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Cell Cycle Dependency of Murine Cytomegalovirus Replication in Synchronized 3T3 Cells

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Synchronized murine 3T3 cells have been used to investigate the possible dependency of murine cytomegalovirus replication upon the cell cycle. The normal latent period of 12 h characteristic of asynchronous 3T3 cells was protracted to more than 24 h after an early G1 infection in synchronous cells. In this case viral progeny were not detected until after the initiation of the host S-phase. Cells maintained in the G1 phase did not replicate virus. This failure could not be explained by a decrease in virus penetration but was apparently due to a requirement for an event associated with the host S-phase. Thymidine-induced inhibition of cell cycle traverse also blocked virus replication. Viral DNA synthesis did not initiate until after the initiation of host DNA. In contrast, herpes simplex virus type 1 replicated in 3T3 cells independently of the cell cycle.

Studies in this laboratory, as well as earlier reports, indicate certain unique features of the murine cytomegalovirus (MCV). These include centrifugal enhancement of infectivity (10, 16), multicapsid morphology (9), and genome size (14). In addition, we have observed that permissive viral replication is contingent upon exponential host cell growth. The dependence of viral growth on the physiological status of the cell suggests that MCV has a predilection for cells in some phase of the cell cycle other than G1.

Although the majority of studies on herpesviruses have involved asynchronously growing cells, a few reports have documented interactions between virus and synchronized cells (4, 5, 12). For example, herpes simplex virus type 1 (HSV-1) was shown to replicate independently of the cell cycle using double-thymidine(TdR)-induced synchrony in human KB cells (4). In another report, equine abortion virus was shown to be dependent upon the S-phase in vitro (12). Thus, the ability of a herpesvirus to replicate independently of the cell cycle appears to be unrelated to genome size. Our analysis with MCV indicates that, like equine abortion virus in vitro, MCV requires events associated with the host S-phase for initiation of viral DNA synthesis.

MATERIALS AND METHODS

Cells. 3T3 cells (Flow Laboratories), HEp-2 cells (Microbiological Associates), and cells obtained from freshly explanted mouse embryos (ME) were grown in Dulbecco modified minimal essential medium supplemented with 10% fetal bovine serum plus 20 μg of gentamicin per ml. Cells were incubated in a humidified atmosphere containing 5% CO_2-95% air at 37°C or in roller bottles at 37°C (Bellco). 3T3 cells were passed a maximum of 20 times, after which fresh cells were obtained from frozen stocks.

Viruses. The Smith strain of MCV was used. Virus stocks were prepared by low-multiplicity passage (<1 PFU/cell) in ME cultures. The P strain of HSV-1 was used after plaque purification on HEp-2 cells.

MCV and HSV-1 were assayed by plaque formation on either freshly plated ME or 3T3 cells and HEp-2 cells, respectively, as described before (14). The multiplicity of infection in these experiments was 20 to 30 PFU/cell. Virus titers are expressed as PFU per milliliter of cells plus supernatant fluid. Intracellular virus was released by freeze-thawing the cells three times.

Synchronization of 3T3 cells. 3T3 cells were synchronized by the "serum-split" method, which entailed serum activation of quiescent G1-arrested cells. 3T3 cells in 90-mm Falcon plates were allowed to reach confluence and left without medium change for a total of 4 to 10 days. Stimulation of quiescent cells was achieved by rinsing the cell sheet with prewarmed medium, trypsinizing for 1 to 2 min, and replating in a five- to eightfold larger volume of fresh minimal essential medium plus 10% fetal bovine serum.

The degree of synchrony achieved was determined by 30-min pulses with 1 μCi of [*methyl*-^{3}H]-TdR (New England Nuclear Corp., 20 Ci/mmol) per ml. The pulse was terminated by the addition of chilled (4°C) TNE (0.01 M Tris-hydrochloride, pH 7.5, 0.1 M NaCl, 0.001 M EDTA). To the resuspended

cells, ice-cold trichloroacetic acid was added to 10% for 1 h at 4°C. The precipitates were then collected on filters, washed twice with 5% trichloroacetic acid and once with ethanol, and dried, and then radioactivity was measured in a liquid scintillation counter.

Cell numbers were determined by trypsinization, gentle syringing to disperse cell clumps, and counting duplicate samples in a Coulter counter.

Mitotic cells were determined by swelling detached cells in hypotonic medium (1% sodium citrate) for 15 min at 4°C. The cells were then fixed by dropwise addition of methanol-acetic acid (3:1), spread onto glass slides, stained, and counted using phase-contrast microscopy. Between 500 and 1,000 cells were scored for mitotic figures.

