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Deoxyribonucleic Acid Synthesis in Synchronized Mammalian KB Cells Infected with Herpes Simplex Virus

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We examined the patterns of host cell and virus deoxyribonucleic acid (DNA) synthesis in synchronized cultures of KB cells infected at different stages of the cell cycle with herpes simplex virus (HSV). We found that the initiation of HSV DNA synthesis, we well as the production of new infectious virus, is independent of the S, G1, and G2 phases of the mitotic cycle of the host cell. This is in contrast to data previously found with equine abortion virus. Because HSV replicates independently of the cell cycle, we were able to establish conditions that would permit the study of rates of HSV DNA synthesized in logarithmically growing cells in the virtual absence of cellular DNA synthesis. This eliminates the need for separation of viral and cellular DNA by isopycnic centrifugation in CsCl. We found that HSV DNA synthesis was initiated between 2 to 3 hr after infection. The rate of DNA synthesis increased rapidly, reaching a maximum 4 hr after infection, and decreased to 50% of maximum by 8 hr. Evidence is also presented which suggests that HSV infection can inhibit both the ongoing synthesis of host DNA as well as the initiation of the S phase.

Lawrence (23; Bacteriol. Proc., p. 170, 1969) showed recently that the synthesis of equine abortion virus (EAV) deoxyribonucleic acid (DNA) in synchronized cultures of KB cells was initated only at the time corresponding to the S phase (DNA synthesis) of KB cells. Thus, when cells were infected during the S phase, viral DNA synthesis began shortly after infection. On the other hand, when KB cells in the G1 or G2 phase were infected, viral DNA synthesis was postponed until the next S phase began. These observations suggested that EAV DNA synthesis depends on certain cellular events occurring only during the S phase of the mitotic cycle.

The above studies raised the possibility that all herpesvirus infections are characteristically linked to the host cell cycle. Indeed, some years ago, Stocker and Newton (33) suggested that herpes simplex virus (HSV) infection might be linked to the events of the mammalian mitotic cycle; and, more recently, Goodheart (12) suggested that latency may be related to mitotic activity in the cell. Furthermore, Nahmias et al. (25) reported that phytohemagglutinin, an inducer of leukocyte proliferation, appeared to be essential for replication of HSV in leukocyte culture, suggesting that leukocytes must be in a particular growth phase to be susceptible to infection with HSV.

In this paper, we examine the patterns of HSV and host cell DNA synthesis in synchronized cultures of KB cells infected at different stages of the cell cycle with HSV. We present evidence here which shows that the initiation of HSV DNA synthesis, as well as the production of new infectious virus, is independent of the S, G1, and G2 phases of the mitotic cycle of the host, in contrast to that found with EAV. Second, because HSV replicates independent of the cell cycle, we were able to establish conditions that would permit HSV DNA synthesis in logarithmically growing KB cells in the virtual absence of cellular DNA synthesis.

MATERIALS AND METHODS

Cell culture. Suspension cultures of KB cells were propagated in Eagle's minimal essential medium (MEM) modified for suspension culture and supplemented with 10% calf serum. The cells were maintained in continuous logarithmic growth phase, by daily feeding at concentrations varying from 10^5 to 3.0×10^5 cells per ml. Cell counts were performed in triplicate in a hemocytometer.

Virus. HSV (HF strain) obtained from John Flana-

gan (Duke Univ.) was plaque-purified. High-titered virus stock preparations to be used for infection of suspension cells or virus purification procedures were prepared as follows. Monolayer cultures of KB cells grown in 32 oz (ca. 960 ml) bottles containing Eagle's MEM supplemented with 10% calf serum were infected at an input multiplicity of 5 to 10 plaque-forming units (PFU) per cell. The cells were harvested after 24 hr, washed with phosphate-buffered saline (PBS), *p*H 7.2, and concentrated by centrifugation (10 min at 800 × *g*). The cell pellet was diluted with PBS to a final concentration of 2×10^7 cells per ml, sonically treated three times at 15-sec intervals in a 60 w MSE Sonifier (Measuring and Scientific Equipment, Ltd., London) and centrifuged at 5,000 × *g* for 30 min to remove cell debris. The resultant supernatant fluids which exhibited titers ranging from 5×10^8 to 10^9 PFU/ml were stored at −100 C until retrieval. Virus preparations were partially purified by a modification of the procedures of Flanagan (9).

Titration of virus. Plaque titrations were performed in plastic petri dishes essentially by the procedure of Flanagan (9).

Cell synchronization. Suspension cultures of KB cells were synchronized by the double-thymidine-block method (4) as modified by Bello (2). In essence, KB cells in suspension culture were treated with 2 mM thymidine for 18 to 20 hr, centrifuged at 800 × *g* for 10 min, resuspended in a fresh warmed medium at 37 C to reverse the thymidine block, and then allowed to grow for an additional 9 to 12 hr. At this time, 2 mM thymidine was again added for 18 to 20 hr. Experiments were started after reversal of the second thymidine block.

The doubling time for randomly growing KB cells is about 24 hr. After synchronization with excess thymidine, however, cell division is considerably shortened, and the average doubling time is only about 16 hr (2).

Rate of DNA synthesis in synchronized cell cultures. Pulse-labeling experiments were carried out by incubating 5.0 ml of infected or uninfected KB cells (generally 1.5×10^5 to 2.0×10^5 cells/ml) with 2.5 μCi of thymidine-*methyl*-^3H (^{3}H-TdR, specific activity 6 Ci/mmole, Schwartz BioResearch, Inc.) in a shaker bath at 36 C. Incorporation of label was stopped at the end of a 60-min period by pipetting the cells into iced PBS. The cells were then collected by centrifugation at 800 × *g* for 10 min and resuspended in 5.0 ml of cold 5% trichloroacetic acid for 20 min. The resulting precipitate was washed three times with 5% trichloroacetic acid and dissolved in 0.1 N NaOH. Samples were added to 10 ml of Aquasol (New England Nuclear Corp.) and counted in a Packard Tri-Carb liquid scintillation spectrometer.

