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Relationship Between Replication of Simian Virus 40 DNA and Specific Events of the Host Cell Cycle

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The relationship between replication of simian virus 40 (SV40) DNA and the various periods of the host-cell cycle was investigated in synchronized CV_1 cells. Cells synchronized through a double excess thymidine procedure were infected with SV40 at the beginning or the middle of S, or in G_2. The first viral progeny DNA molecules were in all instances detected approximately 20 h after release from the thymidine block, independent of the time of infection. The length of the early, prereplicative phase of the virus growth cycle therefore depended upon the period of the cell cycle at which the cells were infected. Infection with SV40 was also performed on cells obtained in early G_1 through selective detachment of cells in metaphase. As long as the cells were in G_1 at the time of infection, the first viral progeny DNA molecules were detected during the S period immediately following, whereas if infection took place once the cells had entered S, no progeny DNA molecule could be detected until the S period of the next cell cycle. These results suggest that the infected cell has to pass through a critical stage situated in late G_1 or early S before SV40 DNA replication can eventually be initiated.

Current knowledge of the biochemical events which occur during the replication cycle of simian virus 40 (SV40) in permissive host cells (for a review, see 18) has been obtained through the use of either resting or randomly growing cell cultures. Depending upon which of these systems was used, even at multiplicities of infection high enough to insure the efficient infection of all the cells in the culture, large variations have been found, both in the duration of the overall virus growth cycle, and in the length of the early period preceding onset of viral DNA replication (28).

Similar observations have been reported in the case of polyoma virus (43, 44). Also, experiments performed with inhibitors of DNA synthesis have shown that replication of the polyoma virus genome is apparently delayed in cells which are infected in the vicinity of the S phase (3, 4, 23).

The present study was, therefore, undertaken to ascertain whether a relationship might exist between the different periods of the host-cell cycle and the time course of SV40 DNA replication.

MATERIALS AND METHODS

Cells and virus. Subcloned epithelioid CV_1 cells (28) were grown without antibiotics in Eagle minimal essential medium (MEM) supplemented with 10% tryptose phosphate and 1% glucose (MCV_1) to which 5% calf serum was added. Infections were performed with 0.2 ml of the required dilution of a stock of the large plaque strain of SV40, at an input multiplicity of 40 to 60 PFU per cell. After 90 min of adsorption at 37 C, the cell monolayers were rinsed twice with a few milliliters, then overlaid with 5 ml of the appropriate medium and incubated at 37 C under 5% CO_2.

Cell synchronization. Synchronization at the beginning of S by the excess thymidine procedure (6, 14, 30, 49) was performed on cells seeded at 1.5×10^5 cells per 5-cm plastic petri dish (Greiner, France). The cells were overlaid with 5 ml of MCV_1 supplemented with antibiotics (100 U of penicillin and 0.1 mg of streptomycin per ml) and 5% calf serum. One day after seeding, the monolayers were rinsed twice with MEM, then overlaid with 5 ml of MEM supplemented with 1% calf serum, antibiotics, and 7 mM thymidine (blocking medium). After 15 to 16 h of incubation at 37 C, the cells were washed twice with MCV_1 supplemented with 5% calf serum, then incubated under 5 ml of MCV_1 supplemented with 5% calf serum, antibiotics, and 10^{-5} M deoxyadenosine,

deoxyguanosine, and deoxycytidine (unblocking medium). Nine to 10 h later, the cells were again blocked with excess thymidine for an additional 15 to 16 h. Release from the second thymidine block was performed as above and was taken as zero time of the experiment. In some experiments, dialyzed calf serum was used in the various media.

