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Aspects of the Encapsidation of Simian Virus 40 Deoxyribonucleic Acid

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Growing subcloned CV*1*-cells were infected with simian virus 40, and the time course of virus formation was determined. When infected cells were fractionated into cytoplasmic and nuclear fractions, most of the progeny virus particles were recovered in the cytoplasmic extract and not in the nuclei. This result was independent of the technique used for the preparation of nuclei and of the time after infection at which the extracts were prepared. Leakage of the virions from the nucleus occurred during the course of cell fractionation, suggesting that the nuclear membrane of the infected cells is damaged. Virions were found to accumulate in a nonlinear fashion, at the time when the number of viral deoxyribonucleic acid (DNA) molecules increases linearly with time after infection. This suggests that the size of the intracellular pool of capsid proteins increases constantly during the late phase of virus replication. Progeny viral DNA to become encapsidated is withdrawn at random from the pool of replicated DNA molecules.

Little is known about the mechanism and time course of the encapsidation of progeny viral genomes in cells productively infected with either polyoma virus (3, 6) or simian virus 40 (SV40) (10). The capsid of both of these viruses is composed of three major proteins (5–7, 9, 12, 18, 27). In addition, mature virus particles contain two to four discrete polypeptides, which appear to be histone-like cellular proteins and which preferentially bind to viral deoxyribonucleic acid (DNA) (5, 7).

Synthesis of capsid proteins is thought to depend upon the transcription of late viral genes (1, 17), and prevention of viral DNA replication by various inhibitors of DNA synthesis precludes the appearance of immunologically reactive capsid proteins. This has been shown through the use of fluorouridine (8, 21, 22), cytosine arabinoside (6, 12, 22, 25), or miracil D (*unpublished results*). Also, the major SV40 capsid protein (molecular weight 45,000) is not detected in extracts of infected cells before viral DNA replication is initiated (6, 19, 27; C. W. Anderson and R. F. Gesteland, Abstr. 3rd Tumor Virus Meeting, Cold Spring Harbor, 1971). Little is known, however, of the actual encapsidation process. It was thought of interest, in this respect, to determine whether encapsidation of SV40 DNA occurs at random from the pool of newly synthesized DNA molecules, and whether the size of the intracellular pool of capsid proteins varies with time after infection. The question of the extent to which such variation might control the kinetics of virus formation also deserved an answer.

The time course of the formation of SV40 virions was determined in the system described in the accompanying paper (15). In this system, infection proceeds simultaneously in most of the cells of the culture and is terminated within 50 hr. As will be shown, encapsidation of a viral DNA molecule can occur at random, either promptly after its synthesis, or much later in the virus growth cycle.

MATERIALS AND METHODS

Cells and infection. Cells from the cloned CV1 subline previously described (16) were grown in minimal Eagle medium supplemented with 10% Tryptose phosphate and 5% calf serum (MCV1 medium). The cells were seeded in either 5×10^5 or 3×10^5 cells per plastic petri dishes (Greiner, France or Nunc, Denmark) depending upon the diameter of the dish (6 and 5 cm, respectively). Infection with SV40 was performed 24 hr after seeding as previously described.

Labeling of DNA and virions. All labeling experiments were performed at 24 hr postinfection or later, i.e., at times when the increase in progeny DNA molecules is linear (15). Virus DNA was labeled with tritiated thymidine (20 Ci/mmole, C. E. A. Saclay) as indicated in the legends to the figures. Labeling of capsid proteins was performed with radioactive amino acids. For this purpose, the infected cells were first

incubated in minimal essential medium containing 1/50 the normal concentration of the amino acid(s) to be used for the label and 2% dialyzed calf serum. Tritiated lysine, leucine, and isoleucine (24, 35, and 28 Ci/mmole, respectively) were purchased from C. E. A. Saclay, and tritiated amino acids mixture from New England Nuclear Corp. The procedures for selective extraction of viral DNA and determination of radioactivities were as previously described (15).