Labeling methods and DNA preparation. Tritium-labeled cellular DNA was obtained from synchronized uninfected or infected KB cells by incubating the culture in medium containing ^{3}H-TdR (0.25 μCi/ml) for various periods of time as indicated. Labeled cells were pelleted, washed, and concentrated by centrifugation. Cellular or viral DNA was extracted from the pellet by a modification of the method described by Marmur (24); in our procedure, Pronase (autodigested for 2 hr at 37 C) was added at a concentration of 1 mg/ml to the sodium dodecyl sulfate (SDS)-disrupted cells (14). Although it has been reported by Becker et al. (1) that SDS completely releases herpes simplex DNA from viral coat protein, Pronase treatment was included to insure the release of as much viral DNA as possible.

Cesium chloride density gradient centrifugation. Cesium chloride density gradient centrifugation in a Spinco no. 40 fixed-angle rotor (8) was employed to separate virus DNA from cellular DNA. Approximately 5,000 to 10,000 counts/min of labeled and purified DNA (1 to 10 μg) was diluted in SSC (0.15 M sodium chloride, 0.015 M sodium citrate, *p*H 7.0) and mixed with 4.0 ml of 0.01 M tris(hydroxymethyl)-aminomethane (Tris; *p*H 8.0)-buffered CsCl. The final buoyant density was adjusted to 1.7111 g/cm^3. After centrifugation at 33,000 rev/min at 25 C for 60 hr, seven-drop samples were collected from tubes punctured at the bottom and deposited on Whatman 3 mm filter discs (diameter, 2.3 cm). The discs were dried, washed with cold 5% trichloroacetic acid, rinsed with acetone, and assayed for radioactivity in 10 ml of Spectrofluor (Amersham/Searle) scintillation fluid. About 70 fractions were collected from each tube. Every tenth fraction was utilized for the determination of refractive index and buoyant density.

RESULTS

To determine whether initiation of HSV DNA synthesis depends on the S phase of the cell cycle, we divided a synchronized suspension of KB cells (2×10^5 cells/ml) into five replicate cultures. The cultures were infected at different times in the cell cycle with an input multiplicity of 200 PFU of HSV per cell. At this input multiplicity, greater than 95% of the cells were infected. Flanagan (9) reported that more than 85% of the KB cells were infected with about 10 times less herpesvirus than we used in our experiments. The rate of DNA synthesis in each of the cultures was measured over a 24-hr period by 1-hr pulses with ^{3}H-TdR.

Figure 1 shows that the cells in an uninfected control culture entered S phase shortly after reversal of the thymidine block. The rate of DNA synthesis in these cells increased rapidly for 3 to 4 hr, then decreased to approximately 8% of the maximum value by 10 hr. This low rate of cellular DNA synthesis remained constant until about 12 to 14 hr after removal of TdR, and then a second round of cellular DNA was initiated. The cell number in this culture remained constant for 9 to 10 hr after thymidine reversal and then doubled. These results are essentially the same as those previously reported by Bello (2) for KB cells.

Figure 2A shows that, when a culture was infected 3 hr after thymidine reversal or during the S phase, the rate of DNA synthesis at 4 hr occurred at only 40% of the rate noted in the unin-

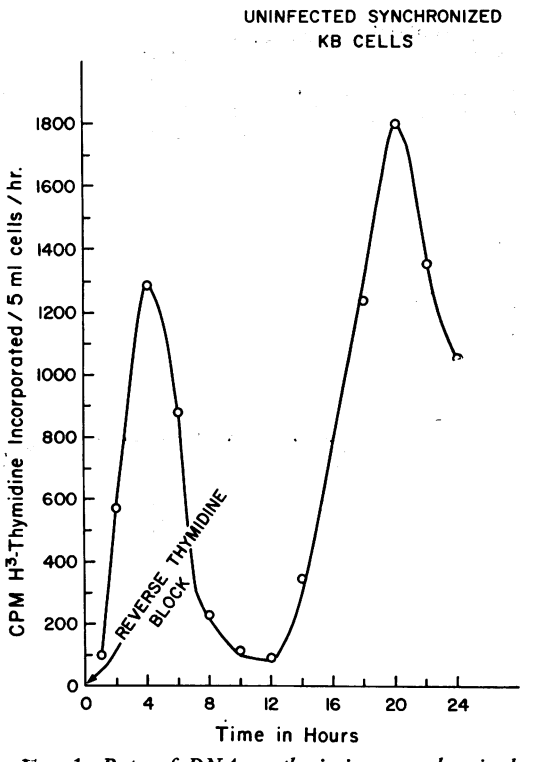

FIG. 1. *Rate of DNA synthesis in a synchronized uninfected culture of KB cells. The rates were determined by pulse-labeling 5.0-ml cell samples with ³H-thymidine for 1 hr and treating as described in the text.*

fected control at this time (Fig. 1). Then, in contrast to the uninfected culture, a second period of DNA synthesis began 5 to 6 hr after thymidine reversal, reached a maximum by 6 to 7 hr, and continued at a decreasing rate until 12 hr into the cell cycle. Figure 2B shows that, when cells were infected 5 hr after synchrony began (late in S phase), a second period of DNA synthesis began 7 to 8 hr into the cell cycle and reached a maximum rate at 9 to 10 hr.