Synchronization in G_1 was obtained by the method of selective detachment of cells in metaphase (33, 34, 42) on randomly growing cells seeded in 2-liter glass Roux bottles. In an attempt to increase the yield of metaphase cells, use of colcemid was tested (11, 39). This procedure gave rise to numerous giant cells in which reinitiation of DNA synthesis occurred without intervening mitoses. The difficulty of obtaining large quantities of viable mitotic cells was finally met by first submitting cell cultures in 2-liter glass Roux bottles to the double synchronization procedure with excess thymidine as described above, then washing them with MEM for suspension culture (SM) supplemented with 5% calf serum, and allowing the cells to proceed to metaphase in SM supplemented with 5% calf serum, antibiotics, and 10^{-5} M deoxyadenosine, deoxyguanosine, and deoxycytidine. Ten to 12 h later, cells having reached metaphase were detached from their support through shaking of the bottles. The resulting cell suspension ($2.5 \times 10^5 - 5 \times 10^5$ cells per ml) was diluted in prewarmed unblocking medium to a final concentration of 3×10^4 to 6×10^4 cells per ml, and 5-ml samples were seeded into plastic petri dishes. Seeding was taken as zero time of the experiment. The great majority of the cells attached to the plastic support within 1 h after seeding.

Labeling of DNA. Labeling of the DNA was performed with 4 μCi of tritiated thymidine per ml (20-25 Ci/mmol, C.E.A., Saclay). At the end of the labeling period, the medium was withdrawn, the cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS) and overlaid with 1 ml of 0.1 M Tris-hydrochloride, pH 7.4, 0.002 M EDTA, 0.6% sodium dodecyl sulfate (SDS). Selective extraction of viral DNA was performed according to Hirt (22) except where otherwise noted. Determination of radioactivity in both extraction pellet (high-molecular-weight DNA) and supernatant fluid (low-molecular-weight DNA) and analysis of viral DNA molecules through sucrose gradient centrifugations were performed as previously described (17, 28).

Counting of cells. Cells were detached from their support with 2 ml of trypsin-EDTA (0.005% trypsin and 1.8×10^{-4} M EDTA), and counted in a hematocytometer. For determination of mitotic index, cells were arrested in metaphase through the use of 0.1 μg of colcemid per ml in SM supplemented with 5% dialyzed calf serum. Metaphase-arrested cells were selectively detached from their support through gentle pipetting and counted as above.

RESULTS

Typical results obtained with the double thymidine block procedure are illustrated in Fig. 1. Release from the second thymidine block resulted in a series of successive S periods (labeled 2, 3, and 4 as indicated in Fig. 1) in between which the cell population doubled (closed circles). The first doubling of the number of cells per petri dish required 4 to 5 h. Colcemid arrest of cells in metaphase showed that more than 95% of the cells entered mitosis within that time (inset to Fig. 1). Percentage of synchronization, as calculated by the method of Engelberg (13), was therefore over 50%. In this and other experiments, duration of S periods number 2 and 3 was approximately 8 and 10 h, respectively. It has been commonly observed that synchronization by excess thymidine results in a shortening of the cell generation time (2, 8, 14, 26, 31, 40). As can be seen in Fig. 1, the interval separating S phases number 2 and 3 was only 16 h, whereas the generation time of randomly dividing cultures is 24 h.

Cells synchronized as above were infected with SV40 at the following stages of their cycle: beginning of S (Fig. 2), middle of S (Fig. 3), and G_2 (Fig. 4). Infection with SV40 usually resulted in a lag of approximately 2 h in the timing of the various S phases, which can probably be accounted for by the 90-min adsorption period, and by the time further required for withdrawal of the nonadsorbed virus and rinsing of the plates. In no case did infection with SV40

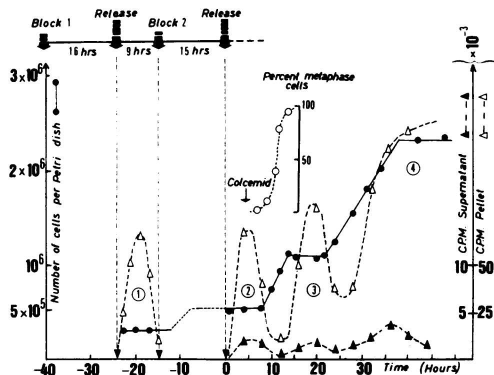

FIG. 1. *Synchronization by double excess thymidine. Subclone CV_1 cells were synchronized by excess thymidine. The rate of DNA synthesis was followed through successive 4-h pulse labelings with 4 μCi of tritiated thymidine per ml. Radioactivity was determined through selective extraction (22) in both high-molecular-weight DNA (△) and low-molecular-weight DNA (▲). Each point represents the trichloracetic acid-precipitable radioactivity recovered from one petri dish. Similar results were obtained with 2-h pulse labelings (not shown). Counting of cells (●) was performed after detachment with trypsin and EDTA. Determination of percentage of metaphase cells (○) was as described in Materials and Methods. Numbers 1 through 4 refer to the successive S phases.*