The fraction of nuclei in the S-phase was also determined by autoradiography. Cells were labeled with 1 μCi of [*methyl*-^{3}H]TdR for 30 min at 37°C, fixed for 30 min with methanol-acetic acid (3:1), washed under cold running water for 30 min, and dried. The plates were then overlaid with Ilford emulsion L4 (Ilford Ltd., Ilford, Essex, England), exposed for 1 week, developed with Kodak Microdol-X, and observed under phase contrast for quantitation.

Double-TdR treatment and mitotic harvest. 3T3 cells seeded at a low density (10^5 cells per plate) were treated with 2 mM TdR for 12 h. The cells were then washed free of TdR and given fresh minimal essential medium plus 10% serum for 8 h, at which time 2 mM TdR was added for an additional 12 h. These cells were infected with MCV in TdR-free medium. After the adsorption period, 2 mM TdR was added and viral growth was assayed. This procedure resulted in a synchronous blockade in the cell cycle such that the majority of the population accumulated at the G1/S border (1, 21).

The method described by Thorn (19) was used for harvesting mitotic 3T3 cells. This procedure was used as a means of achieving selection-type synchrony.

Extraction and purification of DNA. Viral DNA, from virus prepared by differential centrifugation, and cellular DNA were purified by the sodium dodecyl sulfate, Pronase, and phenol methods described previously (14).

Nucleic acid hybridization. The technique for nucleic acid hybridization is essentially the same as described previously (14, 15), with minor modifications. Briefly, non-radioactive CsCl gradient-purified viral DNA, or non-radioactive DNA from other sources, was fixed to nitrocellulose filters (Millipore Corp., type HAWP; 0.45-μm pore size) by gravity filtration in 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), and dried at 80°C for 4 h. The test solution of purified, labeled cellular or viral DNA was prefiltered through a 0.45-μm nitrocellulose filter, sheared by sonication, and denatured by heating at 100°C for 10 min. The annealing reaction was then carried out in 0.1% sodium dodecyl sulfate plus 6× SSC at 65°C for 12 to 24 h, after which the filters were washed extensively with 3 × 10^{-3} M Tris-chloride (pH 9.3). Filters were dried at 80°C, and radioactivity was measured in a liquid scintillation spectrometer.

RESULTS

Synchrony in uninfected 3T3 cells. Mouse 3T3 cells were synchronized by the serum-split method as described above. Cells left without medium change became density inhibited, forming a confluent cell sheet of unit thickness containing predominantly G1-arrested cells (8). Any time after 4 days without medium change, the cells could be stimulated to re-enter the cell cycle in a synchronous fashion by dilution into fresh serum plus medium. Quantitation of synchrony with 30-min [^{3}H]TdR pulses, followed by autoradiography, demonstrated greater than 85% positive nuclei by 20 h post-serum-split. The resulting synchrony is shown in Fig. 1. The main features of this synchrony are as follows: (i) one synchronous S-phase initiating at 12 to 14 h post-serum-split with a peak at 20 h; (ii) a second S-phase peak at 36 to 38 h, followed by rapid desynchronization; (iii) an approximate doubling of cell numbers between 22 and 28 h; (iv) an increase in the mitotic fraction around 26 h.

MCV replication in synchronous and asynchronous exponential 3T3 cells. Figure 2 shows a comparison of viral growth curves in asynchronous exponential 3T3 cells and synchronized 3T3 cells infected in the early G1 period (1 to 2 h post-serum-split). MCV in randomly growing cells replicated with a latent period of 10 to 12 h. However, the latent period in the synchronized cells was protracted to more than 24 h. The final yield of virus per cell was approximately equal for synchronized and randomly growing cells. The time course of viral growth in G1-infected 3T3 cells was similar with both the standard and centrifugal modes of infection, and the protracted latent period could not be altered by increasing the

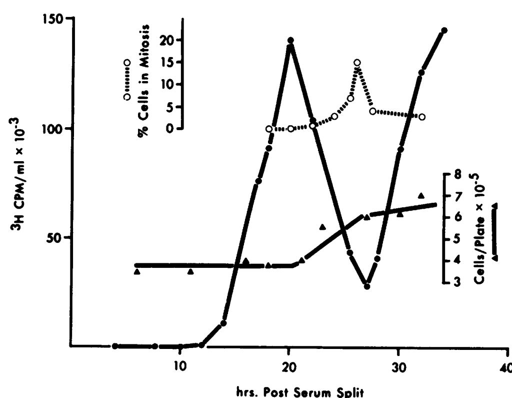

FIG. 1. *3T3 cells synchronized by the serum-split method. DNA synthesis (●), cell numbers per plate (▲), and mitotic fractions (○) were all determined as described in the text.*

