Enhancement of Uptake of Simian Virus 40 by Nuclei of Permissive Cells (37686)

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The involvement of the cell nucleus early in the replication of simian virus 40 (SV40) was recently underlined by observations demonstrating nuclear uptake of intact SV40 and intranuclear uncoating of parental virus (1, 2). Intact SV40 was recovered from the nuclei of permissive monkey kidney cells up to 4 hr after infection (1). Virus replication was most likely initiated by virions which penetrated into the cell nuclei (1, 2). Because only a relatively small proportion of input SV40 gets into the nuclei of infected cells, conditions were sought to enhance nuclear uptake of SV40 in order to facilitate biochemical studies on the nucleus-associated virus, such as the fate of uncoated viral proteins.

Materials and Methods. Cells and virus. Secondary cultures of African green monkey kidney cells were prepared in 50-mm diam petri dishes as described previously (3). Purified SV40 labeled with either [^{3}H]thymidine or [^{3}H]amino acids was prepared as described by Tan and Sokol (3).

Infection of cells with purified infectious virus. Unless stated otherwise in *Results,* monolayer cell cultures (4–6 days after reaching confluency) were washed twice with culture medium containing 2% calf serum, and then infected with SV40 suspended in 0.2 ml of culture medium containing 2% calf serum and buffered with 0.02 *M* Tris–HCl at pH 7.5. The input m.o.i.[1] was 100 PFU of virus/cell (the proportion of virus which penetrated into the nucleus was similar for m.o.i. from 80 to 500 PFU/cell). Infected cells were incubated at 37° for 90 min and agitated every 15 min to redistribute the inoculum. After infection, cells were washed twice with 0.32 *M* sucrose, 0.002 *M* $MgCl_2$ in 0.001 *M* potassium phosphate buffer, pH 6.8, scraped off the glass, and fractionated into nuclear and cytoplasmic fractions (4).

Quantitation of virus uptake.[2] Trichloroacetic acid was added to both cytoplasmic and nuclear fractions to a final concentration of 5%. After standing at 0° for 1 hr, precipitates were collected by centrifugation, dissolved in 0.4 ml NCS (Nuclear–Chicago, Des Plaines, IL), and added to 10 ml of toluene–Permafluor (Packard Inst., Downers Grove, IL) containing 0.1% acetic acid. Radioactivity was measured in a liquid scintillation spectrometer. The acid-precipitable radioactivity associated with the nuclear and cytoplasmic fractions was related to that of the input acid-precipitable radioactivity. The latter was determined by inoculating cells with radioactive virus and immediately determining the total acid-precipitable radioactivity in the inoculum, cell washes, and cytoplasmic and nuclear fractions.

Reagents. Calf thymus histones, type II-A (unfractionated), type III (lysine-rich), type IV (arginine-rich), DEAE-dextran, and cycloheximide (Sigma, St. Louis, MO), hydroxyurea (Nutritional Biochemical, Cleveland, OH), Ara-C (Upjohn, Kalamazoo, MI), BUdR (Calbiochem, La Jolla, CA), FUdR (Hoffmann-la-Roche, Nutley, NJ), and actinomycin D (Merk, Sharp and Dohme, Rahway, NJ) were used.

Results. Effect of $MgSO_4$ and polycations

[1] Abbreviations: m.o.i., multiplicity of infection; PFU, plaque forming unit; Ara-C, cytosine arabinoside; BUdR, 5-bromodeoxyuridine; FUdR, 5-fluorodeoxyuridine; AGMK, African green monkey kidney.

[2] In the present study, "virus uptake" includes both virus adsorption and penetration.