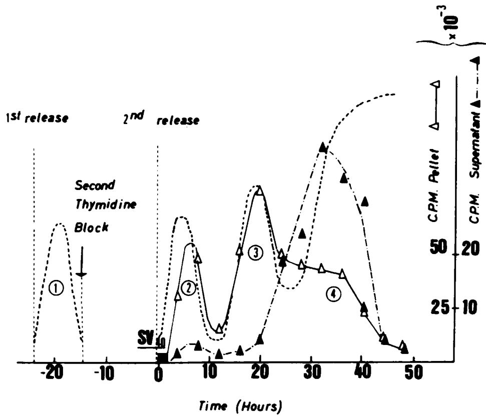

FIG. 2. *Time course of SV40 DNA replication upon infection of cells at the beginning of S. Cells synchronized by excess thymidine as described for Fig. 1 were infected at zero time with 0.2 ml of SV40 (■). At the end of the adsorption period, the cells were overlaid with unblocking medium, and incubated at 37 C. The rate of DNA synthesis was followed as described for Fig. 1. The dashed line, taken from Fig. 1, represents the rate of high-molecular-weight (nuclear) DNA synthesis in uninfected control cells, and numbers 1 to 4 refer to the successive S phases in such cells. Symbols: Δ, high-molecular-weight DNA; ▲; low-molecular-weight DNA.*

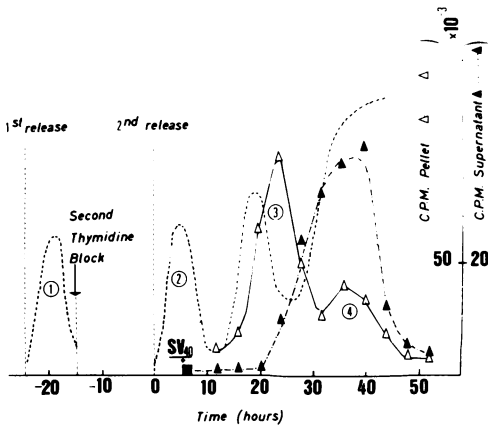

FIG. 3. *Time course of SV40 DNA replication upon infection of cells in the middle of S. Cells synchronized as described for Fig. 1 were infected with SV40 4.5 h after release from the second thymidine block. At the end of the adsorption period, the cells were overlaid again with unblocking medium and further incubated at 37 C. Determination of radioactivity was as described for Fig. 2.*

prevent the normal occurrence of S periods number 2 and 3, irrespective of the time at which the cells had been infected. However, in all three experiments, S phase number 4 was inhibited. This agrees with the observation that in randomly growing cells inhibition of cellular DNA synthesis occurs only at late stages of SV40 multiplication (28). Similarly, while mitoses occurred normally in the infected cells between S phases number 2 and 3, none were detected after S period number 3 (not shown).

Radioactivity in the Hirt extraction supernatant fluids started increasing at approximately 20 h after release from the second thymidine block (closed triangles, Fig. 2–4). Analysis of the labeled material through sucrose gradient centrifugation at both neutral and alkaline pH showed that this increase corresponded to labeling of supercoiled SV40 DNA component I. In all three experiments, the first progeny viral DNA molecules were detected at the same time, corresponding approximately to the middle of S phase number 3. Therefore, the earlier the cells were infected after release from the thymidine block, the more delayed was the onset of replication of SV40 DNA. In the three experiments, viral DNA synthesis lasted approximately from the end of S phase number 3 to that of S phase number 4.