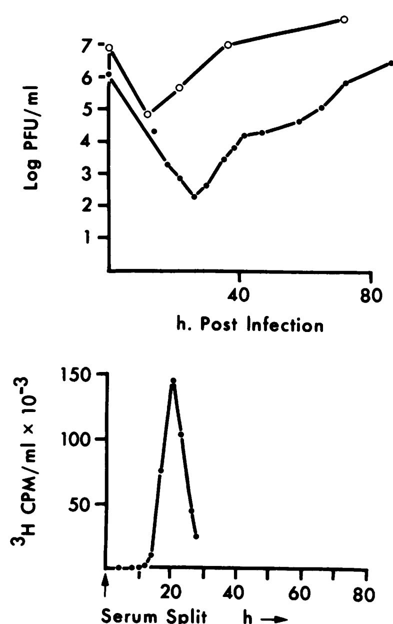

FIG. 2. *MCV growth kinetics in synchronous and asynchronous 3T3 cells. (Top) Synchronous 3T3 cells (●) were "serum split" at a density of 10^5 cells per plate and infected between 1 and 2 h later at a multiplicity of infection (MOI) of 20 PFU/cell. Asynchronous cells (○) were present at a density of 8.2 × 10^5 cells per plate and infected at the same MOI (20 PFU/cell). Virus assays represent total intracellular plus extracellular virus. (Bottom) Rate of DNA synthesis in mock-infected synchronous 3T3 cells (●).*

input multiplicity. The trypsinization procedure used to induce synchrony was not responsible for the protracted latent period, since the same results were observed with cells synchronized without trypsinization, and trypsinization (2 h prior to infection) did not protract the viral growth curve in asynchronous cells. Furthermore, in both an early G1 infection (at 2 h post-serum-split) and a late G1 infection (at 11 h post-serum-split), progeny virus appeared at the same time (i.e., during the S-phase). Thus, a delay in the G1 infection by 9 h resulted in a corresponding decrease in the latent period of viral growth.

Atlhough the protracted latent period after infection in G1 was independent of the multiplicity of infection, it was necessary to ascertain if there were quantitative differences in attachment or penetration of virus between G1, S, and asynchronous cells. Since trypan blue exclusion assays on G1-arrested 3T3 cells (prior to serum-split) demonstrated that greater than 90% of the cells were viable, altered viral growth could not be ascribed to a preponderance of moribund cells in the synchronous population. In addition, the data in Table 1 argue against selective uptake of MCV by G1, S, or exponential asynchronous 3T3 cells. A similar proportion of radioactive virus was taken up by the three types of culture. Furthermore, infectious-center assays from G1-phase cells and asynchronous cells (infected at the same multiplicity of infection) indicated >90% of the cells were susceptible to MCV in both cases. These data are consistent with the notion that the protracted latent period is a postpenetrational event.

Replication of virus in the presence of 2 mM TdR. If MCV requires events associated with the host S-phase, there should be a correlation between cell cycle traverse and viral growth. Table 2 shows the dose response relationship between MCV replication and the concentration of TdR in the medium. Viral replication was severely inhibited under conditions that also inhibited cellular DNA synthesis and division (1), and this inhibition was dose dependent. Consistent with this finding, we observed that 3T3 cells maintained in the G1 phase of the cell cycle did not support viral replication (unpublished observation). These results imply an S-phase dependency for MCV.

Initiation of viral DNA synthesis in synchronized 3T3 cells. One explanation for delayed viral growth in synchronous cells is that replication of MCV DNA requires S-phase events.

TABLE 1. *Uptake of [^{3}H]TdR-labeled MCV into G1, S, and asynchronous 3T3 cells*[a]

Cells (2 × 10^5)	Input cpm	cpm adsorbed	Input adsorbed (%)
G1 phase	2.5 × 10^3	1.85 × 10^3	74
S-phase	1.76 × 10^3	1.20 × 10^3	68
Asynchronous	1.89 × 10^3	1.36 × 10^3	72

[a] [^{3}H]TdR-labeled MCV was prepared free of detectable soluble radioactivity by differential centrifugation and DNase treatment. Radioactive virus was added to synchronous or asynchronous cultures and counts per minute (as trichloroacetic acid-precipitable material within cells) were determined. The multiplicity of infection was 20 PFU/cell for all three types of culture.

