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Replication of Simian Virus 40 Deoxyribonucleic Acid: Analysis of the One-Step Growth Cycle

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The time course of replication of simian virus 40 deoxyribonucleic acid (DNA) was investigated in growing monolayer cultures of subcloned CV1 cells. At multiplicities of infection of 30 to 60 plaque-forming units (PFU)/cell, first progeny DNA molecules (component 1) were detected by 10 hr after infection. During the following 10 to 12 hr, accumulation of virus DNA proceeded at ever increasing rates, albeit in a non-exponential fashion. The rate of synthesis then remained constant, until approximately the 40th hour postinfection, when DNA replication stopped. Under these conditions, the duration of the virus growth cycle was approximately 50 hr. The time needed for the synthesis of one DNA molecule was found to be approximately 15 min. At multiplicities of infection of 1 or less than 1 PFU/cell, the onset of the linear phase of DNA accumulation was delayed, but the final rate of DNA synthesis was the same, independent of the input multiplicity. This was taken as a proof that templates for the synthesis of viral DNA multiply in the cell during the early phase of replication. However, the probability for every replicated DNA molecule to become in turn replicative decreased constantly during that phase. This could be accounted for by assuming a limited number of replication sites in the infected cell.

It is generally assumed that the duration of simian virus 40 (SV40) growth cycle is approximately 48 hr (for a review, see 11 and 15). However, most if not all experiments with SV40 reported in the recent literature present evidence for much longer cycles, even at multiplicities of infection high enough to insure the efficient infection of the totality of the cell culture: virus deoxyribonucleic acid (DNA) synthesis proceeds for 70 hr or more, while virus-induced cytopathic changes are not usually detected until several days after infection (1, 4, 19, 22, 24).

The reason for such discrepancy lies partly in the nature of the cells used (25, 26) and mostly in the state of the cells at the time of infection. Thus, in preliminary experiments with CV1 cells, we observed that infection of resting cells resulted in slow viral multiplication and that 96 hr was required before any typical viral cytopathic effect occurred, whereas infection of the same type of cells, in their growing phase, resulted in short viral cycles, all the cells being detached or lysed in approximately 50 hr. The final yield of infectious virus particles per cell was approximately the same under both conditions. This observation prompted us to define reproducible conditions for short multiplication cycles, hoping in that manner to come as close as possible with natural means to a synchronization of viral development.

The CV1 line was cloned, and a homogenous, epithelioidal subline was selected. Cells from this subline were infected with SV40 in their growth phase, and the time course of viral DNA replication was investigated. As will be shown, the duration of the virus growth cycle under these conditions is reproducibly 50 hr.

MATERIALS AND METHODS

Cells and infection. CV1 cells grown in minimal essential medium were a gift from P. Tournier. The cells were cloned under 0.25% agarose by L. Montagnier. A clone of epithelioidal cells was selected. The cells from this clone were grown in 250-ml Falcon flasks in minimal essential medium supplemented with 10% Tryptose phosphate, 1% glucose, and 5% normal calf serum (MCV1 medium) without added antibiotics. This cloned subline was

repeatedly found mycoplasma free, as judged either through microscope examination after staining with Giemsa, or through examination in the electron microscope.

The large-plaque strain of SV40 was a gift from P. Tournier. Virus stocks were made through infection of subcloned CV1 cells at an input multiplicity of 0.05 plaque-forming unit (PFU)/cell. Cells and medium were collected at 9 to 10 days after infection and frozen and thawed three times. Stocks were kept frozen at −30 C. For the experiments reported here, subcloned CV1 cells were seeded in 6-cm plastic petri dishes (Greiner, France) at 3×10^5 to 5×10^5 cells per dish in MCV1 medium supplemented with 100 international units of penicillin and 100 μg of streptomycin per ml. Infection with SV40 was performed 24 hr after seeding. The culture medium was removed, and 0.2 to 0.4 ml of the appropriate virus suspension was added to each petri dish. Input multiplicity was 30 to 50 PFU/cell except where noted. Virus adsorbtion was for 1.5 hr at 37 C, after which an overlay of 5 ml of fresh MCV1 medium containing penicillin and streptomycin was added to each culture.