To show that these results were not due to different degrees of adsorption or penetration of the virus, synchronized cells were infected at different stages of their cycle with tritiated thymidine-labeled purified SV40 virions. Multiplicity of infection was adjusted to 50 PFU per cell by appropriate dilution of the labeled virus stock with nonradioactive virus. At the end of the 90-min adsorption period, the cells were rinsed three times with PBS, detached from their support through treatment with trypsin and EDTA, centrifuged, and both the cell number in the sample and the cell-associated

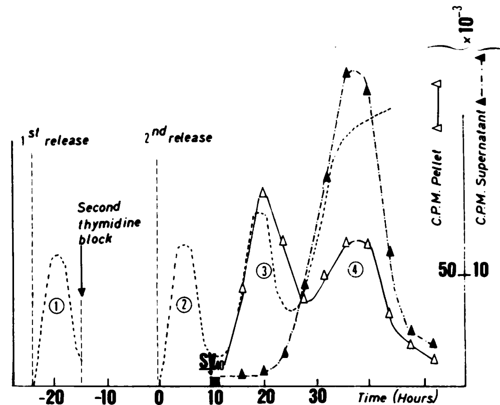

FIG. 4. *Time course of SV40 DNA replication upon infection of cells in G_2. Cells synchronized as described for the preceding figures were infected with SV40 9 h after release from the second thymidine block (see details in the legends to the preceding figures).*

TABLE 1. *Influence of the various periods of the cell cycle on adsorption of SV40*

Period of the cell cycle	Input virus adsorbed per 3×10^6 cells (%)
G_1	12.0
Beginning of S	12.0
	7.5
S	10.5
	11.0
G_2	6.8
	6.3
M	5.3
	4.6
Metaphase cells in suspension	16.4
	17.25

[a] Tritiated thymidine-labeled virus was used to infect a series of duplicate petri dishes of synchronized cell cultures at the various indicated periods of their cycle (see text). Input counts per minute per petri dish was 29,000. Cells in G_1 were obtained through seeding of metaphase cells. They were infected 1 h after seeding. Beginning of S refers to cells taken immediately after release from a second thymidine block, and M refers to cells blocked in metaphase through the addition of 0.1 μg of colcemid per ml. Metaphase cells in suspension were obtained through the use of low-calcium medium and infected while kept in suspension by gentle agitation in a 37 C water bath.

trichloroacetic acid-precipitable radioactivity were determined. As can be seen in Table 1, the highest percentage of adsorption was achieved with metaphase cells in suspension and the lowest with metaphase cells under colcemid. Variations between G_1, S, and G_2 were, however, much less significant, being at the most twofold. This difference is probably meaningless when related to the high input multiplicity of infection used throughout these experiments. Moreover, had actual multiplicities of infection played a role in the results of Fig. 2 to 4, differences should have been noted between the three cultures, both regarding the duration of viral DNA synthesis, and the overall amount of viral DNA synthesized per culture (28). This was obviously not the case. A 25 to 30% decrease in the average rate of viral DNA synthesis was observed in the culture infected in G_2 (Fig. 4), but this was not found in later experiments. Variations in the extent of virus adsorption to cells at different stages of their mitotic cycle were, therefore, apparently negligible. A similar conclusion has been reached in the case of polyoma virus, which was shown to adsorb equally well to BHK cells through the various periods of their cycle (1).

The possibility was next investigated that the treatment of the cells with excess thymidine might have rendered them temporarily unable to sustain the replication of the viral genome. In order to demonstrate that this was not the case, cells were synchronized once only with thymidine, infected with SV40, then synchronized again through excess thymidine overnight. Figure 5 shows that removal of the second thymidine block resulted in the immediate initiation of viral DNA replication, thus confirming that, upon infection of cells in G_2, onset of viral DNA replication occurs during the S period of the following cell cycle. The immediate triggering of viral DNA synthesis upon removal of the thymidine block (Fig. 5) suggests that the early viral genes were expressed under excess thymidine. This hypothesis was strengthened by the observation that at least 80% of the blocked infected cells were positive for T antigen at the time when the block was released (not shown). The results of Fig. 5 also show that blocking of cells with excess thymidine does not preclude the replication of SV40 DNA during the cell cycle which follows removal of the block. The delay in onset of viral DNA synthesis in the cultures infected at the beginning or in the middle of S (Fig. 2 and 3), cannot therefore be attributed to inability of the infected cells to