Preparation of cytoplasmic extracts. The method used was devised after that of Borun et al. (4) as modified for monolayer cultures (by M. Fiszman). Two of three petri dishes of infected cells were used at a time. The cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), and then each petri dish was overlaid with 0.6 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.2), 0.14 M NaCl, 0.01 M $MgCl_2$ containing 0.5% NP40, P40 (NP40, Shell). After 10 min of incubation on ice, the cytoplasmic extract and floating nuclei were scraped from the dish with a piece of silicone rubber. In most experiments, the extract was further homogenized by Dounce homogenization (20). Nuclei were pelleted by centrifugation at 800 × *g* for 2.5 min, resuspended in 0.01 M Tris-hydrochloride (*p*H 8.2), 0.14 M NaCl, 0.01 M $MgCl_2$ containing 0.5% NP40, homogenized, and pelleted again. The supernatant fluids from both centrifugations were mixed and constituted the cytoplasmic extract. The nuclear pellets were resuspended in the same buffer as above, sonically treated for 30 sec with a Branson sonifier, and treated with 20 μg of ribonuclease-free deoxyribonuclease per ml. Both nuclear and cytoplasmic extracts were then made 0.02 M with respect to ethylenediaminetetraacetate (EDTA) and 0.5% with respect to sodium deoxycholate (DOC). They were analyzed by centrifugation through 5 to 20% sucrose gradients in 0.01 M Tris-hydrochloride (*p*H 7.4), 0.01 M NaCl, 0.02 M EDTA (NEB).

RESULTS

Infected cells were labeled with tritiated thymidine at 24 hr after infection. Thirteen hours later, nuclei were separated from cytoplasma with NP40 in isotonic buffer as described in Materials and Methods. The nuclear pellet was treated with DOC and deoxyribonuclease and sonically disrupted in order to free intranuclear virions. Both cytoplasmic and nuclear extracts were then analyzed by sucrose gradient centrifugation. A peak of radioactivity, characterized by its resistance to deoxyribonuclease, its presence exclusively in extracts from infected cells, and its cosedimentation with infectivity, was found at the position expected for SV40 virions. The sedimentation coefficient was approximately 180*S* under the conditions used (as compared to the rate of sedimentation of purified poliovirus). Quite unexpectedly, however, the overwhelming majority of the virions were recovered from the cytoplasmic extract, and not from the nuclei (Fig. 1, left hand panel).

Since it is known, from the electron microscopy work of Granboulan et al. (10), that SV40 leaks from the cell nucleus and accumulates inside cytoplasmic vacuoles at late times of infection, the question was raised of whether the preferential recovery of virions in the cytoplasmic extract was related to the time at which the extraction was performed. Figure 1 (right hand panel) shows that this is partly the case. However, at all times tested, the majority of the virus was recovered in the cytoplasmic fraction of the cells, where it was found to increase in a nonlinear fashion. The doubling time for accumulation of virions was 4 hr in this experiment (inset to Fig. 1). In this and other experiments, the earliest incorporation of labeled precursor to DNA into virions was recorded at approximately 20 hr postinfection.

Various other methods for the preparation of nuclei were tested in order to rule out the possibility that the accumulation of virus particles in the cytoplasmic fraction of the infected cells was due to the extraction procedure used. However, neither Dounce homogenization in low-ionic-strength buffer in the absence of detergent (20), or in 0.25 M sucrose, nor preparation of nuclei in nonaqueous solvents (28), nor homogenization by high nitrogen pressures (26), nor addition of 3 mM Ca^{2+} to the extraction buffer succeeded in changing the pattern shown in Fig. 1. In all cases, the majority of the virions were recovered with the cytoplasmic extract. It was therefore tentatively concluded that, although virus particles are localized inside the cell nucleus in vivo, they leak into the cytoplasm as soon as the cell is broken open. This might be due to increased fragility of the nuclear membrane of the infected cell.

Such a hypothesis was strengthened by electron microscopy examination of both infected cells and nuclei. Figure 2 (bottom panel) is an electron micrograph (courtesy of W. Bernhard) of part of a cell from a culture infected 32 hr previously with SV40. Numerous full and empty virus particles can be seen inside the nucleus. However, the photograph in the top right panel shows that, when nuclei are prepared from part of the same culture and examined, most of the virus particles seem to be gone and the nuclei look half empty. Some of the virions are found oriented along membrane-like filamentous structures outside the nuclei. The proof that leakage of virions is not due to the NP40 procedure is found in the