Labeling and analysis of viral DNA. Except where otherwise stated, labeling of viral DNA was performed by the addition of 4 μCi of tritiated thymidine per ml (25 Ci/mmole, C.E.A. Saclay) to each of the infected cultures. At the end of each labeling period, viral DNA was extracted by the method of Hirt (13). For this purpose, the labeled medium was removed, the cell cultures were washed twice with 2 to 3 ml of phosphate-buffered saline (PBS), and 0.6 ml of 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (*p*H 7.4), 0.01 M ethylenediaminetetraacetate (EDTA), 0.6% sodium dodecyl sulfate (SDS) was added. The viscous cell lysate was scraped with a piece of silicone rubber and poured into 2-ml nitrocellulose tubes containing 0.15 ml of 5 M NaCl. The content of each tube was homogenized by slowly inverting the tube four times. The samples were stored at 4 C overnight and then centrifuged for 35 min at 15,000 rev/min, 4 C, in a Spinco S40 rotor. The SDS pellets, which contained the cellular DNA, were resuspended in 0.1 M NaOH and then neutralized, and trichloroacetic acid-insoluble radioactivity was determined. The supernatant fluids, which contained both mitochondrial and viral DNA, were assayed for acid-insoluble radioactivity or further analyzed by sucrose gradient centrifugation as described in the legend to Fig. 1. Trichloroacetic acid-insoluble material was collected by filtration onto glass-fiber filters GF/C (Whatman) and washed three times with 5% Trichloroacetic acid. Radioactivity was determined by liquid scintillation counting in 10 ml of Bray solution.

RESULTS

Time course of the replication of SV40 DNA. The synthesis of SV40 DNA was first followed by continuous labeling of infected cells with tritiated thymidine beginning at the time of infection, and by determining the amount of label incorporated into viral DNA at various times thereafter. The cells in each sample were lysed with SDS, and viral DNA was separated from the bulk of cell DNA through selective precipitation of the latter with 1 M NaCl by the method of Hirt (13). Essentially all of the virus DNA is extracted by this method, whether already encapsidated into new virions or free inside the cell (13, 19).

The validity of the continuous labeling procedure was ascertained from two different standpoints. It was first checked that the supply of labeled precursor was sufficient to allow a constant rate of incorporation during the time period studied. This was verified by showing that, when added to noninfected control cells, the amount of tritiated thymidine used in these experiments (20 μCi per petri dish in 5 ml of MCV1 medium) allowed continuous incorporation of radioactivity into cellular DNA for periods of time in excess of 30 hr. Secondly, it was checked that no degraded cellular material was found in Hirt extraction supernatant fluids after long periods of labeling. Thus, the results of continuous labeling were essentially similar to those of pulse labeling experiments, during at least the first 30 hr after infection.

The Hirt extraction supernatant fluids from the continuous labeling experiment were analyzed by sucrose gradient centrifugation at neutral *p*H, with the results illustrated in Fig. 1. The sedimentation profiles showed a peak of heavy material in fractions 4 to 7. This was probably mitochondrial DNA, since the same peak was also present in extracts from noninfected cells (not shown). In addition, a peak of radioactivity sedimenting at the position of SV40 DNA component I (fractions 11 to 15 in Fig. 1) was detected in the extracts from infected cells beginning at approximately 11 hr after infection (bottom left panel, Fig. 1). The material in that peak had the same sedimentation coefficient at alkaline *p*H and the same density in CsCl-ethidium bromide as DNA extracted from purified SV40 virions (not shown). The amount of radioactivity in the peak at 21*S* in the gradients shown in Fig. 1 was therefore taken as a measure of the amount of viral DNA synthesized at the various indicated time points.

A second experiment was then performed, in which the amount of SV40 DNA synthesized after 20 hr of infection was determined through a series of successive pulse labelings with tritiated thymidine. Nonspecific labeling was measured by labeling of noninfected cells.