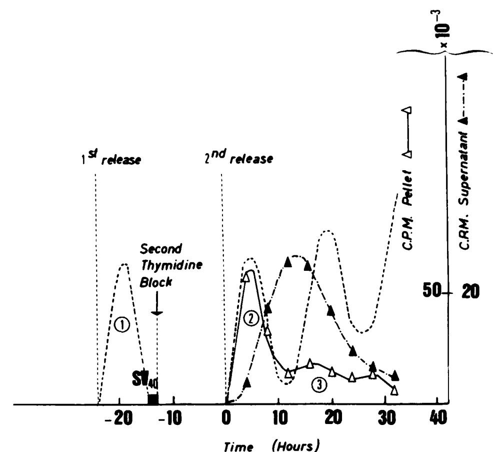

FIG. 5. *Time course of SV40 DNA replication upon infection of cells at the end of S phase number 1. Cells synchronized once only with excess thymidine were allowed to proceed through S, infected with SV40(■), then blocked again under excess thymidine. Upon release from the second thymidine block, host cell (△) and viral (▲) DNA syntheses were followed as described in the legends to the preceding figures.*

replicate viral DNA at this time of their cycle.

Such a delay might reflect the time necessary for the synthesis of the early viral gene products which are known to be required for the initiation of viral DNA synthesis (41). On this basis, it could be argued that in the case of the cells infected at the beginning of S (Fig. 2), and a fortiori of those infected in the middle of S or in G_2 (Fig. 3 and 4), the cells were no longer in the appropriate period of the S phase when sufficient synthesis of early viral proteins had finally occurred. This hypothesis was tested by infecting cells at the beginning of S as for Fig. 2. Part of the culture was them immediately unblocked (curves A, Fig. 6), whereas another part was maintained under excess thymidine for an additional 10 h to allow time for the expression of the early viral genes (curves B, Fig. 6). Viral DNA replication in culture B still did not begin until S phase number 3. It was, therefore, delayed by 10 h by comparison with that in culture A. Appearance of T antigen was, however, identical in both cultures (not shown). This suggests that the delay in culture B was not due to lack of synthesis of early viral proteins. Expression of the early viral genes, albeit mandatory, does not seem sufficient to promote the initiation of viral DNA synthesis.

To further demonstrate this point, synchronized CV_1 cells infected at the time of S phase number 2 were artificially prevented from entering S phase number 3 through arrest in metaphase by treatment with 0.1 μg of colcemid per ml (11, 39). Under these conditions, S phase number 3 was prevented to the extent of 80%. Inhibition of viral DNA replication was also 80%, but the appearance of T antigen was not appreciably inhibited (data not shown). Colcemid added to randomly growing, SV40-infected cells had no effect on the replication of SV40 DNA once it was already under way. These results, therefore, lead to suggest that the initiation of SV40 DNA synthesis is under control of cellular events associated with the normal onset of the host cell S phase.

The time course of viral DNA synthesis in cells infected during G_1 was also investigated. Since the double excess thymidine procedure used above was not very appropriate for obtaining cells in G_1 (see Fig. 1), the procedure of selective detachment of cells in metaphase (33, 34, 42) was used instead. Figure 7 shows that the seeding of cells in metaphase was followed by a 6-h lag before synthesis of DNA could be detected. Mitoses began 18 h after seeding and lasted for approximately 6 h. Cells synchronized by this procedure were infected with SV40 1 h after seeding, i.e., at the beginning of G_1 (inset to Fig. 7). Contrary to what was observed with cells infected during S or G_2, onset of viral DNA replication in these cells was not postponed until the next cell cycle, but occurred between the hour 10 and 20 after seeding, i.e., during the S phase immediately after infection. Here again, most of viral DNA synthesis took place after cellular DNA synthesis, but the first progeny viral DNA molecules were detected when the cells were still in S. Note that in this experiment replication of viral DNA was followed during 15 h only, although it actually lasted for approximately 30 h. Use of cells obtained in G_1 upon arrest in metaphase through the use of colcemid and detachment from the glass in low-calcium medium yielded similar results (data not shown).