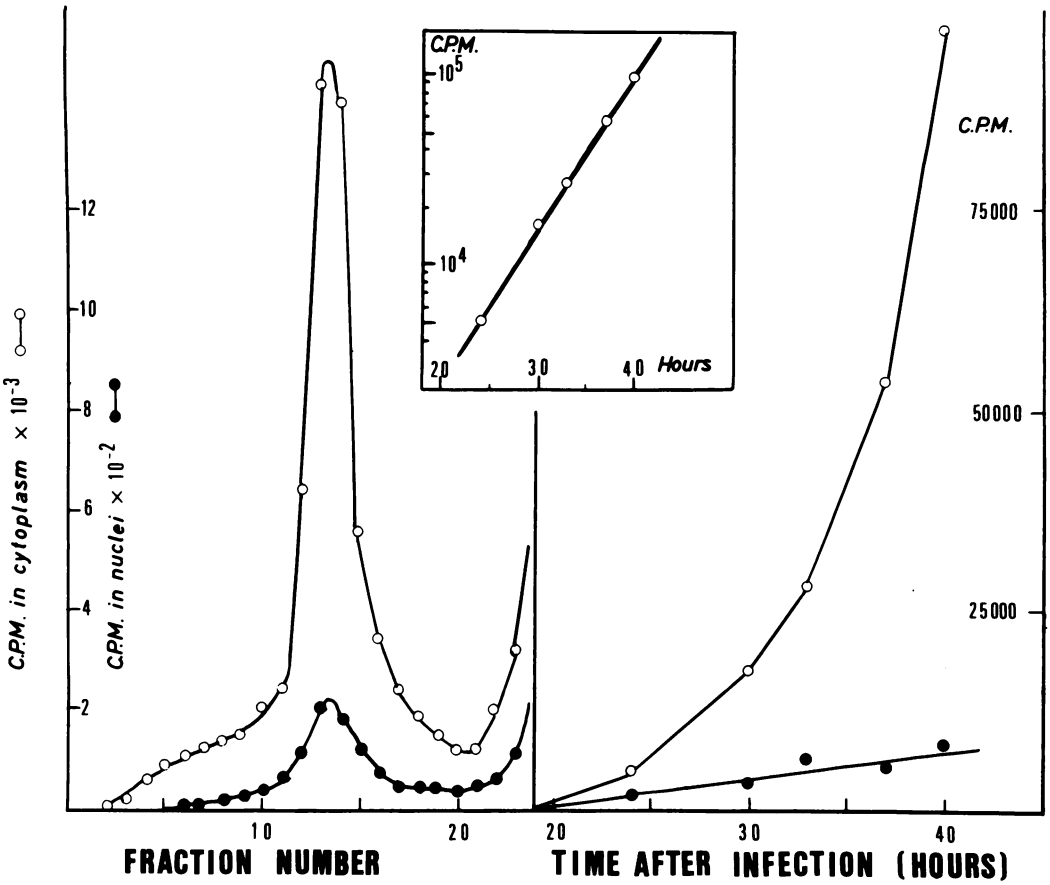

FIG. 1. *Recovery of virions in cytoplasmic extracts. Infected cells were labeled with 4 μCi of tritiated thymidine per ml from 11 to 24 hr, 24 to 30, 33, and 37 hr, and 37 to 40 hr after infection, respectively. At the end of the labeling period, cytoplasmic extracts were prepared from two petri dishes at a time, as described in Materials and Methods. The nuclei were treated with 40 μg of deoxyribonuclease per ml for 15 min at 37 C. Both nuclear and cytoplasmic extracts were made 0.5% with respect to DOC and 0.02 M with respect to EDTA, sonically disrupted, and then layered on gradients from 5 to 20% sucrose (w/w) in NEB. Centrifugation was for 110 min at 27,000 rev/min in a Spinco SW27 rotor. Acid-precipitable radioactivity was determined on each fraction of the gradients. Left: Sedimentation pattern of the labeled virions in both cytoplasmic (O) and nuclear (●) extracts from the cells labeled from 24 to 37 hr after infection. Only the first 23 fractions of each gradient are shown. Note that the scale of radioactivity used for the cytoplasmic extract has been divided by 10 as compared to that used for the nuclear extract. Right: Radioactivity in the peak of virions was determined in both cytoplasm and nuclei as illustrated in the left hand panel of the figure. The data obtained for each of the successive labeling periods were plotted in a cumulative fashion to yield the curve of virion accumulation. Symbols: O, radioactivity of virions in the cytoplasmic extracts; ●, radioactivity of virions in the nuclear extracts. Inset: Counts in cytoplasmic virions were plotted on a logarithmic scale as a function of time after infection.*

top left photograph of Fig. 2, which is an electron micrograph of nuclei prepared from noninfected cells by using the same procedure: the morphological aspect of the nuclei is quite normal. It is not known whether weakening of the nuclear membrane might occur at the time of infection when the parental virions penetrate inside the nuclei (2) or later, as one of the consequences of SV40 development.