The data presented in Fig. 2C show that, when infection took place 8 hr after reversal of the thymidine block, a time when cellular DNA synthesis was at, or approaching, a minimum rate, an unscheduled round of DNA synthesis began approximately 2 to 3 hr after infection of the cells. Essentially similar results were obtained when infection took place at 7 or 9 hr after reversal. In cells infected 11 hr post-thymidine reversal (Fig. 2D), a round of DNA synthesis began at about the time when the second S phase normally occurred in uninfected cultures; however, neither the rate nor the amount of DNA synthesized was as great as that observed in the control culture (Fig. 1). It is apparent, therefore, that an unscheduled round of DNA synthesis (presumable viral) always occurs 2 to 3 hr after infection of synchronized KB cell cultures with HSV, regardless of when in the cell cycle infection takes place.

Viral growth cycle. To substantiate the idea that initiation of HSV DNA synthesis was independent of the phases of the cell cycle tested, a one-step growth curve was carried out in synchronized cultures infected at different stages of the cell cycle. If the initiation of viral DNA synthesis were dependent upon some event occurring only during cellular S phase, infection of synchronized cells at times other than S (for example during G2) would delay the synthesis of viral DNA until the cells reached the next S phase. This delay would be reflected by a prolongation of the latent period in the viral growth cycle.

Synchronized cultures of KB cells were infected at 3, 5, 8, and 11 hr after thymidine reversal, and the time-course of infectious virus formation was

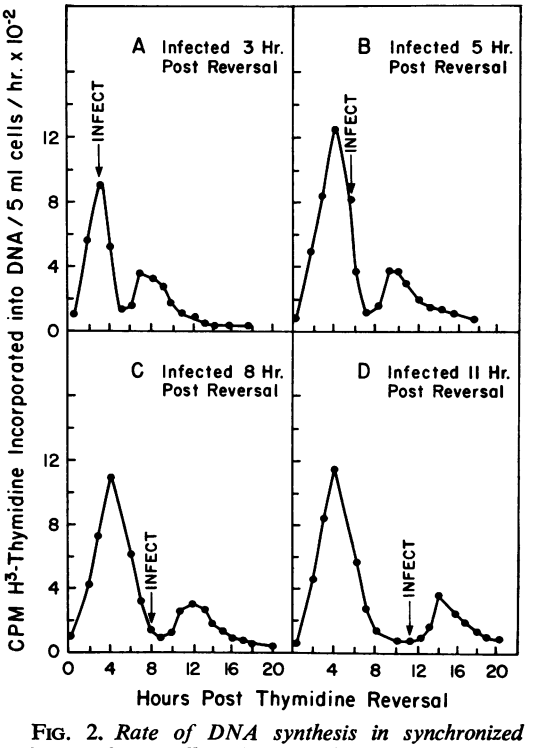

FIG. 2. *Rate of DNA synthesis in synchronized cultures of KB cells infected with HSV at different times in the cell cycle. A suspension culture of KB cells was synchronized by the double-thymidine-block method. The cells were then divided into four replicate cultures in fresh medium at a cell concentration of 2 × 10⁵ per ml. Cultures were infected at the different stages of the cell cycle with an input multiplicity of 200 PFU per cell. The rate of DNA synthesis was determined by pulse-labeling 5.0-ml samples with ³H-thymidine for 1 hr.*

followed. In each case, increases in progeny virus were detected 6 hr after infection, and the maximum titer was reached by 11 or 12 hr after infection. Furthermore, there were no significant differences in the maximum titer measured in each preparation. Thus, no matter when in the mitotic cycle the cells were infected, a normal cycle of viral replication ensued. For the sake of simplicity, only one representative viral growth cycle (cells infected at 8 hr after thymidine reversal) is shown in Fig. 3. These data reflect the idea that KB cells can support HSV replication during any period of the cell cycle tested. It therefore seems likely that the second round of DNA synthesis (shown in Fig. 2A–D) actually represented viral DNA synthesis.

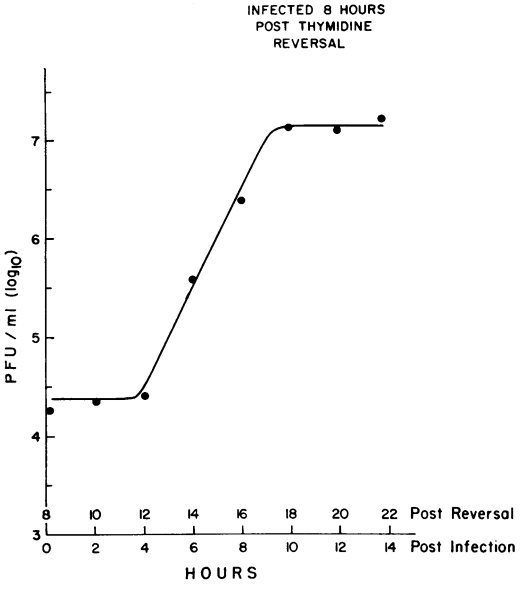

FIG. 3. *Growth curve of HSV in synchronized KB cells infected 8 hr after thymidine reversal. The curve was constructed by removing cell samples at the time 0, infection and every 2 hr thereafter for 14 hr. The cells were washed three times in PBS, sonically treated, and assayed for infectious virus.*

Separation of cellular and viral DNA. The next series of experiments was performed to determine the identity of the DNA made after infection of synchronized cells. Separation of herpes DNA from cellular DNA was first reported by Kaplan and Ben-Porat (18). We obtained better resolution of these two classes of DNA by using the methods of Flamm et al. (8). Purified DNA, extracted from uninfected and HSV-infected KB cells, as well as from partially purified virus, was submitted to equilibrium sedimentation in a CsCl gradient under the conditions described above. DNA from uninfected synchronized KB cells labeled with ^{3}H-TdR from 1 to 9 hr after thymidine reversal (Fig. 1) showed a single tritium band in the CsCl gradient which had an average buoyant density of 1.700 g/cm^3 (Fig. 4A). This density, which corresponds to a guanine plus cytosine (GC) content of 42% is identical to that reported for KB cell DNA by Ginsberg et al. (11).