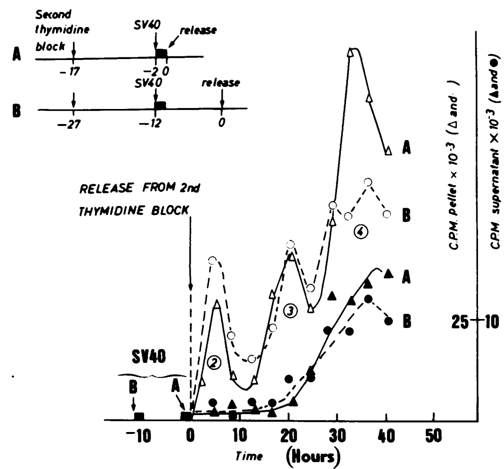

FIG. 6. *Effect of the temporary prevention of cellular DNA synthesis in cells infected at the beginning of an S period. Cells blocked for the second time with excess thymidine were infected with SV40 after 15 h under blocking medium. The virus stock used was diluted with blocking medium to insure that no cell DNA synthesis would occur during the adsorption period. As illustrated by the diagram in the top part of the figure, part of the cells was then immediately unblocked (A), whereas another part was further incubated for 10 h under blocking medium before release (B). Synthesis of cellular and viral DNA (open and closed symbols, respectively) was followed as described before. Symbols: Δ and ▲, culture A; O and ●, culture B. Note that, in order to superpose the successive S periods in both cultures, the actual time course for culture B has been shifted by 10 h, zero time for both experiments being taken as that of unblocking, and not as that of infection.*

It was verified that, in this system also, infection at the beginning of S would result in postponing the onset of viral DNA replication by one cell cycle. To better distinguish between the successive S periods, the cells which had

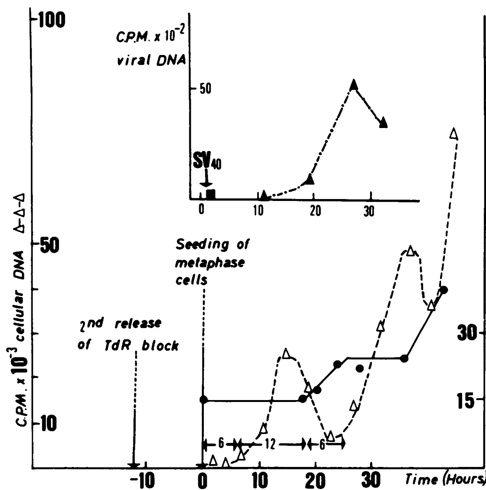

FIG. 7. *Time course of SV40 replication upon infection of cells in* G_1. *Metaphase cells were seeded in petri dishes at zero time. Both the rate of cellular DNA synthesis* (Δ) *and the number of cells per dish* (●) *were then followed (lower panel). Part of the cell monolayers were infected with SV40 1 h after seeding* (■) *and the rate of viral DNA synthesis* (▲) *was determined by sucrose gradient analysis of the Hirt extraction supernatant fluids (upper panel).*

been seeded while in metaphase were further synchronized through treatment with excess thymidine at the end of G_1 (Fig. 8). Duration of the G_1 period was 10 to 12 h in this experiment (inset to Fig. 8). Cells were infected at either 2, 6, or 10.5 h after seeding (samples A, B, and C, respectively), then overlaid with blocking medium until hour 24. Onset of viral DNA replication in these three cultures was found to occur during the S phase immediately after the release from the thymidine block, and the time course of viral development was identical in the three cultures, independent of the time of infection. Control cells synchronized in parallel were infected with SV40 immediately before removal of the thymidine block, then allowed to proceed through S. SV40 DNA replication in these cells did not occur until the S phase of the next cell cycle (Fig. 8, sample D).

DISCUSSION

The present study demonstrates that the time course of SV40 DNA synthesis is related to the particular stage of the cell cycle when infection occurs, and that duration of the early, prereplicative phase of the virus growth cycle varies widely, depending upon the timing of infection with regard to the host cell S phase. In cells infected at the beginning or middle of S, or in G_2, initiation of viral DNA synthesis always took place at the same time of the host cell cycle, namely, the middle of the S period of the next cell cycle. In cells infected at the beginning of G_1, viral DNA synthesis was initiated at some stage of the next S period. The onset of viral DNA replication, with respect to the timing of