The time course of DNA encapsidation was determined at 30 hr postinfection by labeling infected cells with tritiated thymidine for various periods of time and analyzing the resulting cytoplasmic and nuclear extracts on sucrose gradients. Even after the shortest periods of labeling, the overwhelming majority of the labeled virions were recovered in the cytoplasmic fractions. Therefore, only the

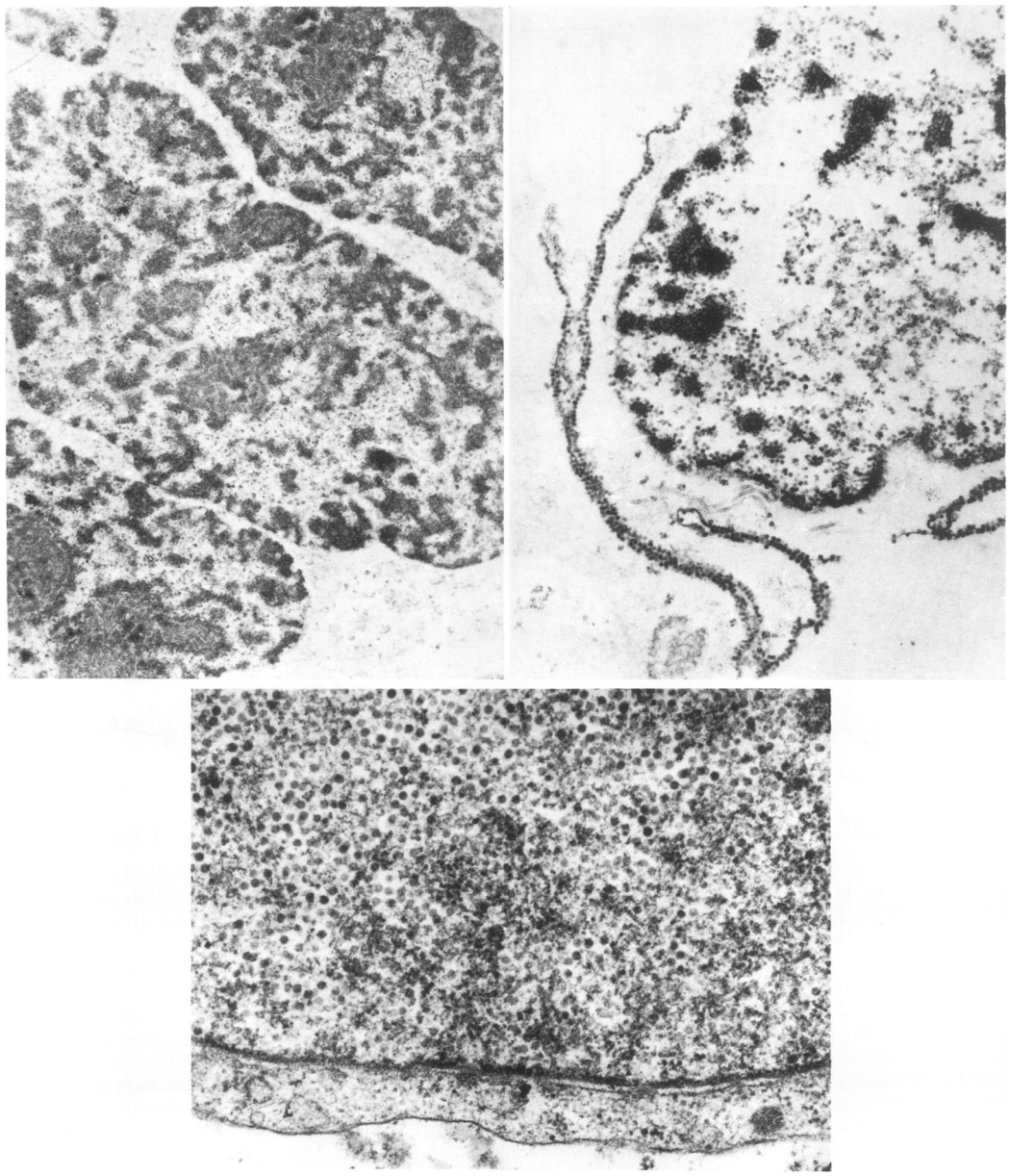

FIG. 2. *Electron micrographs of cells and nuclei. Bottom: Subcloned CV1 cells infected 32 hr previously with SV40 at a multiplicity of infection of 30 PFU/cell were scraped into ice-cold PBS and centrifuged at 800 × g for 5 min. The pellet was fixed in gluteraldehyde and processed for electron microscopy. × 20,000. Top right: A fraction from the same infected cells was washed with PBS and resuspended in isotonic buffer containing 0.5% NP40 (see Materials and Methods). The nuclei were centrifuged for 2.5 min at 800 × g. The crude nuclear pellet was fixed in gluteraldehyde and processed as above. ×10,000. Top left: Nuclei from control uninfected cells were processed in parallel with those from the infected cells. ×5,000.*

results of these have been shown in Fig. 3. Approximately 20 min elapsed before the first labeled virions could be detected. Radioactivity of the virus particles then accumulated in a nonlinear fashion. Since it was previously determined that, in this system, the apparent time for synthesis of one DNA molecule is approximately 20 min (15), it follows that there is little lag, if any, in the encapsidation of newly made viral DNA molecules. Encapsida-

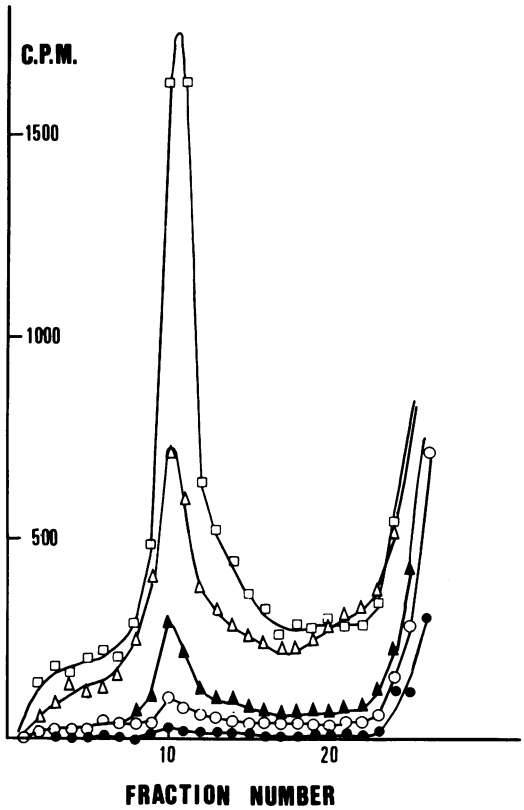

