FIBRILS ATTACHED TO THE NUCLEAR PORE PREVENT EGRESS OF SV40 PARTICLES FROM THE INFECTED NUCLEUS

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Simian virus 40 (SV40) has been reported to penetrate the nucleus of CV1 cells as a whole virus. The viruses were found in the nucleus in small clumps 1 h after infection (14), with only very few in the cytoplasm. Most nuclei were packed with virus particles 24–48 h after infection but only a few particles were in the cytoplasm. The virus was seemingly released only through lysis of the cell. The question raised, therefore, was how can the virus particle enter the nucleus despite a very low gradient from cytoplasm to the nucleus but not leave despite an enormous difference in concentration relative to the cytoplasm? Since the size of the SV40 virus is too large (45 nm) for diffusion through the 4.5-nm patent part of the pore complex (19), we focused our attention on the nuclear pore complex as the size-limiting factor in the exit of the virus progeny.

MATERIALS AND METHODS

The monkey kidney cells, CV1, obtained from the American Type Culture Collection, were grown to confluence, 7–9 days after subculturing in 3.5-cm plastic petri dishes (Falcon Plastics, Div. of Bio-Quest, Oxnard, Calif.) in Dulbecco's modification of Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) in a CO_2 incubator and used between passage 30 and 40. They were infected at confluence with SV40 stock virus (RH 911) at a multiplicity of 100 plaque-forming units/cell for 1 h at 37°C. After 1 h, the unabsorbed virus was removed and the monolayers were refed with MEM without serum to prevent cells from traversing the cell cycle. At different times after infection, samples for electron microscopy were fixed for 1 h at room temperature in 3% glutaraldehyde (phosphate buffered, pH 7.4) and post-fixed for 1 h with 1% OsO_4 (phosphate buffered, pH 7.4). The cells were covered with uranyl acetate in water for 16 h at 60°C (16) and then rapidly dehydrated in ethanol. They were then flat-embedded in Epon according to the method of Brinkley et al. (7). For analysis of cross-sectioned cells, the Epon-embedded sheets were glued together to obtain two lines of cells in each section. Rather long sections were picked up on collodium- and carbon-coated, single-hole grids. Sections were observed either unstained or with the contrast increased by lead citrate. The cells were observed with a Hitachi HU-11E electron microscope at 75 kV.

RESULTS

In uninfected control cells (Fig. 1), euchromatin can be distinguished from heterochromatin. Granules of the same size and electron opacity as SV40 particles (arrow pointing down) can be distinguished from other perichromatin granules (arrow pointing up) by their slightly smaller size. The inset shows a clump of these particles which, however, lack the perfect roundness of the progeny particles (Fig. 2).

During the productive phase of virus assembly, evident at 24 h after infection, SV40 particles accumulate in the nucleus and tend to replace the euchromatin; later at 48 h, they replace the heterochromatin (Fig. 2). The particles are highly concentrated in the nucleus and are only occasionally found in the cytoplasm. At low magnification, no chromatin can be seen.

After absorption of the nonenveloped SV40 particles to the cell membrane (Fig. 3), they seem to become enveloped by membrane and thus enter the cytoplasm. The micrograph taken from a productively infected cell 48 h after infection (Fig. 2) shows a cytoplasmic particle that must have entered from another lysed cell and not from the nucleus of this cell, since no coating of the SV40 particle with the nuclear membrane or any release through the pore complex could be seen. This micrograph also shows some heavily stained material directly attached to the nuclear membrane which may be remnants of the heterochromatin. Some fibrous material can also be found throughout the nucleus and at the nuclear pore complexes. Around most of the nuclear pore complexes there seems to be an area that contains no SV40 particles. The density of the particles in any given micrograph, however, was relatively low so that such absence of SV40 particles may simply reflect the random distribution of particles. We then used our micrographs with nuclear pores in cross section and transferred the location of each particle relative to the nuclear pore on a glycine sheet. The

glycine sheets were then laid over a blackened negative with the nuclear membrane and pore outlined and the location of SV40 punched in with a fine needle. The accumulated data are reproduced in Fig. 4*a*. It is clear from this graphical display that there is an area around the nuclear pore complex that contains no SV40 particles. If one plots the accumulated SV40 from this negative within a 100-nm strip parallel to the nuclear membrane at 20-nm class intervals, one finds that the increase in virus particles reaches a maximum at a distance of ~80 nm from the nuclear membrane but that there are not particles until 80 nm at the nuclear pore area. The same maximum as that at the nonpore areas is eventually reached for the pore area at a distance of 200 nm from the membrane (Fig. 4*b*).