TABLE 2. *Effect of TdR concentration on MCV yield*

Concn of TdR (mM)[a]	Final yield (PFU/ml)	Maximum yield (%)
0	6.7 × 10^8	100
0.002	7.1 × 10^7	10.6
0.02	4.2 × 10^7	6.3
0.2	3.7 × 10^7	5.5
2	6 × 10^6	0.9

[a] 3T3 cells were given a double-TdR block as described in the text and infected with MCV, and various amounts of TdR were added back immediately after the adsorption period.

Therefore, the appearance of viral DNA in G1 and S phases was examined by DNA-DNA annealing, using DNA extracted from cells continuously labeled with ^{32}P. Viral DNA was not detected at 16 h postinfection, by either annealing on filters (Table 3) or CsCl gradient centrifugation (data not shown). By 20 h, however, ^{32}P-labeled MCV DNA was detected, which suggests that DNA synthesis began in the period between 16 and 20 h postinfection (approximately mid-S-phase). Between 20 and 24 h postinfection, viral DNA synthesis was clearly maximal. Thus, viral DNA synthesis could not be detected in synchronized cells until after 16 h postinfection, in contrast to asynchronous cells, which produce infectious virions within 12 h postinfection.

Other methods of cell synchronization. To verify our results with serum-split-induced synchrony, we attempted to synchronize the cells by other methods, namely, the double-TdR block (1, 21) and mitotic harvest. The preceding results (Table 2) indicate that synchrony induction through a nucleoside blockade is not ideal for MCV.

Selection synchrony by mitotic harvest was therefore attempted (19). Unfortunately, our line of 3T3 cells was not amenable to this procedure due to the tenuous attachment of interphase cells. This resulted in a large contamination of mitotic cells by interphase cells and a subsequent low degree of synchrony. However, we found that secondary- or tertiary-passage ME cells could be synchronized by the serum-split method. Our results with these ME cells were the same as with synchronized 3T3 cells; namely, after an early G1 infection, viral growth did not occur until S-phase (after 24 h post-serum-split) in both cell systems. Thus, the long latent period observed with 3T3 cells is not unique to that cell line.

Replication of HSV-1 in serum-split synchronous 3T3 cells. To be certain that the protracted G1 period from 0 to 12 h post-serum-split was not artifactual, we determined the ability of a known cell cycle-independent her-

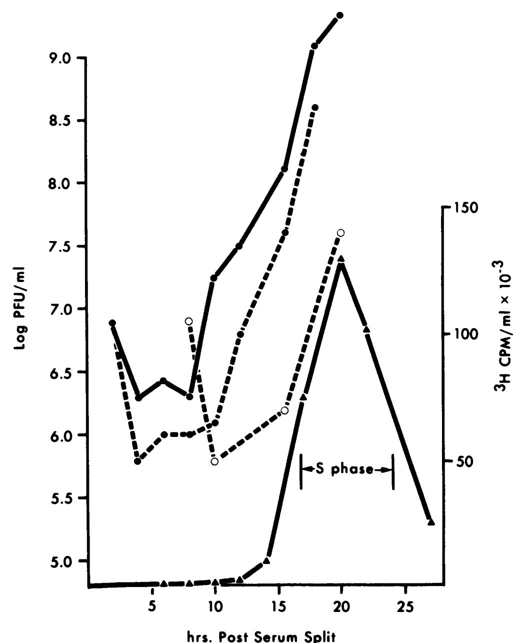

FIG. 3. *Replication of HSV-1 in synchronous and asynchronous 3T3 cells. 3T3 cells were synchronized by the serum-split method and infected in early G1 (●----●) or late G1 (○----○). Asynchronous 3T3 cells were infected (●——●) for comparison. Total virus was assayed on 3T3 cells. The host S-phase ([^{3}H]-TdR incorporation in uninfected synchronous cells) is also shown (▲).*

TABLE 3. *DNA-DNA annealing*

Source of ^{32}P-labeled DNA in solution[a]	Specific radioactivity (cpm/μg)	Input μg added (cpm)	cpm hybridized to 1.0 μg of DNA on filters[b]		
			3T3 cells	Calf thymus	MCV
Synchronized infected 3T3 cell DNA harvested at 16 h p.i.	54	65.7 (3.55×10^3)	82	8	3
Synchronized infected 3T3 cell DNA harvested at 20 h p.i.	382	74.7 (2.85×10^4)	407	30	435
Synchronized infected 3T3 cell DNA harvested at 24 h p.i.	810	92.7 (7.51×10^4)	370	100	3,333
Synchronized mock-infected 3T3 cell DNA harvested at 16 h p.i.	719	67.0 (4.82×10^4)	483	51	5

[a] Carrier-free ^{32}P (New England Nuclear Corp.) was added to mock- or virus-infected cells (250 μCi/culture) immediately after serum activation. DNA was extracted and purified at the indicated times and annealed to non-radioactive DNA on filters. p.i., Postinfection.