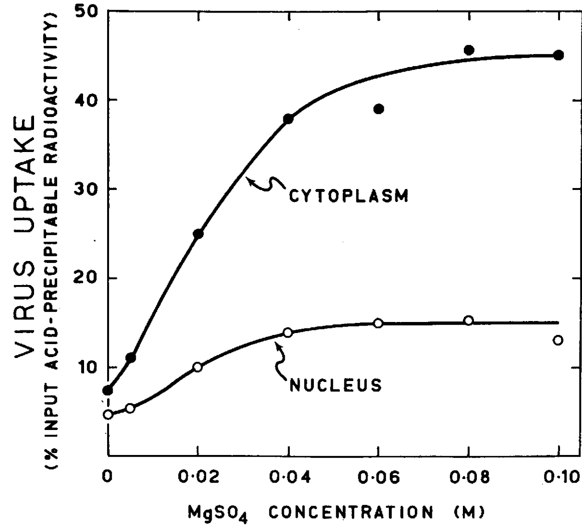

FIG. 1. Effect of $MgSO_4$ on the uptake of SV40 by AGMK cells. Uptake of [^{3}H]thymidine-labeled SV40 (input of 16,500 cpm/culture) by AGMK cells was determined at different concentrations of $MgSO_4$ after incubating infected cultures at 37° for 90 min.

on virus uptake. Joklik (5) reported that the uptake of rabbit pox virus by HeLa cells was enhanced in the presence of $MgSO_4$. Similarly, both the cytoplasmic and nuclear uptake of SV40 increased with increasing concentrations of $MgSO_4$ up to about 0.04 *M* (Fig. 1). The extent of enhancement of nuclear uptake of virus by 0.04 *M* $MgSO_4$ was usually

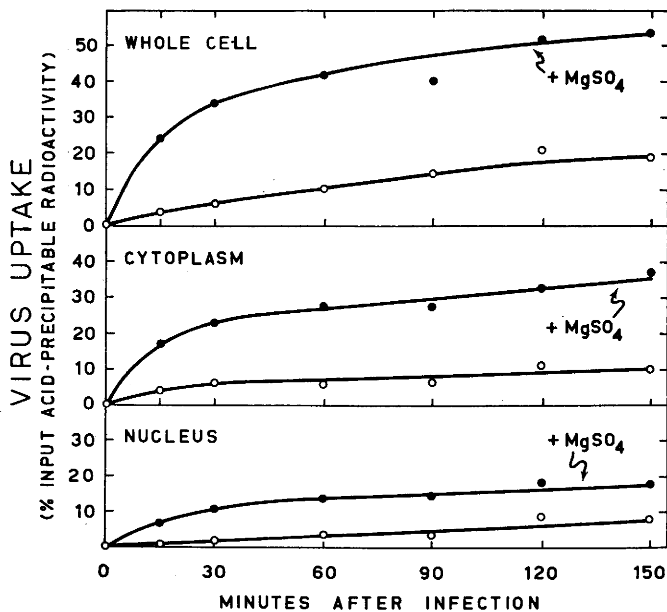

FIG. 2. Kinetics of SV40 uptake by AGMK cells in the presence and absence of $MgSO_4$. Secondary AGMK cells were infected with [^{3}H]thymidine-labeled SV40 (input of 14,600 cpm/culture) in the presence (●—●) and absence (○—○) of 0.04 *M* $MgSO_4$. Virus uptake was determined at intervals after infection.

TABLE I. SV40 Uptake by AGMK Cells in the Presence of Polycations.[a]

Addition to culture medium		Virus uptake (% input acid-precipitable radioactivity) by:	
		Cytoplasm	Nucleus
None		15.7	9.1
DEAE-dextran	100 μg/ml	33.4	26.7
	500 μg/ml	15.3	18.6
Unfractionated histones	100 μg/ml	24.7	12.8
	500 μg/ml	24.4	40.0
Arginine-rich histones	100 μg/ml	28.3	22.1
	500 μg/ml	14.9	49.1
Lysine-rich histones	100 μg/ml	15.6	8.8
	500 μg/ml	27.1	11.3

[a] Secondary AGMK cells were infected with [^{3}H]thymidine-labeled SV40 (input of 15,000 cpm/culture) in the presence of polycations. Virus uptake was determined after 90 min incubation at 37°.