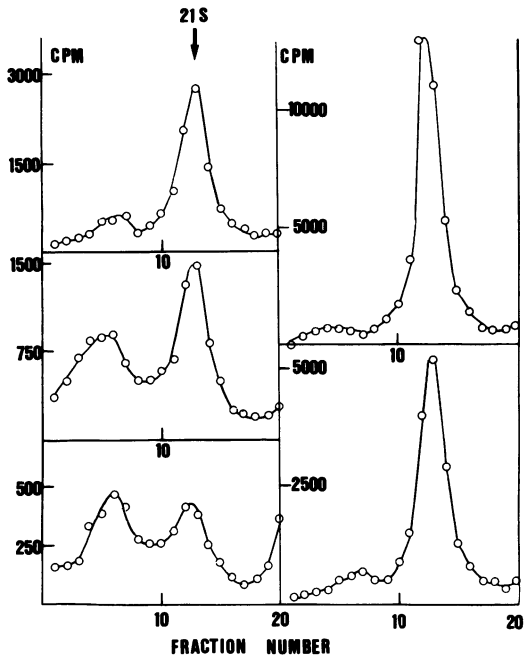

FIG. 1. *Cumulative labeling of SV40 DNA. Subcloned CV1 cells were seeded at 5 × 10⁵ cells per petri dish and infected 24 hr later with SV40 at a multiplicity of 50 PFU/cell. Tritiated thymidine (25 Ci/mmole, 4 μCi/ml) was added immediately upon infection. Extraction of viral DNA was performed by the method of Hirt (13) at various times thereafter. The Hirt extraction supernatant fluids were analyzed by centrifugation for 5.5 hr at 40,000 rev/min, 4 C, in a Spinco SW41 rotor, through 5 to 20% gradients of sucrose in 0.01 M Tris-hydrochloride (pH 7.4), 1 M NaCl, 0.02 M EDTA. Fractions were precipitated with 5% trichloroacetic acid, and their radioactivity was determined. Left panel, bottom to top: samples withdrawn at 11, 13 and 15 hr postinfection, respectively. Bottom right panel: 17 hr postinfection. Top right panel: 20 hr postinfection. Only the first 20 fractions of each gradient are shown. The arrow labeled 21S shows the position of purified SV40 DNA in a gradient run in parallel.*

The difference between total acid-precipitable label in the Hirt extraction supernatant fluid from infected cells and that from noninfected cells was taken as a measure of the radioactivity incorporated into viral DNA. The validity of this procedure was quantitatively checked by showing that the number of counts thus obtained was similar to that found by gradient analysis of the Hirt supernatant fluids (compare also open and closed circles in Fig. 2). A cumulative curve for the synthesis of viral DNA was then reconstructed, by adding, to the number of counts found in viral DNA at the end of the cumulative labeling of the preceding experiment, those successively incorporated during the series of pulse labelings (Fig. 2). As may be seen, SV40 DNA replication was initiated approximately 10 to 11 hr after infection. The rate of viral DNA synthesis constantly increased from then on to about the 20th hour after infection, when it reached its maximum. It then remained

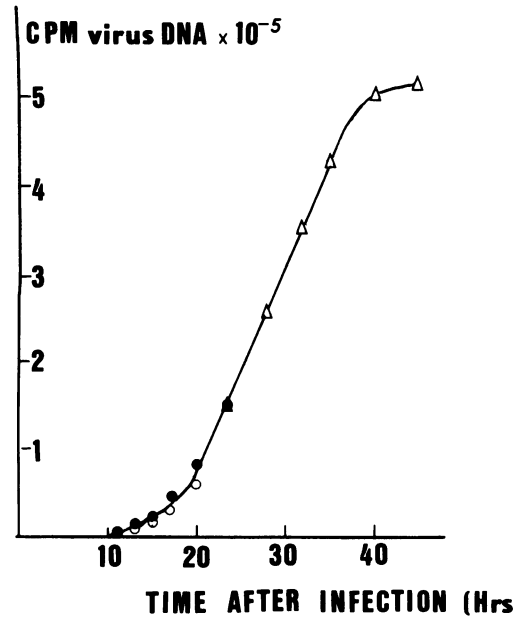

FIG. 2. *Time course of the accumulation of SV40. Cumulative labeling of SV40 DNA for the first 24 hr after infection was determined from the radioactivities recovered in the peak of viral DNA in the gradients shown in Fig. 1 (●). Each point represents the radioactivity recovered from one petri dish. The total radioactivity of each of the Hirt supernatant fluids from this experiment, minus that recovered from Hirt supernatant fluids from noninfected cells cumulatively labeled in the same way, is also shown (○). A similar correction was performed in a series of successive pulse labelings with 4 μCi of tritiated thymidine per ml performed at various times between 20 and 45 hr after infection (see text). Radioactivities thus obtained were successively added one after the other to the preceding ones, in order to reconstruct the cumulative curve for SV40 DNA labeling after the 20th hour of infection (Δ). A 20% difference was found, however, between the amount of radioactivity incorporated between hours 20 and 23.5 in the pulse labeling experiment and the increase in radioactivity of SV40 DNA as measured during the same period by sucrose gradient analysis of the samples from the cumulative labeling experiment. The values from the pulse labeling experiment were therefore corrected accordingly. These have been plotted at the time corresponding to that of the end of each of the successive pulse labeling periods.*

constant for the following 16 to 20 hr, and replication ceased by approximately 40 hr after infection.