FIG. 3. *Encapsidation of viral DNA. Infected cells were labeled with 30 μCi of tritiated thymidine per ml at 30 hr after infection. Cytoplasmic extracts were prepared at various times after labeling as described in the legend to Fig. 1, using three petri dishes at a time. The extracts were centrifuged for 120 min at 27,000 rev/min, 4 C, in a Spinco SW27 rotor, through 36-ml gradients of 5 to 20% sucrose in NEB. Symbols: ●, 15 min; ○, 20 min; ▲, 30 min; △, 45 min; □, 60 min of labeling. For the sake of clarity, radioactivity at the top of the gradients is not shown.*

tion can therefore occur very quickly, if not immediately, after replication of the DNA.

In view of this result, it was questioned whether recently synthesized molecules alone were encapsidated, in which case previously made DNA would be excluded from progeny virions, or whether all DNA molecules, newly as well as previously synthesized, were part of a same pool from which maturation occurred at random. To answer this question, an experiment was devised in which DNA replication was halted through the use of cytosine arabinoside (ara-C). Infected cells were first labeled with thymidine for 2 hr. DNA synthesis was then stopped by the addition of 40 μg of ara-C per ml, and subsequent formation of virions was investigated. As can be seen in Fig. 4 (right panel), incorporation of thymidine into viral DNA was quickly halted upon the addition of ara-C (results to be published elsewhere have shown that ara-C at such concentration completely inhibits SV40 DNA replication within 20 min). In spite of the arrest of DNA synthesis, radioactivity continued to accumulate into virus particles, but the rate of virion formation was changed and the increase in labeled particles became linear Fig. 4, left hand panel). Therefore, viral DNA molecules synthesized between 26 and 28 hr postinfection can still continuously be encapsidated 3 hr later. A similar result has been reported by Ozer (18), who showed that interruption of DNA synthesis still permitted the assembly of viral capsid proteins into virions.