Most of the tritium-labeled DNA extracted from partially purified virus (Fig. 4B) had an average buoyant density of 1.725 g/cm^3 in CsCl (67% GC content) and was considered to represent HSV DNA. This value conforms with the

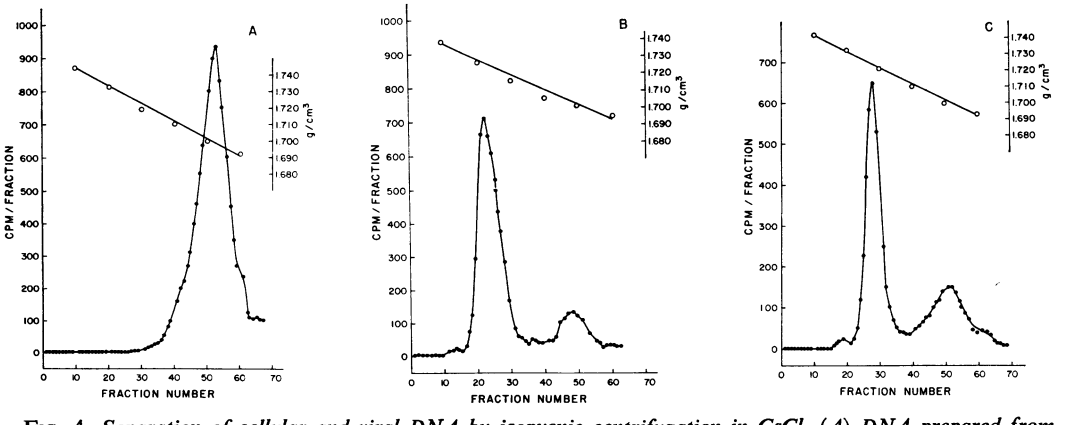

FIG. 4. *Separation of cellular and viral DNA by isopycnic centrifugation in CsCl. (A) DNA prepared from KB cells incubated in ^{3}H-thymidine-containing medium for 9 hr after reversal of thymidine block, i.e., DNA made during the S phase. (B) DNA prepared from partially purified virus isolated from infected randomly growing KB cells incubated in ^{3}H-thymidine-containing medium for 24 hr. (C) DNA prepared from KB cells infected at the time of thymidine reversal and incubated for 9 hr in ^{3}H-thymidine containing medium.*

density reported for HSV DNA by Roizman and Roane (29) as well as Russell and Crawford (30). Approximately 20% of the labeled material extracted from viral DNA preparations banded at about 1.700 g/cm³. This second peak may represent small molecules of cellular DNA which were not degraded completely by deoxyribonuclease added during purification of the virus (22).

Figure 4C shows the results of isopycnic CsCl centrifugation of DNA extracted from synchronized KB cells which were infected 1 hr after thymidine reversal and labeled with ³H-TdR for 9 hr. After infection, two bands of tritium label were detected, one corresponding in buoyant density to KB DNA (Fig. 4A) and another corresponding in buoyant density to viral DNA (Fig. 4B).

These results clearly demonstrate that our methods are quite adequate for the separation and identification of host and viral DNA.

CsCl analysis of the DNA of synchronized cells infected at different times in the cell cycle. Synchronized cultures of KB cells were infected with HSV at an input multiplicity of 200 PFU/cell at 5, 8, and 11 hr after reversal of the TdR block, times which corresponded to S, G1, and G2, respectively. Labeled ³H-TdR was added to the cultures 1 hr after infection. The infected cells were then incubated in the presence of label for an additional 8 hr. The 1-hr delay in adding the label after addition of virus meant that none of the DNA synthesized during the first hour after infection (presumably all host) would be labeled. The DNA from infected preparations was extracted, purified, and analyzed by isopycnic centrifugation n CsCl.

The radioactive profiles obtained from the CsCl gradients are shown in Fig. 5A–C. As the time of infection was delayed after the cells were synchronized, there was a progressive increase in the amount of viral DNA synthesized in proportion to the amount of cellular DNA. When cells were infected 5 hr after thymidine reversal, 64% of all newly synthesized DNA was viral DNA. When the cells were infected 8 hr after thymidine reversal, the percentage of newly synthesized DNA that was viral increased to 86%. When cells were infected at 11 hr, it appeared that only viral DNA was synthesized. These data substantiate the idea that the second round of DNA that occurs in synchronized KB cells after infection (Fig. 2) is mainly viral.

All of the data presented in this study indicate that the initiation of HSV DNA synthesis, as well as the production of new infectious virus, is independent of the host cell cycle. Therefore, KB cells are capable of supporting HSV replication during all phases of the mitotic cycle studied. Our findings for HSV are in direct contrast to those reported for EAV (23).