Morphological alterations of the infected cells were first detected by optical microscopy at approximately 36 hr postinfection. By the 50th hour, the majority of the cells in the culture were either detached or lysed, and pronounced cytoplasmic vacuolization was observed in the few remaining cells still attached to their support. SV40 replication was practically simultaneous in most of the cells, since more than 90% of the cells were strongly positive for V antigen 24 hr after infection, as judged by immunofluorescence assays.

To better detect the first replicated molecules, virus DNA replication was also followed by pulse labeling infected cells at various times after infection with 40 μCi of tritiated thymidine per ml. DNA was extracted at the end of each labeling period and analyzed by sucrose gradient centrifugation as in Fig. 1. However, no progeny DNA molecules could be detected under these conditions before 10 hr after infection. Also, the rate of accumulation of daughter molecules was similar to that shown in Fig. 2.

Influence of the multiplicity of infection. The effects of the multiplicity of infection on the time course of SV40 DNA replication and on the synthesis of host cell DNA were next investigated. As may be seen in Fig. 3 (left hand panel), although infection by SV40 constantly inhibited host cell DNA synthesis, the extent of the inhibition was dependent upon the multiplicity of infection. Thus, at 30 hr after infection, rates of host cell DNA synthesis were decreased by 80, 60, and 20% in cultures infected at multiplicities of 60, 6, and

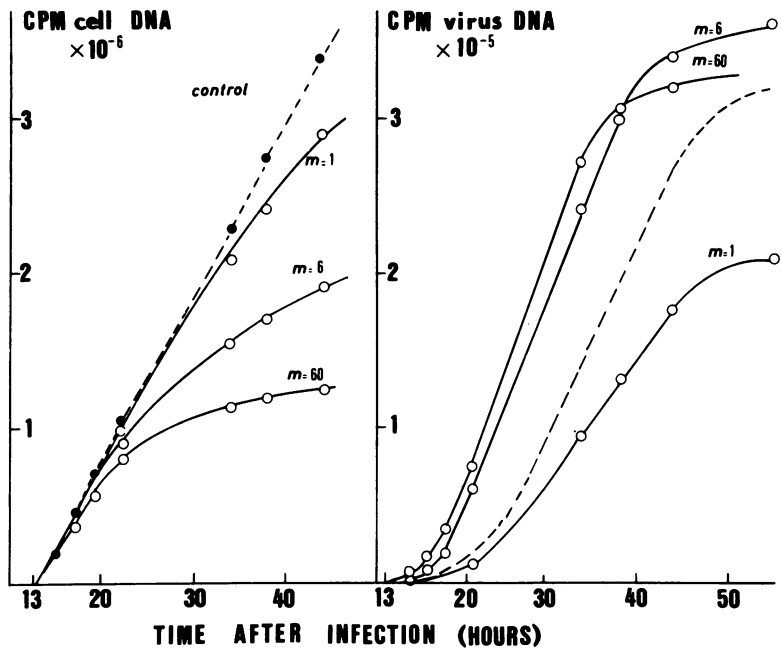

FIG. 3. *Effect of various multiplicities of infection on the synthesis of host cell and virus DNA. Left panel: host cell DNA synthesis. Cells were infected with 0.2 ml of serial dilutions of the same stock of SV40. Cellular DNA synthesis was followed by labeling the cells with 4 μCi of tritiated thymidine per ml either under conditions of continuous labeling beginning 13 hr postinfection or through successive pulse labels after the 20th hour. At the end of each labeling period, selective extraction of viral DNA was performed on one petri dish at a time, and host cell DNA synthesis was measured by determining the amount of acid-precipitable radioactivity in the Hirt extraction pellets. The data have been plotted in a cumulative fashion as for Fig. 2. Symbols: ●----●, control uninfected cells; O——O, cells infected at multiplicities of 1, 6, and 60 PFU/cell as indicated. Right panel: SV40 DNA synthesis. The counts recovered in the Hirt extraction supernatant fluids from the same experiment were corrected by the incorporation in extracts from noninfected cells. Some of the infected cell supernatant fluids were also analyzed on sucrose gradients as for Fig. 1. Both procedures yielded identical data. These were plotted in a cumulative fashion versus time after infection. The data from the culture infected at a multiplicity of 1 PFU/cell were then corrected for the number of infected cells in the culture, assuming that only 67% of the cells had been infected, and plotted in parallel (dashed line).*

1 PFU/cell, respectively. Even at the highest multiplicity tested, however, the inhibition was barely detectable during the first 20 to 24 hr after infection. It only became preponderant after replication of virus DNA reached its constant rate (see Fig. 2, and right hand panel, Fig. 3). No stimulation of cell DNA synthesis was ever observed in this system. This is most likely due to the fact that the cells are actively growing at the time of infection (29). Also, mitochondrial DNA synthesis does not seem to be stimulated, contrary to what has been observed with resting cells (18).

