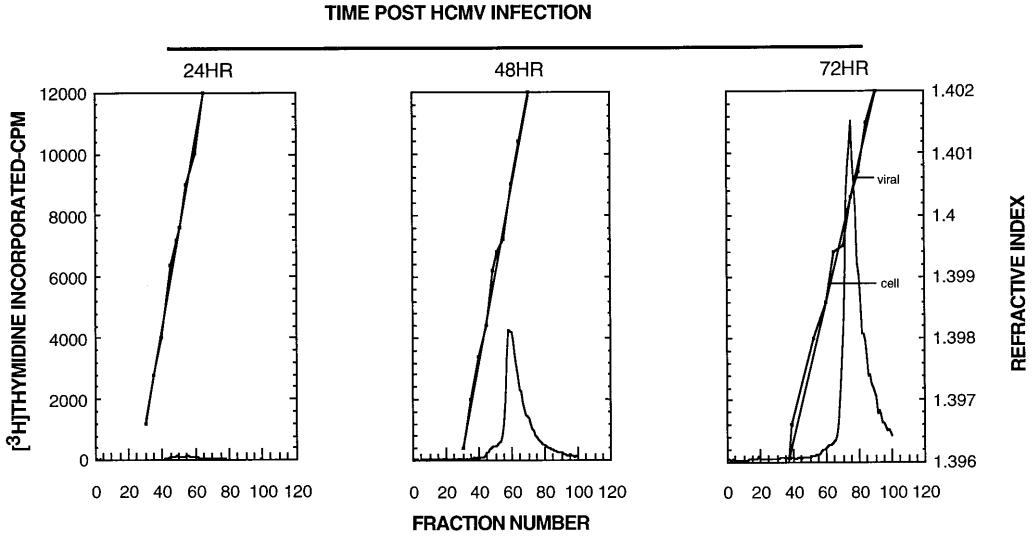

FIG. 1. Exogenous [^{3}H]thymidine is preferentially incorporated into HCMV DNA in quiescent, growth-arrested, infected human fibroblasts. Cultures were pulse-labeled with [^{3}H]thymidine and harvested at different times after infection. Viral DNA and cellular DNA were separated by equilibrium centrifugation in CsCl and counted. The buoyant density of cellular DNA was confirmed with DNA isolated from uninfected cells that had been metabolically radiolabeled with [^{14}C]thymidine.

(Fig. 1). Similar results were observed when infected cells were metabolically radiolabelled and harvested 36, 42, 54, 66, 68, or 78 h after infection (data not shown).

These results confirm the recent finding that an early function of HCMV infection blocks cell division of growth-arrested, serum-stimulated fibroblasts but that cellular DNA synthesis continues over the first 72 h, resulting in a DNA content per cell equivalent to 3 N (10). Furthermore, our results extend these results by demonstrating that this increase in cellular DNA content after infection also occurs in quiescent, growth-arrested cells that are not stimulated to divide by serum, indicating that viral infection alone can initiate the response. These results also resolve the apparent disagreement between the conventional view that HCMV infection stimulates the synthesis of cellular DNA (1, 12, 14) and persistent reports that HCMV infection actually inhibits the incorporation of [^{3}H]thymidine into cellular DNA (5, 8) in permissive cells. If stimulation of cellular DNA synthesis and the diversion of exogenous thymidine to viral DNA are separate viral functions, it might be expected that metabolic radiolabelling experiments with different cell types could yield variable results. This might be especially true if the establishment of viral control over cellular thymidine metabolism is an important criterion for permissivity. The complex interaction of HCMV with its host is known to significantly alter the metabolism of exogenous thymidine (3, 6, 9, 18). Nevertheless, the almost complete diversion of exogenous thymidine into viral DNA that is demonstrated here would seem to require additional viral functions that have not yet been described, possibly including mechanisms to control intracellular transport and localize key enzymatic pathways within virally specified subcellular compartments.

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