It is of some interest that when cells were infected 11 hr post-thymidine reversal (Fig. 5C), i.e., 2 hr before initiation of the second S phase in uninfected KB cells (Fig. 1), there was no detectable host cell DNA synthesis in the infected cell. This finding implies that HSV infection prevents the normal initiation of cell DNA synthesis in synchronized KB cells. This idea was suggested by Roizman and Roane (29) who studied HSV infection of randomly growing cells. In contrast to our finding, Kaplan and Ben-Porat (18) showed that the decrease in the rate of cellular DNA

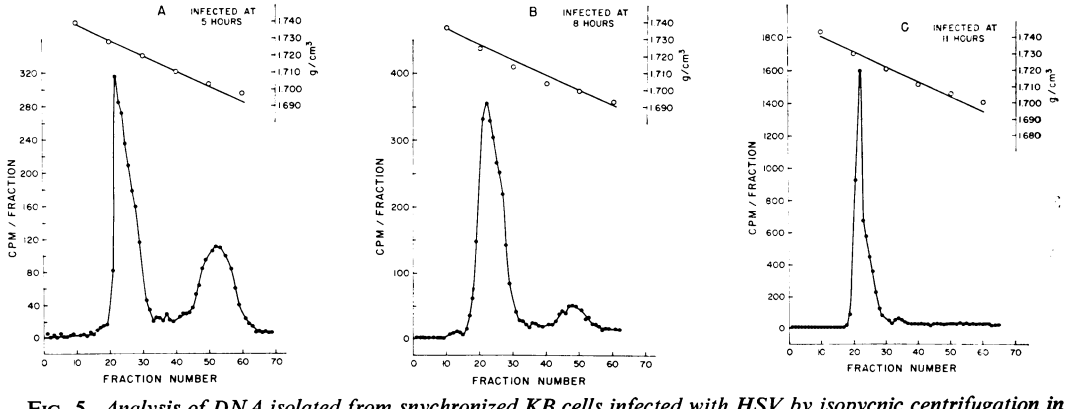

FIG. 5. *Analysis of DNA isolated from synchronized KB cells infected with HSV by isopycnic centrifugation in CsCl KB cells were divided into three separate cultures at a cell density of 2 × 10⁵ per ml and infected as in the text. (A) DNA isolated from cells infected at 5 hr after thymidine reversal. Tritium-labeled thymidine was added at 6 hr, and the cells were incubated for an additional 8 hr. (B) DNA isolated from cells infected at 8 hr post-thymidine reversal, labeled at 9 hr, and incubated for an additional 8 hr. (C) DNA isolated from cells infected at 11 hr after thymidine reversal, label added at 12 hr, and incubated for an additional 8 hr.*

synthesis after infection with pseudorabies (a herpesvirus) was not due to the inability of the infected rabbit kidney cells to enter into the S period of the growth cycle. They found instead that only ongoing cellular DNA synthesis was inhibited. Our observation that host DNA synthesis is inhibited by HSV during the first S period (Fig. 2A) suggests that HSV infection can inhibit both the ongoing synthesis of host DNA as well as the initiation of S phase.

Replication of HSV DNA in synchronized KB cells. The experiment shown in Fig. 5C indicated that only viral DNA was synthesized after infection of synchronized KB cells at 11 hr into the cell cycle. This suggested that employing these techniques we might be able to quantitate various aspects of HSV DNA synthesis directly in log-growing cells and eliminate the need for separation of the two classes of DNA (viral and cellular) by isopycnic centrifugation in CsCl.

To verify that our pulse-labeling technique could actually be used as a direct measure of viral DNA synthesis, we combined the methods of pulse-labeling and isopycnic centrifugation in one experiment. For this experiment, we chose to study the rate of DNA synthesis because this parameter has been well characterized (28, 29, 31). The conditions employed were identical to those described for Fig. 5C. Cultures of KB cells were infected with 200 PFU/cell 11 hr (G1) after synchrony began, a time when residual host cell DNA synthesis was at a minimum. Samples were processed at 1-hr intervals for the determination of rates of DNA synthesis and infectivity. Figure 6A shows that by 9 to 10 hr into the cycle DNA synthesis in uninfected cells is reduced to approximately 6% of the maximum. The initial increase in the rate of viral DNA synthesis was detected in pulse-labeled samples within 2 to 3 hr after infection (13 to 14 hr post-thymidine reversal) and rapidly reached a maximum by 4 hr after infection (Fig. 6B). At this point there was still no detectable increase in infectious virus (Fig. 6B). By 8 hr after infection, the rate of viral DNA synthesis had decreased to 50% of the maximum, and approximately 75% of the total new infectious virus particles were already made. KB cells which had been infected 11 hr after thymidine reversal were also incubated in the presence of ^{3}H-TdR from 1 to 9 hr after infection and isopycnic centrifugation studies were carried out on the purified DNA. The results of this experiment were indentical to those previously presented in Fig. 5C. Virtually all of the tritium counts recovered had the buoyant density of viral DNA. In parallel experiments, cells were infected with HSV 11 hr into the cell cycle and pulse-labeled with ^{3}H-TdR during three successive periods of the infectious cycle: 1 to 3, 3 to 6, and 6 to 9 hr after infection. Figures 7A–C illustrate that only