The multiplicity of infection had no major effect on the time course of viral DNA replication between 6 and 60 PFU per cell (Fig. 3, right hand panel), but synthesis was delayed by approximately 2 hr at the lowest multiplicity. At a still lower multiplicity (1 PFU/cell), the time course of SV40 replication seemed at first glance to be quite different (Fig. 3, right panel). However, since at such input multiplicity only 67% of the cells are infected, the number of counts determined in viral DNA should be corrected by a factor of 100:67 to account for the presence of 33% uninfected cells. The normalized curve which can thus be reconstructed (dashed line in Fig. 3) shows that the overall kinetics of virus replication per infected cell is identical at the multiplicity of 1 PFU/cell to that obtained with the higher multiplicities of infection, both in the rate of synthesis during the linear increase in viral DNA and in the final extent of DNA replication. The time required to reach the linear phase is increased, however, at the multiplicity of 1.

Similar observations were made at still lower multiplicities. Figure 4 (left hand panel) shows the cumulative incorporation of radioactive thymidine into viral DNA in cells infected at input multiplicities of 5, 1, 0.35, and 0.17 PFU/cell (A, B, C, and D). The results were corrected as above according to the Poisson distribution, in order to take into account the number of uninfected cells in the various cultures. The resulting normalized curves were all parallel, but synthesis was delayed at the three lowest multiplicities (Fig. 4, right hand panel). The maximal rate of viral DNA synthesis in SV40-infected cells is therefore independent of the multiplicity of infection.

The biphasic pattern obtained here for the time course of SV40 DNA replication has common features with that of other viruses, the replication of which proceeds first in an exponential fashion and then at a constant rate. In the case of SV40, the question was therefore raised of whether replication of the DNA

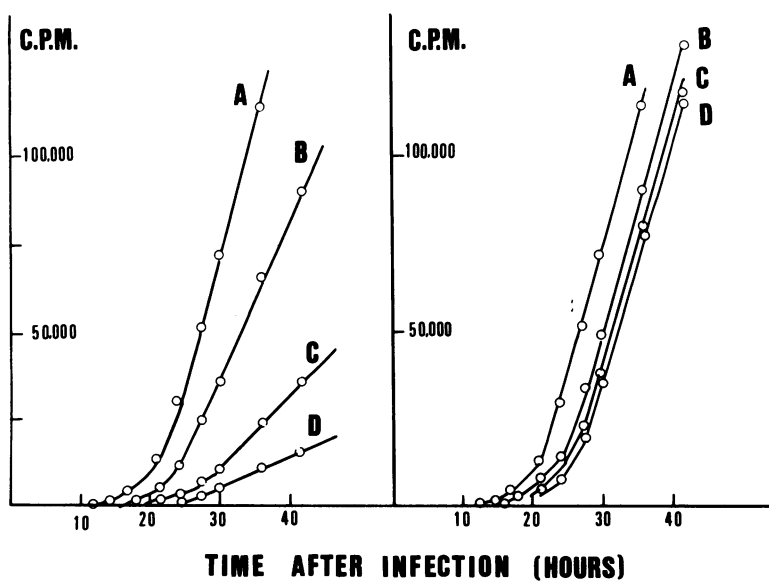

FIG. 4. *Time course of SV40 DNA synthesis at low multiplicity of infection. Left: Cells were infected with 0.4 ml of serial dilutions of the same stock of SV40. Viral DNA replication was monitored as for Fig. 2. Input multiplicities of infection were: A, 5; B, 1; C, 0.35; and D, 0.17 PFU/cell. Right: The results from the left hand panel of the figure were corrected for the actual number of cells infected at each of the various input multiplicities, as calculated from a Poisson distribution.*

also occurred in an exponential fashion in the first part of the cycle. If so, one could have hoped to determine the precise time for the onset of replication, through due extrapolation of the logarithmic curve towards the earliest times of infection. The normalized data in Fig. 4 were accordingly plotted on a semilogarithmic scale. As may be seen in Fig. 5, the nonlinear increase in viral DNA molecules which was observed during the early phase of replication did not lend itself to this type of interpretation. The resulting family of curves showed that replication proceeds in the same fashion at the various multiplicities tested, but is delayed when multiplicity is lowered. The lag between replication in cells infected with 5 PFU/cell and that in cells infected with 1 PFU was approximately 4 hr in this experiment. However, although the overall rate of DNA synthesis during the early phase of replication was ever increasing in all of the infected cultures, none of the data could be fitted with an exponential accumulation of daughter molecules.