Closer inspection of the nuclear pore complex in serial face on sections (Fig. 5*a*, *b*) reveals a fibrous ring with connections radiating to the heterochromatin (arrows in Fig. 5*a*, *b*). These fibrous extensions of the nonmembranous part of pore complex must be closed (Fig. 5*b*, double arrow) like a "fish trap" to exclude SV40 particles.

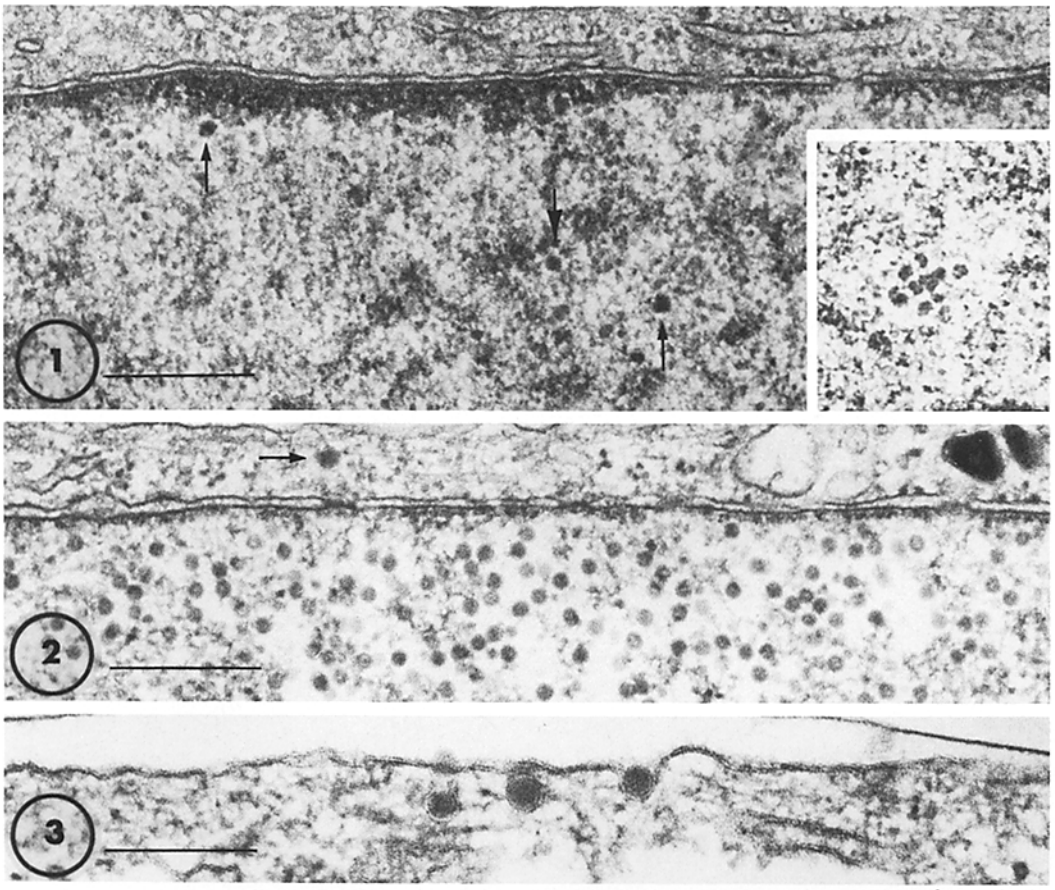
DISCUSSION

This investigation sought to clarify the apparent paradox of SV40 particles that penetrate the nucleus as whole virions with only a slight concentration gradient from the cytoplasm to the nucleoplasm but cannot leave the nucleoplasm despite an extraordinary concentration gradient. The ultrastructure of CV1 cells during the infection cycle was basically the same as that described by Granboulan et al. (11) except that, in the approximately 900 cross-sectioned cells analyzed,[1] we observed no SV40 particle inside the two nuclear membranes. This seems to exclude penetration of the nuclear membrane as a regular route of nuclear cytoplasmic virus ingress and egress. Only later during the infectious cycle were cytoplasmic particles seen, and nearly all of them were membrane enveloped. They are thought to be due to SV40 reinfection after release into the medium by cell lysis. The approximately 4,000 pore complexes per nucleus (Maul, G. G., and L. Deaven, manuscript in preparation) should allow virus escape, and such escape should be able to be visualized. The nuclear pore complex did not seem to be a route of virus release into the cytoplasm, however, because no virus was observed apparently in transit and because an area of exclusion of SV40 particles exists around the nucleoplasmic side of the pore complex. The structure limiting the viral particle egress does not seem to be chromatin, as all euchromatin and heterochromatin (except for some nucleolar material) had been replaced by viral particles. Rather, it may be part of the fibrous lamina (1, 2), the nuclear matrix (6), or the detergent-resistant, interporous "skeleton" meshwork (20).