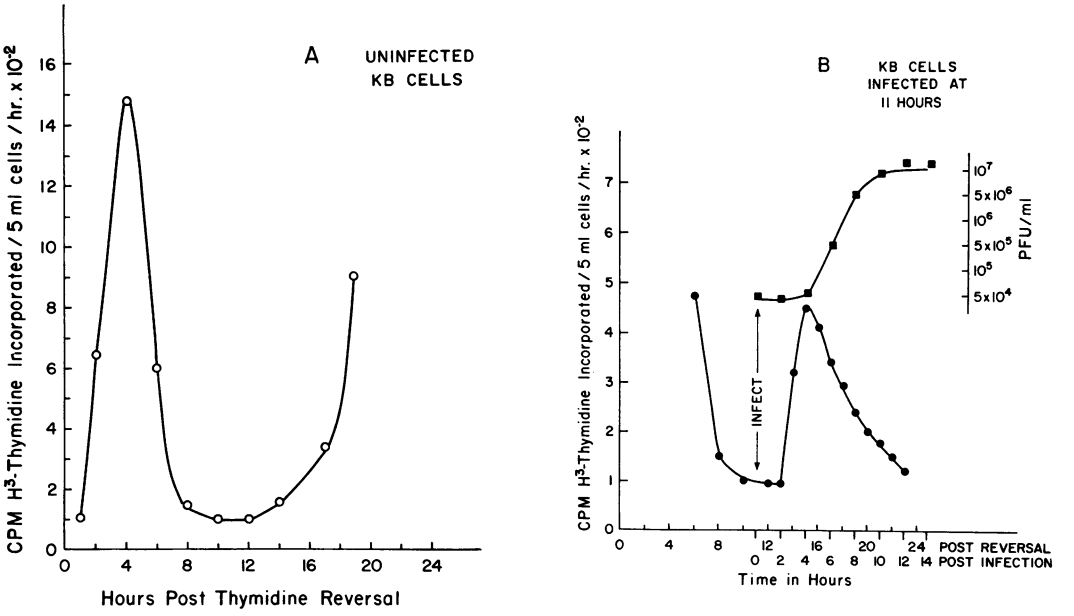

FIG. 6. *Rate of DNA synthesis in uninfected and infected synchronized cultures of KB cells. The conditions employed were essentially the same as in Fig. 1 and 2. (A) Uninfected KB cells. (B) Culture infected at 11 hr post-thymidine reversal. The viral growth curve was constructed as in Fig. 3.*

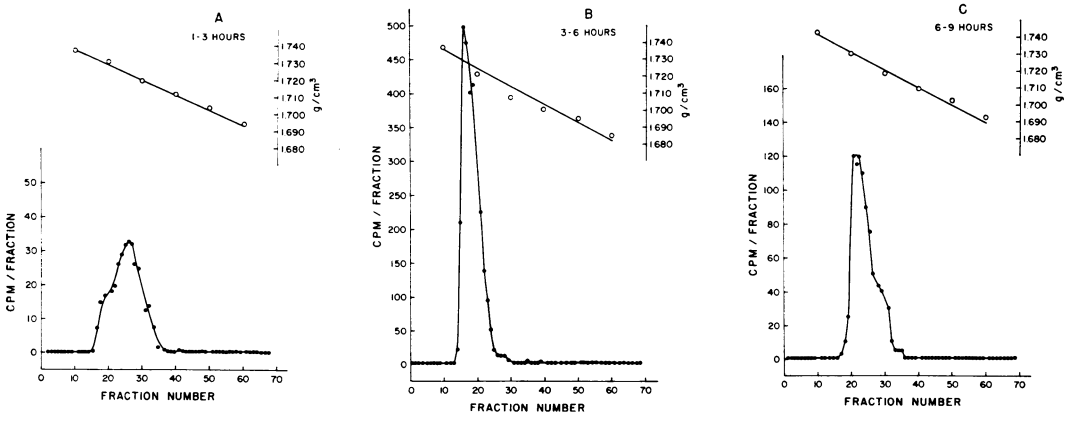

FIG. 7. *Analysis of DNA isolated from synchronized KB cells infected with HSV by isopycnic centrifugation in CsCl. Cells were infected with HSV at 11 hr after synchrony began. At various times, 200-ml portions were incubated in ^{3}H-thymidine-containing medium for the following periods: (A) DNA extracted from cells incubated 1 to 3 hr postinfection; (B) 3 to 6 hr postinfection; (C) 6 to 9 hr postinfection.*

HSV DNA was synthesized during these time periods of labeling. No newly synthesized host DNA was detected. Furthermore, this experiment indicates that HSV DNA synthesis began 1 to 3 hr after infection of the cells. This time agrees with the time indicated by our direct pulse-labeling experiments (Fig. 6B) which also indicated that HSV DNA synthesis began 2 to 3 hr after infection. Identical experiments were performed on synchronized cells infected with HSV 8 hr into the cell cycle. All of the cellular DNA which was synthesized during the 1 to 9-hr period (Fig. 5B) was actually synthesized within the first 3 hr after infection. Approximately 10% of the total DNA synthesized during this early period had the buoyant density of viral DNA. Furthermore, only HSV DNA was synthesized during the 3 to 6 and 6 to 9 hr periods of labeling. These experiments give us confidence that we can follow HSV DNA synthesis exclusively in pulse-labeled synchronized cells infected 11 hr into the cell cycle.

DISCUSSION

This communication presents evidence that the initiation of HSV DNA synthesis is independent of the events of the KB cell cycle. Our data indicate that, no matter when infection took place during the mitotic cycle, viral DNA synthesis began 2 to 3 hr after infection in each case and proceeded at approximately the same rate. Similarly, the formation of new infectious particles of HSV was also shown to be independent of the host cell cycle.

These findings are in general agreement with the work of other investigators on HSV and other members of the herpesvirus group. For example, Ben-Porat and Kaplan (3) working with pseudorabies virus showed that contact-inhibited rabbit kidney cells, which are presumably in G1 phase, supported viral growth with a normal time course for viral DNA synthesis. In addition, it has been shown that HSV is capable of replicating during the late G2 period or in metaphase (26, 34) of the cell cycle. However, it has been reported that mitotic HEp-2 cells will absorb but not replicate HSV (27). Such cells regain their capacity to support viral multiplication after mitosis.