about three-fold but was as high as six-fold in some experiments. Therefore, in all subsequent experiments, cells were infected in the presence of 0.04 *M* $MgSO_4$, unless stated otherwise. At this $MgSO_4$ concentration, the initial rates of virus uptake both by the cytoplasm and nuclei of infected cells were increased significantly (Fig. 2). The virus inoculum containing $MgSO_4$ is slightly hypertonic. Hypertonicity is, however, probably not the cause of enhanced virus uptake, because rendering the inoculum hypertonic with other salts, such as $MgCl_2$, $MnCl_2$, $MnSO_4$, or Na_2SO_4, did not enhance virus uptake by cells. Polycations present in the virus inoculum devoid of $MgSO_4$ also enhanced virus uptake (Table I). Nuclear uptake of virus was greatly stimulated by the presence of 100 μg DEAE-dextran/ml or 500 μg unfractionated histones/ml. Arginine-rich histones (500 μg/ml) markedly increased nuclear uptake of SV40, resulting in about 50% of the input virus becoming associated with the nuclei. In contrast, lysine-rich histones did not enhance nuclear uptake of SV40. High concentrations of histones are toxic to cells (6, 7), but under the conditions used in these experiments, cells treated with histones for 2 hr were similar in morphological appearance to untreated cells.

Effect of cell age and metabolic inhibitors on virus uptake. To determine whether the age of the cells affects virus uptake, AGMK cells were seeded to form confluent monolayers in 2 days. Virus uptake was determined at intervals subsequent to cell monolayer formation. Figure 3 shows that the age of the cell monolayer has a marked effect on virus

TABLE II. Effect of Metabolic Inhibitors on Uptake of SV40 by AGMK Cells.[a]

Inhibitor		% Inhibition of virus uptake by:	
		Cytoplasm	Nucleus
FUdR	15 μg/ml	5.3	10.1
BUdR	5 μg/ml	18.0	20.0
Ara-C	15 μg/ml	8.5	0.0
Hydroxyurea	5 μg/ml	12.0	17.7
Actinomycin D	1 μg/ml	13.4	0.0
Cycloheximide	5 μg/ml	11.1	4.8

[a] Secondary AGMK cells were pretreated with the inhibitors for 3.5 hr at 37° before infection with [^{3}H]thymidine-labeled virus (input of 23,000 cpm/culture) suspended in the same concentration of inhibitor, 0.04 *M* $MgSO_4$, and 2% calf serum. Trichloroacetic acid-precipitable radioactivity associated with the cytoplasm and nuclei was determined after 90 min incubation at 37°. In the untreated cells, 36 and 18% of the input virus were recovered from the cytoplasm and nucleus, respectively.

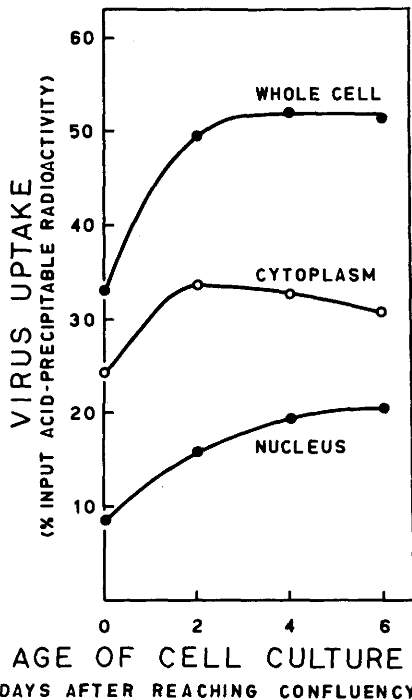

FIG. 3. Influence of age of monolayer cultures of AGMK cells on uptake of virus. All monolayer cultures were prepared from the same batch of AGMK cells. When the cultures reached confluency, time was designated as 0 day. The number of cells/culture at 0, 2, 4, and 6 days was 2.9×10^6, 2.6×10^6, 2.4×10^6, and 2.8×10^6, respectively. Uptake of [^{3}H]thymidine-labeled SV40 (input of 15,000 cpm/culture) was determined as described in *Materials and Methods*. The virus inoculum was frozen in small aliquots which were thawed only once just before infection at the indicated times.

uptake in that total virus uptake by cells in monolayers immediately after reaching confluency was poor, and that nuclear uptake of SV40 increased with the age of the monolayer. This increase in nuclear uptake was not a reflection of an increase in cell number. Since a correlation between the rate of virus uptake and the age of the cell monolayer may be the result of a change in the physiology of the cells, it was of interest to determine if the inhibition of cellular protein, RNA, and DNA synthesis would affect virus uptake. Inhibition of the synthesis of cellular protein by 92%, RNA by 94%, and DNA by 95% did not markedly affect either nuclear or cytoplasmic uptake of virus (Table II).