Time required for the synthesis of viral DNA molecules. The time required to synthesize one molecule of SV40 DNA has been determined by Nathans and Danna (21) and can be inferred from the work of others (19, 28). These authors, however, used barely or slowly growing cells. Since the system described here which uses actively growing subcloned CV1 cells gives a much shorter virus growth cycle, the question was raised of whether this was due to a shortened synthesis time for the virus DNA molecules. Cells were therefore pulse labeled with tritiated thymidine for various lengths of time at 30 hr after infection, i.e., at a time when the rate of virus DNA synthesis is constant (see Fig. 2 and 3). The radioactivity in each of the Hirt extraction supernatant fluids was analyzed by sucrose gradient centrifugation. Some representative results are shown in Fig. 6 (left hand panel). After 10 min of labeling (upper graph), most if not all of the incorporated label was found in a peak at 25*S* corresponding to the structure described by Levine et al. (19) as the replicative intermediate (R.I.). Label later appeared and eventually accumulated in a peak at 21*S* corresponding to mature SV40 DNA (middle and bottom graphs; 20 and 60 min of labeling, respectively). It was verified by sucrose gradient analysis at alkaline *p*H (indifferently 11.8 or 13) that the labeled molecules in the R.I. had the expected size for nascent DNA strands (7 to 16*S*) and that those sedimenting at 21*S* at neutral *p*H behaved as supercoiled component 1 DNA (14, 19, 28).

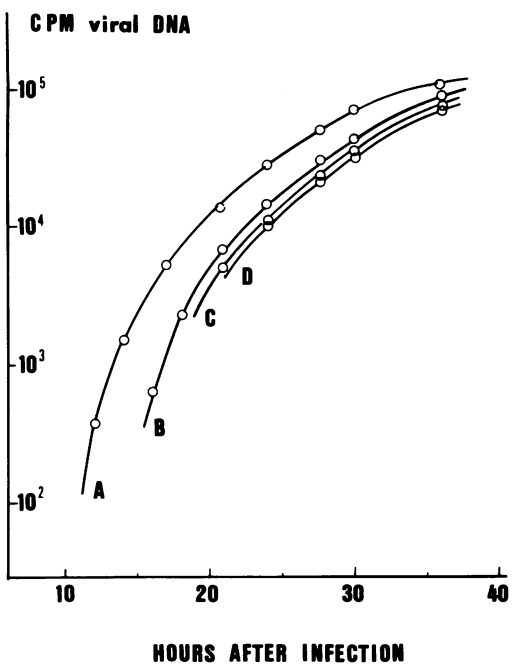

FIG. 5. *Absence of a logarithmic phase at the beginning of infection. The normalized data from Fig. 4 (right hand panel) were plotted on a semilogarithmic scale. Symbols: A, 5; B, 1; C, 0.35; and D, 0.17 PFU/cell.*

The time course of labeling of both R.I. and 21*S* DNA was determined and expressed as the percentage of counts in R.I. (dashed line, right hand panel in Fig. 6). In such an experiment, the time for synthesis of one DNA molecule (component 1) is the delay after which 50% of the incorporated counts are in R.I. (6; M. Girard, D. Baltimore, and J. E. Darnell, Fed. Proc. **24**:379, 1965). As can be seen in Fig. 6, it experimentally required 21 min for R.I. and 21*S* DNA to become equally labeled. However, the overall time course of incorporation of the tracer thymidine into the various DNAs showed a lag of several minutes before a constant rate of labeling was reached (closed circles, Fig. 6). This result is in agreement with those of Hirt (12) and Levine et al. (19). A similar lag of about 7 min was also found before the percentage of counts in R.I. started decreasing (open circles, Fig. 6). This lag, which can presumably be attributed to a delay in the equilibration of exogenous label with intracellular precursors to DNA, should be taken into account. The time for the synthesis of one molecule of SV40 DNA in this system can therefore be estimated at 15 min