The question of whether progeny genomes synthesized early in the virus cycle could be encapsidated at late as well as at early times was

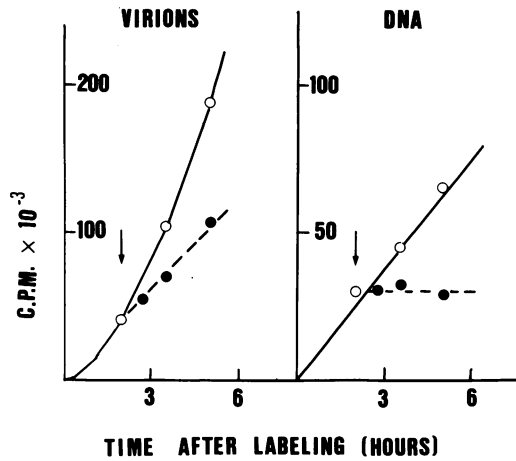

FIG. 4. *Continuing formation of virions after interruption of DNA synthesis. At 25 hr postinfection, infected cells were overlaid with 1 ml of minimal essential medium supplemented with 2% dialyzed calf serum. One hour later, tritiated thymidine (40 μCi/ml) was added. Left: Cytoplasmic extracts were prepared at various times thereafter and analyzed for radioactivity in virions as described in the legends to the preceding figures. Two petri dishes were used for each extraction. At 2 hr after labeling (arrow), a second batch of cells received 40 μg of cytosine arabinoside (ara-C) per ml and was processed as above to determine residual encapsidation. Symbols: ○——○, control; ●----●, ara-C-treated. Right: Petri dishes from both batches, labeled and treated as above, were used for Hirt extractions. Acid-precipitable radioactivity was determined in 0.1 ml of the supernatant fluids. Symbols: ○, control; ●, ara-C 2 hr after labeling.*

also investigated by pulse labeling infected cells with radioactive thymidine at 24 hr after infection and then chasing the label with cold medium and following its eventual appearance into virions. Figure 5 shows that the chase was quite effective, as judged from the fact that the number of counts recovered in viral DNA (closed circles) remained fairly constant during the 8 hr of the chase. The proportion of viral DNA molecules to become encapsidated (open circles) continued, however, to increase at an apparently constant rate during the same time. It can therefore be concluded that DNA molecules synthesized early in the replication cycle can mature to virions at later times as well.

The percentage of newly made viral DNA molecules which are encapsidated at various times after infection was determined. Infected cells were pulse labeled for 2 hr with tritiated thymidine at the various indicated times (Table 1), and the percentage of labeled viral DNA recovered as virions was determined after each of the labeling periods. Table 1 shows that the probability for a newly made DNA molecule to the encapsidated is always approximately the same, independent of the time after infection at which its replication occurs. In the sample labeled from 36 to 38 hr postinfection, viral DNA replication was turning off, as judged by the radioactivity recovered in DNA molecules. However, 16% of the labeled DNA was encapsidated by the end of the labeling period, which is not very different from the percentage of encapsidated DNA found at the various other sampling times.

Measurement of the size of the capsid proteins pool at 29 hr postinfection was also accomplished by pulse labeling infected cells with radioactive lysine and then chasing the label and interrupting further protein synthesis by addition of 50 μg of cycloheximide per ml. After a brief period of labeling and chase, two species of particles were found, the fastest of which sedimented as mature virions at ca. 180*S*, and the slowest of which had a sedimentation coefficient of approximately 150*S* (Fig. 6). The latter are probably analogous to those which were observed by Ozer in isopycnic density gradients (18). Accumulation of label in the lighter particles ceased after 15 to 20 min of chase, whereas labeled virions accumulated for more than 100 min. Similar results were obtained through the use of labeled leucine, isoleucine, or amino acids mixture.

The preferential labeling of the 150*S* particles after short periods of exposure to radioactive amino acids suggests that some of them at least might be precursors to the virions (18). However, no major buildup of such particles could be detected upon arresting viral DNA replication with a variety of inhibitors of DNA synthesis. This is not surprising in view of the above finding that interruption of DNA replication with ara-C does not preclude the continuous encapsidation of previously synthesized SV40 genomes. An experiment similar to that of Jacobson and Baltimore (13), accumulating poliovirus procapsids through the use of guanidine, therefore does not seem feasible in the case of SV40.