Our present results are in direct contrast to those reported for EAV by Lawrence (23) who observed that initiation of EAV DNA synthesis could only take place during the S phase of the KB cell cycle. Although these viruses are both classified as members of the herpesvirus group on the basis of a number of similar features, there are also significant differences between EAV and HSV. For example, there is a large difference in the DNA base composition of HSV and EAV. The molar percentage of GC is 67% for HSV but only 56% for EAV (7). It is apparent from all of these results that one cannot generalize about the biochemical events that occur during infection by members of the herpesvirus group as it is presently constituted.

As for other DNA viruses, the data of Cairns (6) and Groyon and Kniarzeff (15) indicate that the synthesis of vaccinia DNA does not depend on the mitotic phase of the host cell. In addition, Salzman et al. (32) showed that in vaccinia-

infected monolayer cells viral DNA synthesis can be initiated in most cells of a population, regardless of whether the cell is making DNA at the time of infection. Hodge and Scharff (16) report that, when synchronized cultures of HeLa cells were infected with type 5 adenovirus, the synthesis of viral DNA was independent of the growth cycle. Our results with HSV infection of synchronized KB cells are in accord with those reported above for vaccinia virus and adenovirus.

The second point of this communication is that we can create conditions that allow us to follow HSV DNA synthesis directly in logarithmically growing KB cells in the virtual absence of cellular DNA synthesis. This situation arises when synchronized KB cells are infected with HSV during G1. As pointed out by Roizman (28) and Kaplan (17), the most significant problem associated with kinetic studies of herpes DNA synthesis in rapidly growing (unsynchronized) cells is that host DNA synthesis persists for several hours after infection. This meant that an accurate determination of viral DNA required separation of viral from host DNA by centrifugation in CsCl. At best only a fraction of each species of DNA is obtained. We analyzed the rate of HSV DNA synthesis by a direct method, trichloroacetic acid precipitation of ^{3}H-TdR pulse-labeled extracts, and compared these data to those obtained from duplicate preparations subjected to isopycnic centrifugation in CsCl. We found HSV DNA synthesis in KB cells was initiated between 2 to 3 hr after infection. The rate of DNA synthesis increased rapidly, reaching a maximum 4 hr after infection, a time when no increase in progeny virus was detectable. Eight hours after infection, the rate of viral DNA synthesis decreased to 50% of the maximum, and 75% of the total new virus was already made. These results agree substantially with those reported by Roizman and Sydiskis (unpublished results appearing in reference 28) and Roizman and Roane (29).

Several other ways are presumably available to study HSV DNA synthesis in mammalian cells in which cellular DNA synthesis is at a minimum. Kit and Dubbs (20) suggested that it would be of great advantage to have a system in which cells retained their capacity to support viral replication while host-cell chromosomal DNA synthesis was either eliminated or at least minimized. They described three potentially useful cell-virus systems which included (i) primary monolayer cultures of rabbit kidney or rabbit embryo cells, (ii) cold-shocked suspension cultures of mouse fibroblasts, and (iii) a cell line of mouse fibroblasts (L-M-TK$^-$) from which the enzyme thymidine kinase has been deleted. As mentioned previously, Ben-Porat and Kaplan (3) used contact-inhibited primary monolayer rabbit kidney cells to advantage to follow pseudorabies DNA synthesis in the virtual absence of host cell DNA synthesis. The second system mentioned (cold shock) has undesirable side effects on cells. Kit and Dubbs (20) found that during the first 7 hr at 37 C after cold shock, the rate of incorporation of ^{3}H-TdR into host DNA was greatly reduced. However, it took 30 hr of incubation at 37 C for the cell population to recover completely from the cold-shock treatment. Furthermore, although these cells supported vaccinia growth, it was at a somewhat retarded rate.

The desirability for studying virus infection in exponentially dividing cells has been pointed out by Ginsberg (10) and by Graham (13). They suggest employing rapidly growing (suspended) cells because optimum synthesis of host as well as viral macromolecules and maximum yield of virus occur in these cultures.

The last system mentioned for studying viral DNA synthesis when cellular DNA synthesis is at a minimum involved the use of exponentially growing L-M cells which are deficient in thymidine kinase (TK$^-$; reference 21). Because the uninfected L-M (TK$^-$) cells lack thymidine kinase, they cannot incorporate ^{3}H-TdR into cellular DNA. However, a virus-specific thymidine kinase (5) is induced in L-M (TK$^-$) cells after infection with vaccinia virus, and, since this virus replicates in the cytoplasm, ^{3}H-TdR is incorporated almost entirely into cytoplasmic viral DNA. However, this system is probably not useful for studying a DNA virus such as HSV which replicates in the nucleus for the following reason. The virus-specific thymidine kinase which is also induced after HSV infection of L-M (TK$^-$; reference 19), would provide a pool of labeled thymidine monophosphate in the nucleus that would be utilized in the synthesis of host as well as viral DNA.

The procedure we have reported in this paper provides a simple and reliable means for studying HSV DNA synthesis in rapidly growing cells without interference by host DNA synthesis.