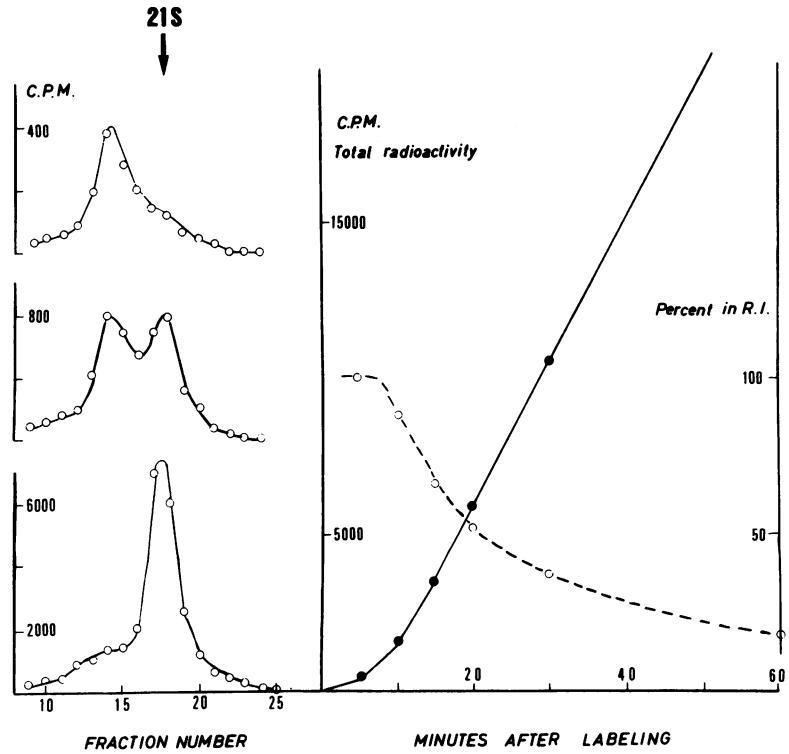

FIG. 6. *Synthesis time of SV40 DNA molecules. SV40-infected cells were overlaid at 30 hr after infection with 3 ml of prewarmed medium containing 35 μCi of tritiated thymidine per ml (25 Ci/mmole). Viral DNA was extracted by Hirt's procedure at various times afterwards and analyzed by centrifugation through gradients of 5 to 20% sucrose in 0.01 M Tris-hydrochloride (pH 7.4), 1 M NaCl, 0.02 M EDTA, for 8 hr at 32,000 rev/min, 4 C, in a Spinco SW41 rotor. Left hand panel: sedimentation profiles of the trichloroacetic acid-precipitable radioactivity recovered in some of the Hirt extraction supernatant fluids. From top to bottom: cells taken after 10, 20, and 60 min of labeling. Only fractions 9 to 25 of each gradient are shown. The arrow labeled 21S refers to the position of purified SV40 DNA sedimented in parallel. Right hand panel: ●——●, total counts in both R.I. and mature viral DNA (component I) at the various indicated times (each point represents the radioactivity from one petri dish); O----O, percent of the counts recovered in R.I. at each of the indicated times.*

or less, which is very close to that determined in resting or barely growing cells. The fact that in the system described here the virus growth cycle is shorter cannot therefore be attributed to a faster rate of replication of the viral chromosome.

DISCUSSION

The experiments reported here show that multiplication of SV40 in growing cultures of subcloned CV1 cells occurs in less than 50 hr. During the first hours after infection, the only detectable change in the infected cell is the appearance of a small fraction of total ribonucleic acid (RNA) which can hybridize to SV40 DNA (1, 22, 23, 27 and *unpublished results* of S. M.) and which probably corresponds to messenger RNA coding for the early viral functions (3, 16, 20; see review by Green [11]). SV40 DNA replication begins approximately 10 hr after infection, and its rate constantly increases during the following 10 to 12 hr, until a maximal rate is reached which then remains constant for approximately 16 additional hours. At the same time, synthesis of host cell DNA is inhibited. Virus DNA replication is terminated by the 40th hour after infection, and most cells are detached or lysed by the 50th hour.

As noted earlier, quite different durations of virus cycles can be obtained depending upon the state of the cells at the time of infection. When resting rather than growing CV1 cells were used, virus cytopathic effect was delayed, and replication of the virus in the culture lasted longer. This is not due to a faster rate of replication in growing cells, since the

time required for the synthesis of one molecule of viral DNA was estimated here to be 15 min or less, as it is in resting cells (19, 21, 28). It is therefore likely that the reason SV40 multiplication seems to be slowed down in cultures reaching high cell density lies not in a slower replication rate, but in a higher degree of asynchrony in virus multiplication among the individual infected cells of the resting cultures. Such an interpretation is supported by the fact that in experiments with resting cultures of standard CV1 cells, less than 50% of the cells were found to be positive for V antigen as late as 50 hr after infection, whereas, with growing cells, this percentage could rise to more than 90% at times as early as 24 hr (*unpublished observations*). It is also supported by the results of experiments performed with synchronized cell cultures, to be reported elsewhere (Manteuil et al., *manuscript in preparation*), which have shown that the onset of SV40 DNA replication occurs during the S phase of the cell cycle. Duration of the virus growth cycle can accordingly vary depending upon the phase of the cell cycle at which infection occurs and upon the time required for each cell to reach the S period.