[b] Corrected for machine background of 20 to 30 cpm.

pesvirus, HSV-1 (4), to replicate outside of S-phase in our synchronous system. Using doubly TdR-synchronized human KB cells, Cohen et al. (4) demonstrated that HSV-1 could replicate without regard for host cell cycle events. Figure 3 shows our results with HSV-1 in serum-split synchronized 3T3 cells infected at two points (early and late G1) and, for comparison, viral growth in asynchronous (exponential) 3T3 cells. The kinetics of viral growth did not vary significantly in any of the three situations, suggesting that, in accordance with Cohen et al. (4), HSV-1 does not have a proclivity for cells in any one particular phase of the cell cycle. Therefore, our results with MCV are not vitiated by an aberrant synchrony induction procedure, and the serum-split method of synchronization is probably valid for the study of cell cycle and herpesvirus interactions.

DISCUSSION

These studies were undertaken to determine if MCV was dependent or independent of the host S-phase for MCV replication. The results fortify the concept that MCV is dependent on the physiological state of the cell, and that the virus requires events associated with the host S-phase for initiation of viral DNA synthesis.

Using synchronous 3T3 cells, we observed that an early G1 infection culminated in an unusually long latent period of viral growth. The normal latent period of 10 to 12 h in asynchronous cells was protracted to 24 h postinfection. The delay in the onset of appearance of progeny virus suggests that G1 cells are not primed for initiation of viral replication. The latent period of the viral growth cycle could be decreased by infecting at a later time in G1, closer to the host S-phase. Thus, the "critical" region of the cell cycle necessary for viral replication was localized in early to middle S-phase.

Other lines of evidence are consistent with the conclusion that MCV requires the host S-phase for initiation of DNA synthesis. First, inhibition of cell cycle traverse by excess TdR prevented viral replication. A similar result was reported for HSV-2 (3, 17). Second, 3T3 cells maintained in G1 did not support viral growth.

In asynchronous 3T3 cells, viral DNA synthesis commenced by 10 to 12 h postinfection, as determined by the appearance of infectious viral progeny by 12 h and the presence of DNA at the density of MCV DNA on CsCl gradients (unpublished data). Henson et al. (7) have also shown that MCV DNA is first synthesized within 12 h postinfection in asynchronous ME cells. Synchronous cells, in contrast, did not produce viral progeny or viral DNA in this period (after an early G1 infection). DNA-DNA annealing studies indicated that MCV cannot initiate viral DNA synthesis until the early to middle region of the host S-phase. Therefore, an event in S-phase (not necessarily host DNA synthesis) must be exploited by the virus as a prerequisite to viral DNA synthesis. This priming event may be located in the period between 16 and 20 h post-serum-split. The precise nature of the priming event is a matter of speculation. However, any function displaying a transient appearance in the cell cycle limited to S-phase would be a candidate. These would include "periodic" enzymes (13) synthesized only in S-phase and involved in DNA synthesis (e.g., TdR kinase, DNA polymerase), as well as deoxynucleotide pools (2, 18). In density-inhibited 3T3 cells, only low levels of deoxynucleotide pools are present (20). The inability of MCV to replicate in G1-arrested 3T3 cells may be linked to this phenomenon.

MCV cell cycle dependency has also been verified in freshly explanted ME cells synchronized by the serum-split method. This demonstrates that cell cycle dependency is not an artifact unique to an established cell line.

We feel that the demonstration of cell cycle dependency is significant since it provides a means of studying other aspects of herpesvirus replication. For example, our observation that MCV cannot replicate in G1-arrested cells is being developed as a model system for latency among the cytomegalovirus group. Although MCV cannot replicate under these conditions, virus may be activated as late as 8 days postinfection, by providing the appropriate stimulus (fresh serum plus medium) for cell cycle traverse (Muller, manuscript in preparation). Recent reports with Epstein-Barr virus and simian virus 40 have indicated that activation of a latent virus is a cell cycle-related process (6, 11).

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