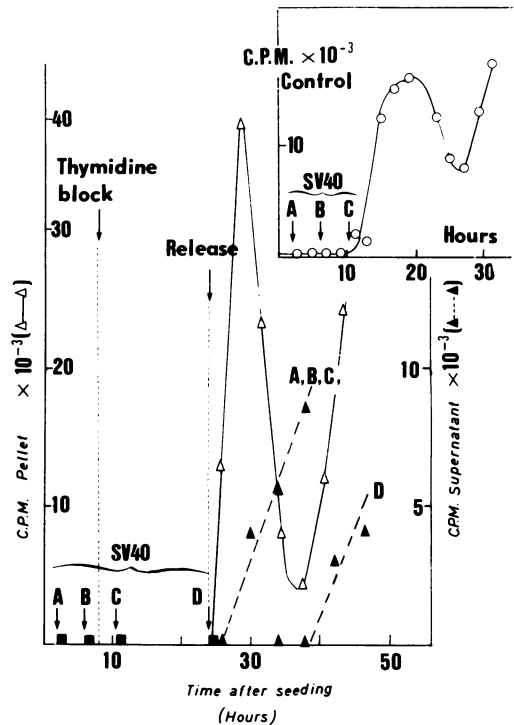

FIG. 8. *Permissiveness of cells infected at various times of* G_1. *Metaphase cells were detached and seeded (1.6 × 10^5 cells per petri dish). Overall DNA synthesis was followed by a series of successive 2-h pulse labelings with 4 μCi of tritiated thymidine per ml. At the end of each labeling period, the cells were lyzed with SDS, and trichloroacetic acid-precipitable radioactivity was determined (open circles, inset to the figure). In another part of the culture, cellular DNA synthesis was temporarily prevented through incubation in blocking medium beginning at 8 h after seeding. Excess thymidine was removed at 24 h, and cellular DNA synthesis was followed by a series of 3-h pulse labelings with tritiated thymidine* (Δ) *as described in the legend to the preceding figures. Cells infected with SV40 at 2 and 6 h after seeding (A and B, respectively) were also blocked at 8 h. Cells infected at 10.5 h after seeding (C) were blocked immediately at the end of the adsorption period. A fourth series of cells were infected with SV40 at the time of release from the thymidine block (D). Arrows labeled A to D refer to the time of infection in the respective cell cultures. Viral DNA synthesis in these four cultures was followed as described in the legend to Fig. 2* (▲). *The time course of viral DNA replication in cultures B and C was identical to that in culture A. Therefore, only one line was drawn for the three cultures.*

the host cell cycle, occurred, therefore, with the shortest delay in cells which had been infected during G_1, and with the longest in cells which had been infected at the beginning of S. In all cases, however, duration of the replicative phase of the virus growth cycle was identical, independent of the time of infection. Most of viral DNA synthesis took place following cellular DNA synthesis and lasted for 25 to 30 h. Infection with SV40 did not interfere with the normal progression of the cell through the successive periods of its cycle, at least until viral DNA replication was well under way.

The fact that the first progeny SV40 DNA molecules were always detected at a time when the cell was in an S period raises the question of whether cell DNA synthesis is required for the onset of viral DNA replication. As an argument in favor of its necessity comes the fact that infection with SV40 induces cellular DNA synthesis in resting AGMK or CV_1 cell cultures, whether contact-inhibited (16, 20, 25, 27, 32) or X-irradiated (16, 48). Similar observations have been made with polyoma virus (12, 15, 24, 45, 47). Yet, resting cultures of at least one cell line of BSC_1 cells are not induced to synthesize DNA upon infection with SV40 (16, 32), although they fully support the growth of the virus. On the one hand, it is worth noting that the time course of SV40 multiplication in BSC_1 cells is delayed by about 20 h as compared to that in AGMK or CV_1 cells (32). This could be due to the fact that virus production in the former has to wait for a small level of cellular DNA synthesis, which is too limited to be detected by overall incorporation of labeled thymidine into bulk DNA. On the other hand, the possibility remains that what is required for the onset of SV40 DNA replication is not cellular DNA synthesis per se, but rather some cellular event related to, and usually followed by, cellular DNA synthesis. The necessity for such an event could account both for the lack of induction of cellular DNA synthesis in SV40-infected BSC_1 cell cultures and for its occurrence in other cell lines. It would also account for the results reported here with synchronized CV_1 cells.