Although this limiting structure may be assumed to function like a fish trap, it is difficult to resolve. In fortuitous sections, it appears to consist of eight traverse fibers (17, 18) but in face on sections it projects with a ring-like appearance (see references 8, 9, 10, 15 for review on pore structure). Most of these rings in uninfected cells can be observed to have several fibrous connections (possibly eight) to heterochromatic materials as was proposed in a model of the pore complex by Hoeijmakers et al. (12). This ring structure cannot be resolved into eight fibers by tilting the section (not shown) and is, therefore, not due to a slightly oblique projection of the traverse fibers. The same type of fibrous connections from the ring structure to the chromatin also connects the pores. They are most often present well below the membrane level. This type of "pore connecting fibrils" may not tear during detergent disruption of the nuclear membrane but act to hold the pore complexes together. Scheer et al. (20) hypothesize a detergent-resistant, interporous skeleton meshwork within the membrane that holds the pore complexes together after disolution of the nuclear membrane.

The initial question of how a structural arrangement of fibers can allow a rather large particle in and prevent it from going out remains unresolved. A fish trap-like structure may exist but that explanation may be too naive. Active transport in one direction only must then be envisaged because the patent hole of the pore complexes was determined to be only 4.5 nm (19). Another explanation could be that the problem as such does not exist. In fact, we did find structures resembling SV40 particles in size and density in our control cells. Most of them were, however, not so round as the progeny virus, although they looked exactly like those found by Hummeler et al. (14), who introduced the concept of whole particles entering the

[1] Maul, G. G. 1975. Annulate lamellae and single pore complexes in normal, SV40-transformed and tumor cells *in vitro*: a semiquantitative analysis. Manuscript submitted for publication.


1
2
3
4a
4b
Number of SV40 particles
1
2
3
Distance from nuclear membrane (nm)
100
200
300