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LITERATURE CITED

1. Becker, Y., H. Dym, and I. Sarov. 1968. Herpes simplex virus DNA. Virology 36:184–192.
2. Bello, L. J. 1968. Synthesis of DNA-like RNA in synchronized cultures of mammalian cells. Biochim. Biophys. Acta 157:8–15.
3. Ben-Porat, T., and A. S. Kaplan. 1963. The synthesis and fate of pseudorabies virus DNA in infected mammalian cells in the stationary phase of growth. Virology 20:310–317.
4. Bootsma, D., L. Budke, and O. Vos. 1964. Studies on synchronous division of tissue culture cells initiated by excess thymidine. Exp. Cell Res. 33:301–309.
5. Buchan, A., D. W. Watson, D. R. Dubbs, and S. Kit. 1970. Serological study of a mutant of herpesvirus unable to stimulate thymidine kinase. J. Virol. 5:817–818.
6. Cairns, J. 1960. The initiation of vaccinia infection. Virology 11:603–623.
7. Darlington, R. W., and C. C. Randall. 1963. The nucleic acid content of equine abortion virus. Virology 19:322–327.
8. Flamm, W. G., H. E. Bond, and H. E. Burr. 1966. Density gradient centrifugation of DNA in a fixed angle rotor: a higher order of resolution. Biochim. Biophys. Acta 129:310–319.
9. Flanagan, J. F. 1967. Virus-specific ribonucleic acid synthesis in KB cells infected with herpes simplex virus. J. Virol. 1:583–590.
10. Ginsberg, H. S. 1969. Biochemistry of adenovirus infection, p. 329–359. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, New York.
11. Ginsberg, H. S., L. J. Bello, and A. J. Levine. 1967. Control of biosynthesis of host macromolecules in cells infected with adenovirus, p. 547–572. *In* J. S. Colter and W. Paranchych (ed.), The molecular biology of viruses. Academic Press Inc., New York.
12. Goodheart, C. R. 1970. Herpes virus and cancer. J. Amer. Med. Ass. 211:91–96.
13. Graham, A. F. 1959. Symposium on the biology of cells modified by viruses or antigens. III. Physiological conditions for studies of viral biosynthesis in mammalian cells. Bacteriol. Rev. 23:231–234.
14. Green, M., and M. Piña. 1964. Biochemical studies on adenovirus multiplication. VI. Properties of highly purified tumorigenic adenovirus and their DNA's. Proc. Nat. Acad. Sci. U.S.A. 51:1251–1259.
15. Groyon, R. M., and A. J. Kniazeff. 1967. Vaccinia virus infection of synchronized pig kidney cells. J. Virol. 1:1255–1264.
16. Hodge, L. D., and M. D. Scharff. 1969. Effect of adenovirus on host cell DNA synthesis in synchronized cells. Virology 37:554–564.
17. Kaplan, A. S. 1969. Herpes simplex and pseudorabies viruses. Virology. Monogr. no. 5. Springer-Verlag, New York.
18. Kaplan, A. S., and T. Ben-Porat. 1963. The pattern of viral and cellular DNA synthesis in pseudorabies virus-infected cells in the logarithmic phase of growth. Virology 19:205–214.
19. Kit, S., and D. R. Dubbs. 1963. Acquisition of thymidine kinase activity by herpes simplex infected mouse fibroblast cells. Biochem. Biophys. Res. Commun. 11:55–59.
20. Kit, S., and D. R. Dubbs. 1963. Biochemistry of vaccinia-infected mouse fibroblasts (strain L-M) IV. ^{3}H-thymidine uptake into DNA of cells exposed to cold shock. Exp. Cell Res. 31:397–406.
21. Kit, S., D. R. Dubbs, L. J. Piekarski, and T. C. Hsu. 1963. Deletion of thymidine kinase activity from L cells resistant to bromodeoxyuridine. Exp. Cell Res. 31:297–312.
22. Knowland, J. S. 1970. Polyacrylamide gel electrophoresis of nucleic acids synthesized during the early development of *Xenopus laevis* Daudin. Biochim. Biophys. Acta 204:416–429.
23. Lawrence, W. C. 1971. Evidence for a relationship between equine abortion (herpes) virus deoxyribonucleic acid synthesis and the S phase of the KB cell mitotic cycle. J. Virol. 7:736–748.
24. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208–218.
25. Nahmias, A. J., S. Kibrick, and R. C. Rosan. 1964. Viral leukocyte interrelationships. 1. Multiplication of a DNA virus-herpes simplex in human leukocyte cultures. J. Immunol. 93:69–74.
26. Rapp, F., and T. C. Hsu. 1965. Viruses and mammalian chromosomes. IV. Replication of herpes simplex virus in diploid Chinese hamster cells. Virology 25:401–411.
27. Roizman, B. 1961. Virus infection of cells in mitosis. I. Observations on the recruitment of cells in karyokinesis into giant cells inudced by herpes simplex virus and bearing on the site of virus antigen formation. Virology 13:387–401.
28. Roizman, B. 1969. The herpes viruses—a biochemical definition of the group. Curr. Top. Microbiol. Immunol. 49:1–79.
29. Roizman, B., and P. R. Roane, Jr. 1964. The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology 22:262–269.
30. Russell, W. C., and L. V. Crawford. 1963. Some characteristics of the deoxyribonucleic acid from herpes simplex virus. Virology 21:353–361.
31. Russell, W. C., E. Gold, H. M. Keir, H. Omura, D. H. Watson, and P. Wildy. 1964. The growth of herpes simplex virus and its nucleic acid. Virology 22:103–110.
32. Salzman, N. P., A. J. Shatkin, E. D. Sebring, and W. Munyon. 1962. On the replication of vaccinia virus. Cold Spring Harbor Symp. Quant. Biol. 27:237–244.
33. Stoker, M. G. P., and A. Newton. 1959. The effect of herpes virus on HeLa cells dividing parasynchronously. Virology 7:438–448.
34. Waubke, R., H. zur Hausen, and W. Henle. 1968. Chromosomal and autoradiographic studies of cells infected with herpes simplex virus. J. Virol. 2:1047–1054.