Of particular significance, in this respect, is the observation that synchronized cells which had already passed the end of G_1 at the time of infection were unable to promote the initiation of SV40 DNA synthesis until their next mitotic cycle, even if enough time was provided for the expression of the early viral genes before allowing the cells to proceed through S. This clearly shows that cellular DNA synthesis was not the key determinant in this case. Lack of competence of cells infected after G_1 was neither due to impaired adsorption of the virus, nor to any intrinsic inability of the cells to sustain replication of the viral genome at that time of their cycle. These observations, taken together with the fact that cells infected during G_1 were able to promote the initiation of SV40 DNA replication at some stage of their next S phase, i.e., within the same mitotic cycle, could best be explained by assuming that the infected cell has to pass through a critical stage situated near the end of G_1 or the very beginning of S, in order to gain competence for the eventual initiation of viral DNA synthesis. This hypothesis would explain why SV40 DNA synthesis was postponed by as much as one cell cycle in cells which were in S at the time of infection and was suppressed when cells infected during an S period were prevented from entering their next mitotic cycle through arrest in metaphase.

It has recently been demonstrated that the yield of polyoma virus from infected Balb/3T3 cells, as measured 48 h after infection, depends on the stage in the cell cycle that the cells have reached when they are infected, and is maximal for cells which are infected at, or near, the beginning of G_1 (44). Previous observations had also shown that the time course of polyoma virus DNA synthesis depended upon the timing of infection with regard to the host cell S phase (3, 4, 23). The similarity between these observations and those reported here for SV40 leads to suggest that a critical stage of the host-cell cycle, situated near the end of G_1 or the very beginning of S, might also be required in the case of polyoma virus for the infected cell to eventually begin replicating viral DNA. This hypothesis might, moreover, explain why dividing cells seem to offer the most favorable environment for the replication of polyoma virus (43) as well as for that of SV40 (28), since the critical stage of the host cell cycle might be expected to occur more quickly in the majority of a dividing cell population than in confluent cell cultures in which the arrest of metabolic processes has to be overcome.

The critical stage of the host cell cycle, as defined here, did not apparently correspond to the time when viral DNA synthesis was initiated, since the first SV40 progeny DNA molecules were usually not detected before approximately the middle of an S period. It could thus be that the cell-controlled event which occurs at the critical stage determines only the permissiveness of the cell to the later replication of the viral genome. However, since the first round or rounds of SV40 DNA replication might well escape detection through labeling with thymidine, and since zero time of replication cannot be inferred from the early kinetics of accumulation of DNA component I (28), actual

onset of viral DNA synthesis might occur earlier than was detected. It is not clear, therefore, whether initiation of viral DNA synthesis occurred at the time of the critical stage, or at a later time of the S period. Onset of viral DNA synthesis was usually detected earlier, with respect to the host cell S phase, in cells synchronized by excess thymidine than in those synchronized by mitotic detachment. Whether this reflects the possible disturbance introduced in the rhythm of the host cell cycle by either or both synchronization procedure(s) is unknown.

The nature of the cell-controlled event which occurs at the time of the critical stage and eventually triggers the initiation of SV40 DNA replication is still obviously a matter of speculation. On the one hand, it could be any of the numerous events which are known to occur immediately before S in noninfected cells and which might be a key to cellular DNA synthesis and cell proliferation. The most important among these are the induction of several enzymes (for a review, see 37 and 46), the synthesis of acidic chromosomal proteins (5, 35-38), that of histones and histone messenger RNA (7), and alterations in the level of both adenosine 3':5' cyclic-monophosphate (9, 10, 29, 50) and of guanosine 3'-5' cyclic-monophosphate (19). The great number of variables warrants further investigation before a clear picture of the sequential events which lead to cellular DNA synthesis in animal cells can be drawn and their possible role in promoting SV40 DNA replication experimentally tested.

On the other hand, one should not overlook the possibility that the cellular event which is required for the initiation of SV40 DNA replication is the formation of specific initiation sites in the cell. Results from previous studies have led to the hypothesis that SV40 DNA replication in CV_1 cells occurs on a limited number of sites (28). The critical event which seems to control the permissiveness of the SV40-infected cell might therefore be connected with the binding of parental SV40 DNA to specific replication sites inside the cell, or to its integration into the host-cell chromosomes (21). Experiments aimed at testing these different hypotheses are in progress.

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