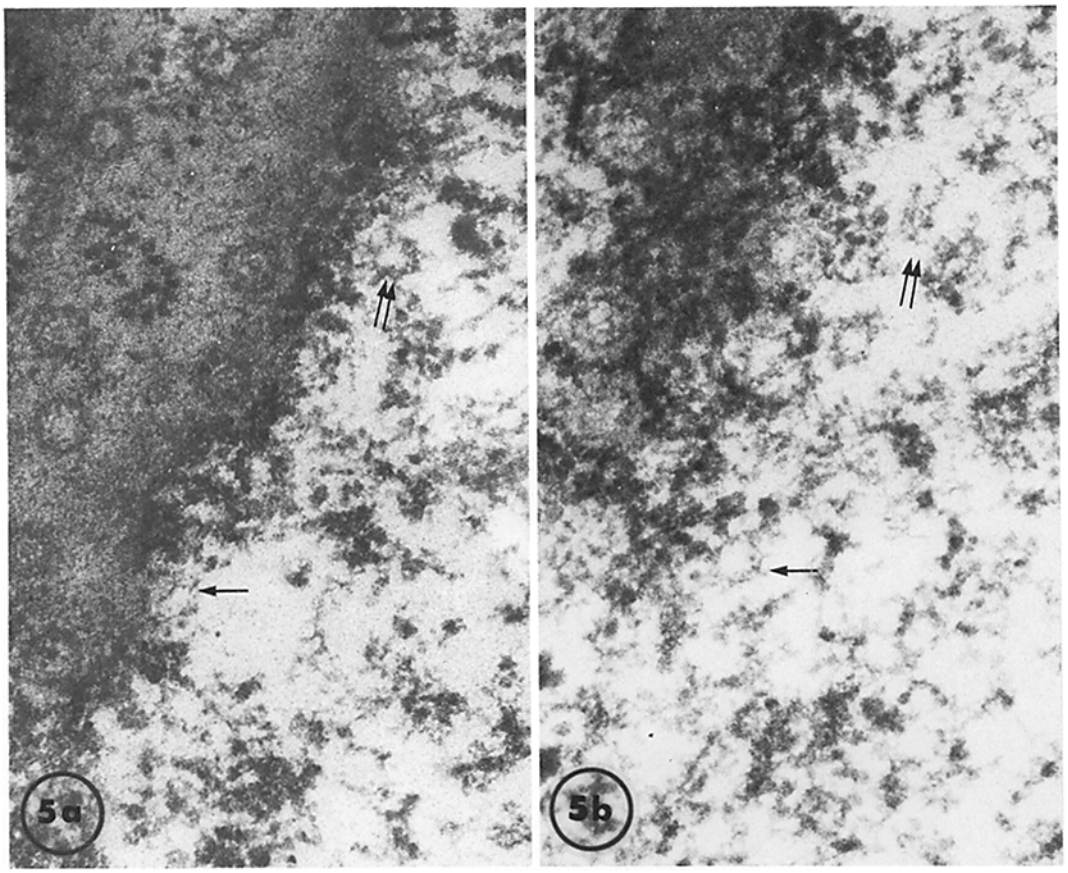


FIGURE 5 Tangential serial sections of the nuclear membrane. Ringlike structures are evident below the membranous part of the pore complex. They also have fibrous attachments to chromatin (arrows). The double arrow in Fig. 5*b* points to the fibrous arrangement below the ring structure in Fig. 5*a*. × 100,000.

FIGURE 1 Nuclear membrane area of a control (uninfected) CV1 cell. Heterochromatin is attached to the nuclear membrane. Arrows point to perichromatin granules and structures equal in size and density to SV40 particles. × 50,000. *Inset:* clumps of particles which have the size of SV40 particles. × 50,000.

FIGURE 2 Nuclear membrane area 48 h after infection. Heterochromatin in productively infected cells has disappeared. Fine fibrous material is present at the nuclear pore complex and between the SV40 particles. One membrane-enveloped particle is present in the cytoplasm (arrow). × 50,000.

FIGURE 3 Sequence of absorption of SV40 particles at the cell membrane and entering the cell. × 100,000.

FIGURE 4*a* The accumulated positions of SV40 particles relative to the nuclear membrane and pore complex are depicted. Each dot represents the center of an SV40 particle. × 100,000.

FIGURE 4*b* The number of particles was counted parallel to the nuclear membrane in 20-nm class intervals perpendicular to the membrane. Particles within the width of 100 nm under the pore with the same 20-nm class interval were also determined and plotted. The particles under the membrane were normalized to the same 100-nm width as those under the pore complex. (●—●) SV40 under the membrane; (□—□) SV40 under the pore area.

nucleus. It may be, then, that SV40 virus penetrates the nucleus already in an uncoated form and not as an infectious virus particle. Such an apparent uncoating has been shown for adenovirus on pore complexes of HeLa cells (8). Again, the virus particles do not approach the pore proper but are left at a certain distance, which is apparently determined by the fibrils of the pore complex. The interpretation by Chardonnet and Dales (8) is that the pore material is injected in a phagelike fashion since empty capsids are found on the pore complex later during the infectious cycle. It is known that uncoated DNA molecules of SV40 alone can be infectious (3, 4, 13, 21). The completed progeny particle may not be able to exit because of physical restrictions of the nonmembranous pore structure. The biochemical evidence suggesting whole infectious particles in the nucleus, however, should not be overlooked (5, 22).

SUMMARY

SV40 particles can apparently enter the nucleus intact. However, they do not leave the nucleus despite the high concentration present during the productive phase. We found structural evidence that SV40 virus is prevented from approaching the most likely site of exit, the nuclear pore complex. From these images, it is concluded that the fibrils attached to the nuclear pore complex prevent egress of SV40 particles from the infected nucleus.

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