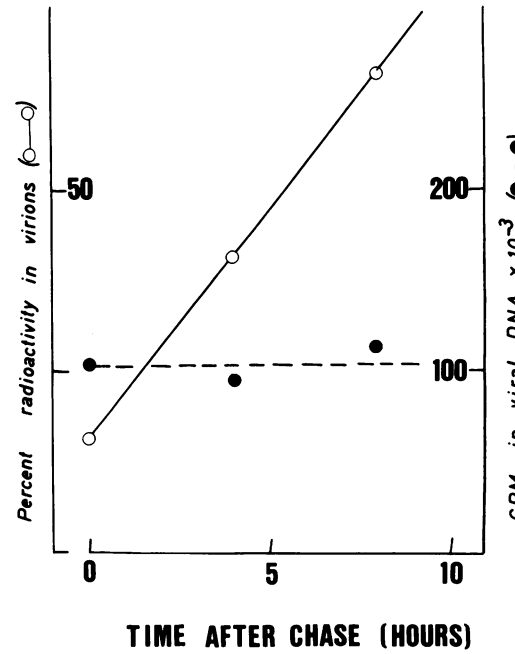

FIG. 5. *Labeling of virions during a chase. SV40-infected cells were overlaid 23 hr after infection with 1 ml of minimal essential medium containing 2% dialyzed calf serum. Tritiated thymidine (40 μCi/ml) was added at 24 hr postinfection. Two hours later, the cell monolayers were washed twice and then overlaid with 5 ml of prewarmed MCV1 medium. Cytoplasmic extracts were prepared at various times thereafter on two petri dishes at a time and analyzed for radioactivity in virions as described in the legends to the preceding figures. Hirt extractions (11) were performed at the same times on a third petri dish, and the resulting supernatant fractions were analyzed for radioactivity in viral DNA by centrifugation through gradients of 5 to 20% sucrose in 0.1 M NaOH, 0.02 M NaCl, 0.02 M EDTA, for 4.5 hr at 41,000 rev/min, 4 C, in a Spinco SW41 rotor. Symbols: ●----●, number of counts in viral DNA (component I); O——O, percentage of such counts recovered as virions.*

TABLE 1. *Percent of newly made DNA encapsidated at various times after infection*[a]

Time of labeling (hr postinfection)	Radioactivity in viral DNA (counts/min)	Radioactivity in virions (counts/min)	Percent labeled viral DNA encapsidated
24-26	95,000	15,900	16.8
28-30	83,000	17,500	21
32-34	85,000	17,000	20
36-38	40,000	6,500	16.2

[a] At the indicated times, three petri dishes of infected cells were pulse labeled for 2 hr with 40 μCi of tritiated thymidine in 1 ml of minimal essential medium supplemented with 2% dialyzed calf serum. At the end of each labeling period, two of the petri dishes were used for preparation of cytoplasmic extracts and analysis by sucrose gradient centrifugations of the radioactivity incorporated into virions, whereas the third was used for Hirt extraction (11) and determination of the radioactivity accumulated into viral DNA molecules (see legend to Fig. 4).