Uptake of infectious virus and "empty" capsids. Simian virus 40 contains both infectious virus and noninfectious "empty" capsids which are essentially devoid of DNA (3). Nuclear uptake of empty capsids was determined by inoculating cells with purified empty capsids. The results (Table III) show that not only were empty capsids taken up by the nuclei, but they were taken up more efficiently than infectious virus.

Fate of parental virus in the cell nuclei. Barbanti-Brodano *et al.* (1) reported that intact parental SV40 could be recovered from the nuclei of infected cells at 4 hr after infection, and at 6 hr after infection virus uncoating was complete. The fate of parental viral polypeptides in the nuclei of AGMK cells was investigated by polyacrylamide gel electrophoresis. The results (Fig. 4) show the amount of intact viral polypeptides in the nuclei decreased with progressive infection, but even at 24 hr after infection, they were present in detectable amounts.

Virus uptake by CV-1 cells. Simian virus 40 uptake was also determined with CV-1

TABLE III. Uptake of Different Forms of SV40 by AGMK Cells.[a]

Virus form	Virus uptake (% input acid-precipitable radioactivity) by: Cytoplasm	Nucleus
Infectious virus	33.6	14.5
Empty capsids	37.1	27.2

[a] Secondary cultures of AGMK cells were inoculated with [^{3}H]amino acid-labeled virus in the presence of 0.04 *M* $MgSO_4$. The m.o.i. with infectious virus was 100 (input of 31,000 cpm/culture). An equivalent amount of viral protein was used to inoculate cell cultures with empty capsids (input of 28,000 cpm/culture). Virus uptake was determined after 90 min incubation at 37°. Similar results were obtained with several different preparations of SV40. When nuclei from cells 90 min after infection with radioactive empty capsids were lysed and subjected to equilibrium sedimentation in CsCl solution (1), all the radioactivity was recovered at the buoyant density (1.30 g/cm^3) of empty capsids (3).

