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Membrane Fusion as a Mechanism of Simian Virus 40 Entry into Different Cellular Compartments

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Permissive and nonpermissive simian virus 40 (SV40)-infected cells were ultrastructurally analyzed. Viral particles were found in the cytoplasm, rough endoplasmic reticulum, nuclear envelope, lysosomes, and mitochondria. Upon entering the cell the virion obtains a tight membrane envelope. It seems to be either released from the envelope upon fusion with other membranes of the cell or aggregated into tubular membrane specializations upon fusion with other membrane-enveloped particles. Reconstructed morphological sequences and the finding of SV40 in different spaces of the cell suggest that entry of SV40 into the different compartments and eventually into the site of replication is facilitated by its capacity for being enveloped by a variety of membranes (notably the cell membrane and the nuclear membrane) and the sequential fusion and fission of these membranes.

The morphological aspects of simian virus 40 (SV40) infection and replication have been described by Hummeler et al. (8) and Granboulan et al. (7), respectively. The sequence begins with adsorption on the cell membrane. The viral particle is then engulfed, gaining a tight membrane envelope in the process, a sequence also presented in detail for polyoma virus (10). Hummeler et al. assumed that fusion of this envelope with the nucleus preceded injection of the viral particle into the nucleus where it would be uncoated. To investigate the possible ways the viral particle could pass into the nuclear compartment, we drew a number of hypothetical sequences (see Fig. 13) based on the reported membrane envelopment of SV40 during entry into the cell and on the assumption that the viral particle enters the nucleus morphologically intact. We then began our search for morphological evidence for each of the steps expected from the different modes of entry. Using permissive CV1 monkey cells and nonpermissive mouse preimplantation embryos and cultured mouse embryo cells, we have made several observations that complete the morphological sequence of the entering particle's passage through the different compartments of the cell. Entry to the site of replication seems to be accomplished by successive membrane fusion and fission events that allow the virion to pass through all membranous barriers of the different cellular compartments.

MATERIALS AND METHODS

Green monkey kidney cells (CV1) (obtained from the American Type Culture Collection) were grown in 75-cm^2 plastic flasks in Dulbecco-modified Eagle medium supplemented with 10% fetal calf serum in a 5% CO_2 incubator. They were subcultured at a split ratio of 1:5 and used for the experiments between passages 30 and 40. For the experiments described, the cells were plated in 3.5-cm plastic petri dishes (Falcon) at a concentration of 10^5 cells per dish. When the cells reached confluence, they were infected with a plaque-purified large-plaque mutant of SV40, strain Rh911, at a multiplicity of 100 PFU/cell for 1 h at 37°C. The unabsorbed virus was then removed, and the monolayers were refed with Dulbecco medium without serum.

Secondary cultures of mouse embryo cells isolated from 15-day-old fetuses of ICR mice were infected with SV40, strain Rh911, at a multiplicity of 2×10^3 to 5×10^3 PFU/cell for 24 or 48 h at 37°C. Mouse preimplantation embryos were isolated and infected with SV40 as described by Abramczuk et al. (2).

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, with 1 mM $MgCl_2$) and postfixed 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 (9), then rapidly dehydrated in ethanol. The dehydrated cells were flat-embedded in Epon according to the method of Brinkley et al. (4). 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 Zeiss 10A elecron microscope at 80 kV.