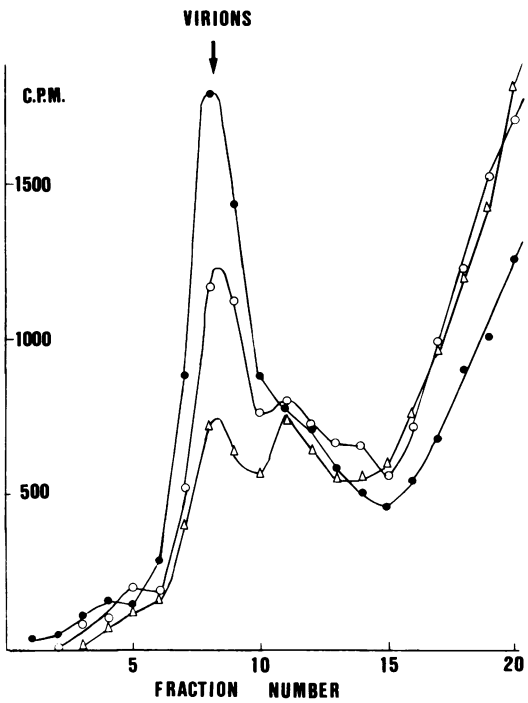

FIG. 6. *Assembly of capsid proteins in the presence of cycloheximide. At 25 hr after infection, cell monolayers were overlaid with 1 ml of medium containing 1/50 the normal concentration of lysine. Four hours later the cells were pulse labeled for 15 min with 10 μCi of tritiated lysine per ml. At the end of the labeling period the medium was aspirated and the cell monolayers were overlaid with 5 ml of MCV1 medium supplemented with twice the normal lysine concentration and 50 μg of cycloheximide per ml. Samples (three petri dishes at a time) were withdrawn at various times thereafter, and cytoplasmic extracts were prepared and analyzed by sucrose gradient centrifugation as in Fig. 3. Only the first 20 fractions of the gradients are shown. The samples shown were withdrawn after: Δ, 15 min of chase; O, 35 min of chase; and ●, 55 min of chase. The arrow labeled "virions" refers to the position of tritiated thymidine-labeled virions centrifuged in parallel.*

DISCUSSION

The most unexpected result reported in this study is that the majority of the SV40 virions are at all times recovered with the cytoplasmic fraction of the infected cells. This result was obtained with a variety of techniques for the preparation of nuclei, all known to yield intact nuclei from uninfected cells. Electron microscopy examination of the infected cells and of their purified nuclei showed that virions leak from the nucleus into the cytoplasm at the time of cell fractionation. This most likely reflects a specific fragilization of the nuclear membrane due to SV40 infection. The minority of virions remaining inside the nuclei are not essentially different from those recovered in the cytoplasmic extract, since between 24 and 30 hr postinfection the great majority of labeled virions were recovered in the cytoplasmic extract, independently of the duration of the labeling period and of the nature of the labeled precursor. However, all constituents of the infected cell nucleus do not leak equally. Thus, 45 and 32*S* precursors to the infected cell ribosomal ribonucleic acids RNAs are normally recovered in the nuclear fraction, whereas some of both viral and cellular DNAs leaks into the cytoplasm (*unpublished results*).

It is clear from these results that experiments in which SV40-infected cells are fractionated into cytoplasmic and nuclear fractions, and where the relative distribution of molecules, particles, or label between these two fractions is determined, should be interpreted with caution.

Thus, when determining the sedimentation profile of the polyribosomes from African green monkey kidney (AGMK) cells infected with SV40, Rapp and Guentzel (23) observed in the cytoplasmic extract the presence of a peak of material which sedimented at approximately 150 to 200*S* and which resisted both

treatments with EDTA and ribonuclease. Complement-fixing activity with antiserum against SV40 viral antigen was associated with the material in that peak. These considerations led the authors to conclude that the peak was nascent SV40 virions maturing on the polyribosomes. It is much more likely, in view of the results reported here, that those were SV40 mature particles leaked from the nucleus into the cytoplasmic extract at the time of cell fractionation. It is not known whether fragilization of the nuclear membrane of the infected cell might be related to the mode of entry of the parental virions into the cell nucleus and to their mode and site of decapsidation (2). But it might explain the progressive accumulation of SV40 virions in the giant cytoplasmic vacuoles which are found late in infection in vivo (10) and which relate to the well known vacuolating property of the virus.

The results presented here also show that the overall synthesis of SV40 virions occurs in a nonlinear fashion, beginning at approximately 20 hr after infection. However, encapsidation of the DNA molecules made at the 26th hour after infection occurred continuously, and at a constant rate, for at least the 8 hr after their synthesis. After the 20th hour of infection, SV40 DNA replication is known to proceed at a constant rate (15). Nevertheless, at all times after infection, equal percentages of newly made viral DNA were recovered in virions (from 15 to 20% of the DNA labeled with radioactive thymidine in the course of 2 hr).

These various apparent contradictions lead to the conclusion that the size of the intracellular pool of capsid proteins should be at all times adjusted to that of viral DNA. According to this hypothesis, the probability of a newly made DNA molecule becoming encapsidated in the space of 2 hr would remain the same, whether its synthesis occurs at late or at early times in the replication cycle, and in spite of its greater dilution at later times by previously synthesized molecules. In the same way, encapsidation of the DNA molecules made at early times should proceed at a more or less constant rate, in spite of their progressive dilution into the pool of accumulating progeny DNA. The overall nonlinear increase in virions could therefore result from the simultaneous and constant increase in size of both the viral DNA and the capsid protein pools. In agreement with this hypothesis is the fact that interruption of DNA replication by ara-C immediately resulted in a shift from a nonlinear to a linear rate of virus particle formation. The adjustment of the sizes of both pools to one another might be accomplished through the coupling of the transcription of late viral genes to the replication of viral DNA. Experiments aimed at testing this hypothesis are in progress.

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