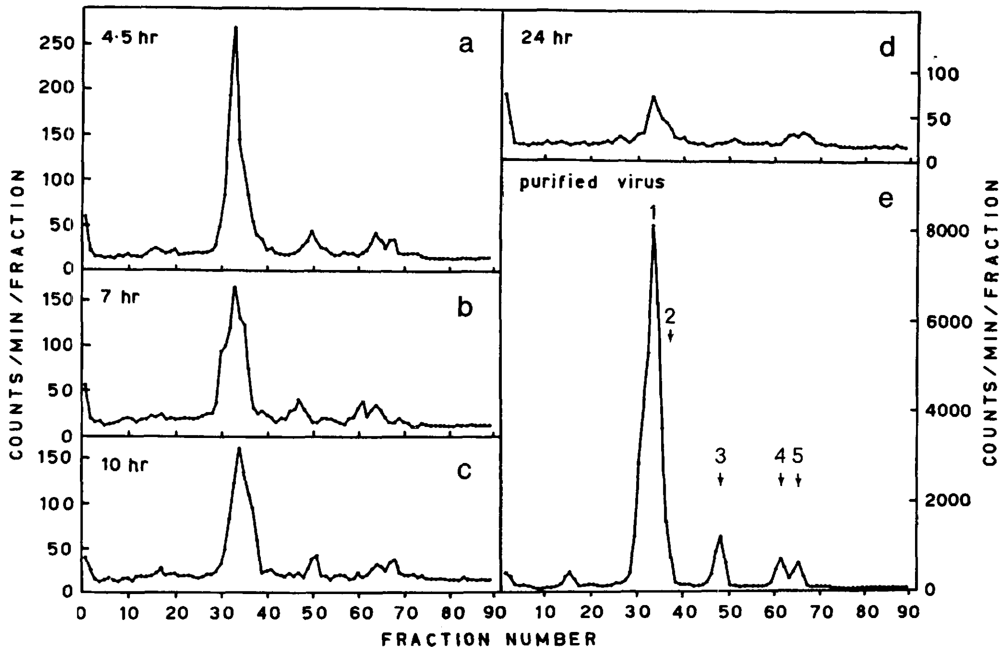

FIG. 4. Persistence of undegraded parental viral polypeptides in nuclei of infected cells. Monolayer cultures of AGMK cells (8.5 × 10^6 cells) were inoculated with 100 PFU of purified infectious SV40 (labeled with ^{3}H-amino acids) per cell and incubated at 37° for 90 min. The virus inoculum was removed, and the cells were incubated at 37° with fresh culture medium. At 4.5 hr (a), 7 hr (b), 10 hr (c), and 24 hr (d) after infection, nuclei were isolated from the same number of cells and solubilized for electrophoresis in 9% SDS–polyacrylamide gels as described previously (3). (e) Purified virus. Electrophoretic migration is from left to right, and polypeptides are numbered as described previously (3). In this experiment, polypeptide 2 was not resolved from polypeptide 1.

cells, an established cell line of AGMK cells (8). The optimum conditions for virus uptake by CV-1 cells were found to be the same as those for secondary AGMK cells. However, both cytoplasmic and nuclear uptake of virus by CV-1 cells were inefficient (2–3-fold lower) in comparison with that in AGMK cells.

Discussion. In permissive cells infected with SV40, virus is rapidly transported into the cell nucleus, and, presumably, this nucleus-associated virus initiates viral replication (1, 2). Detailed biochemical studies on the fate of the parental virus in the nuclei of infected permissive cells are lacking. One of the factors which make such studies difficult is that only a small proportion of infectious virus reaches the cell nucleus. Our data show that SV40 uptake by nuclei of permissive AGMK cells could be enhanced by infecting monolayer cultures, several days after reaching confluency, in the presence of $MgSO_4$ or polycations. Although maximum enhancement of nuclear uptake of SV40 was effected by arginine-rich histones, this procedure is not recommended for studying the fate of nucleus-associated virus at late times after infection because histones are toxic to cells, although this toxicity is reversible (6). However, arginine-rich histones could be used to enhance nuclear uptake of SV40 to study the early events of infection in the nucleus. $MgSO_4$ (0.04 *M*) is not toxic to cells and is therefore suitable for enhancing nuclear uptake of virus for studying the fate of parental virus at both early and late times after infection. The observed enhancement of nuclear uptake of SV40 in the presence of 0.04 *M* $MgSO_4$ probably does not relate to the uptake of viral aggregates because more

plaques were obtained in assaying the infectivity of SV40 in the presence of $MgSO_4$ than in the absence of this salt (M. McFalls and K. B. Tan, unpublished data).

Our preliminary results on studies on the fate of parental SV40 in infected AGMK cell nuclei showed that all viral polypeptides, in an undegraded form, were detectable up to 24 hr postinfection. At this time, no intact parental virus is detectable in the infected cell nuclei (1). We have also shown that empty capsids are efficiently taken up by the cell nuclei. In view of these results, the possibility of the protein moiety of the parental virus, whether from empty capsids or infectious virus, playing a role in the regulation of viral functions in infected cell nuclei, whether at early or late times after infection, should be considered.

Summary. Simian virus 40 uptake by the nuclei of permissive monkey kidney cells was enhanced by infecting in the presence of $MgSO_4$, DEAE-dextran, or arginine-rich histones, but not in the presence of lysine-rich histones. Nuclear uptake of virus was found to be more efficient in aged monolayer cultures than in freshly formed cell monolayers. Inhibition of cellular protein, RNA, or DNA synthesis did not prevent virus uptake. "Empty" capsids, which are essentially devoid of viral DNA, were taken up by the nuclei of permissive cells even more efficiently than infectious virus. Undegraded polypeptides of parental virus could be detected in the nuclei up to 24 hr after infection.

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