In this respect, CV1 cells appear to differ greatly from primary African green monkey kidney (GMK) cells, since experiments reported by Carp and Gilden (4) showed that replicating GMK cells infected with SV40 produced very low yields of virus, continued to replicate, and could be subcultured at least 32 times after infection, whereas stationary GMK cells produced up to 100 PFU of SV40 per cell and were destroyed to the extent of 80% by the fifth day following infection. Similar results were reported by Fischer and Munk (7). In the CV1 system described here, the total yield of infectious particles per cell is approximately 50 and seems to be independent of whether the cells are actively growing or not, but virus replication and appearance of virus-induced cytopathic effects are delayed in cells reaching confluency.

One conclusion from this study is that the templates for synthesis of SV40 DNA multiply in the cell during the first 10 hr of replication. This is drawn from the fact that the overall rate of DNA synthesis constantly increased from 10 to 20 hr after infection even in cells infected with multiplicities of 1 or less than 1 PFU/cell (see Fig. 3 to 5). The pattern obtained for the time course of SV40 DNA replication is therefore apparently similar to that of other viruses, e.g., poliovirus (2), or RNA phages (10). However, contrary to what has been observed with these viruses, the early nonlinear part of the SV40 replication cycle is not truly exponential, and no constant doubling time could be found for the accumulation of viral DNA molecules during the first 10 hr of virus replication. This, combined with the fact that the overall rate of synthesis nevertheless kept increasing at the same time, implies that the percentage of *replicated* molecules which become *replicative* constantly decreases with time after the onset of DNA synthesis. The probability for every DNA daughter molecule to become in turn replicative therefore progressively decreases during the whole early phase of replication.

This can hardly be explained through encapsidation of some of the newly made DNA molecules since, as will be shown in the accompanying paper (9), no virions could be detected in this system before the beginning of the linear phase. A more likely explanation is that there might be competition between replication and transcription. According to this hypothesis, more and more of the newly made DNA molecules might be withdrawn with time from the pool of DNA, to serve as templates for the transcription of the late viral genes. Indeed, as will be reported elsewhere, the rate of SV40 messenger RNA synthesis rapidly increases between 10 and 20 hr after infection and then remains constant until the end of infection (Manteuil and Girard, *to be published*). However, another likely hypothesis which would account for the observed time course of viral DNA synthesis is that replication occurs on specific sites of the cell, which could be limited in number. Thus, at the earliest times of replication, DNA synthesis would proceed for a brief period of time in a truly exponential fashion, but the probability of a newly made DNA molecule finding an empty site would progressively decrease with the extent of replication. The overall rate of synthesis would keep increasing, although not exponentially, because more and more sites would progessively be occupied by replicating molecules until the time when, all sites being occupied, a maximal rate of replication would be reached, which would then remain constant until the end of infection. Although such a hypothesis is, for the time being, quite speculative, it finds support in the work with $\phi \times 174$ (17), which shows that a limited number of sites can exist inside a cell for the replication of a virus genome. Attempts at demonstrating that replicative SV40 DNA can be recovered in association with membranes of the infected cell are in progress.

In the system described here, the final yield of infectious SV40 particles per cell is approximately 50, and the majority of progeny viral DNA molecules are encapsidated by the end of infection (9). The actual value of the ratio of physical to infectious particles is not known. That reported for polyoma virus is 100 (5). If a similar figure applied to SV40 also, it would follow that the number of SV40 DNA molecules synthesized per infected cell should very approximately be 10,000. As was shown here, 90% of these molecules are made in the course of 16 hr (see Fig. 1). Therefore, the rate of synthesis per infected cell during the linear increase in viral DNA can tentatively be estimated at 600 molecules per hour. Since the time required for the synthesis of one DNA molecule is approximately 15 min, it follows that the average number of replication sites per cell should be 150. It is obvious that this figure can only be taken as suggestive, in view of the lack of precision on several of the numbers which were used for its determination. It shows, nevertheless, that the actual number of replicating SV40 DNA molecules and, therefore, of replication centers is probably limited to a few hundred in the infected cell.

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