RESULTS

Morphological sequence of transfer of SV40 to the nucleus. We have repeated the

observations made by Hummeler et al. (8) on CV1 cells during the first hour of infection but will describe our observation on other systems which showed certain aspects more clearly and more frequently. Figure 1 shows several viral particles enveloped at the cell membrane of a cultured mouse embryo cell 24 h after infection. There were no particles adsorbed at the membrane and only a few enveloped particles in the cytoplasm. Other cells in the same preparation showed many enveloped particles in the cytoplasm, but few, if any, at the cell membrane or in the rough endoplasmic reticulum (RER). A third group of cells had unenveloped particles in what appeared to be RER-derived membrane sacs (Fig. 2). These sacs usually contained very few or no ribosomes and were flat or only slightly distended. Few of the cells had many viral particles localized in the intracisternal parts of the nuclear envelope. The inner nuclear membrane was occasionally seen apparently enveloping the viral particle as on the cell membrane (arrow, Fig. 3). The inner nuclear membrane was usually apposed by heterochromatin, the densely staining material seen in Fig. 3, but at the apparent site of viral entry into the nucleus the continuous stretch of heterochromatin was interrupted. If the viral particle is enveloped by the inner nuclear membrane, then the nucleus should contain enveloped particles. Accordingly, enveloped particles were found in the nucleus (Fig. 4 and 5), often in the vicinity of those without membrane envelopes. The stripping away of the membrane must then be a nuclear function. Figure 5c shows an enveloped SV40 particle attaching to the outer nuclear membrane. This is interpreted as the step immediately before fusion of the two membranes.

Free or enveloped SV40 particles were found in the cytoplasm of CV1 cells; only once were both types of virion observed in the same cell (Fig. 6). There are two possible explanations for free viral particles in the cytoplasm. Floating membrane vesicles containing SV40 from lysed cells may fuse with the cell membrane, releasing the SV40 particles (Fig. 7), or single SV40 virions enveloped before or during lysis (arrow, Fig. 7) may fuse with the membrane and be released in the cytoplasm. It was observed that the released SV40 was tightly attached to large sheets of free membranes as has previously been reported by Granboulan et al. (7) and Oshiro et al. (14).

Virus-specific membrane specialization. A tubular membrane specialization containing SV40 was found in CV1 cells infected at high multiplicities and over long periods of time. It apparently contained most of the virus in the cell since few particles were seen in other areas of these particular cells. Figure 8 shows this structure in CV1 cells. The membranes tightly surround the virus but are continuous as tubules (Fig. 8). Since the section thickness far exceeded the dimensions of the membrane-enveloped virus, such continuities could only be seen occasionally.

Although at early times after infection of CV1 cells it was more common to see images such as those in Fig. 9, in which a single viral particle seems to be attached to the membrane of two sheets of RER, tubular membrane specializations were also observed. The earliest observation of membrane specializations after infection was 6 h (a single observation). Membrane specializations seen at this time were in smaller aggregations, whereas those observed later were large and filled the height of the monolayer cells.

Quantitation of the tubular membrane specializations in CV1 cells, counting only those cross-sectioned cells that had a section of a nucleus, indicated that the number of these structures increases progressively beginning with h 6 (Table 1). Single viral particles attaching to RER as in Fig. 4 were not counted, but the number of cells with viral particles in the cytoplasm (membrane enveloped as well as without membranes) and the number of cells with virus in the nucleus were counted. In both cases there was an increase until h 6 and then a decrease to h 24. At h 48 the number of virus-producing cells was so great that the number of cells with few particles became meaningless, and the number of cells with virus in the cytoplasm increased from very few to 66%. This was interpreted to be due to reinfection or continuous infection from virus incompletely washed off. Twenty-four hours after infection about 27% of the nuclei already had a high concentration of viral particles. In a repeat of this experiment we found only 50% (versus 69% in the first experiment) of the cells producing virus after 48 h. This lower number could be traced to a larger number of overlapping cells. The cells growing under those that were productively infected did not produce SV40, which indicates low or insufficient initial infection.

To determine whether the SV40-containing membrane specializations are specific for the permissive state, cell cultures derived from mouse embryos were incubated with SV40 for 24 and 48 h before fixation. Large membrane specializations with SV40 were found closely resembling those of the permissive CV1 cells. These membranes appear like smooth endoplasmic reticulum tubules, but are often continuous with RER as seen in Fig. 8. Cells infected briefly and then grown in the absence of virus were found to contain this membrane specialization only infrequently, and when present it was much

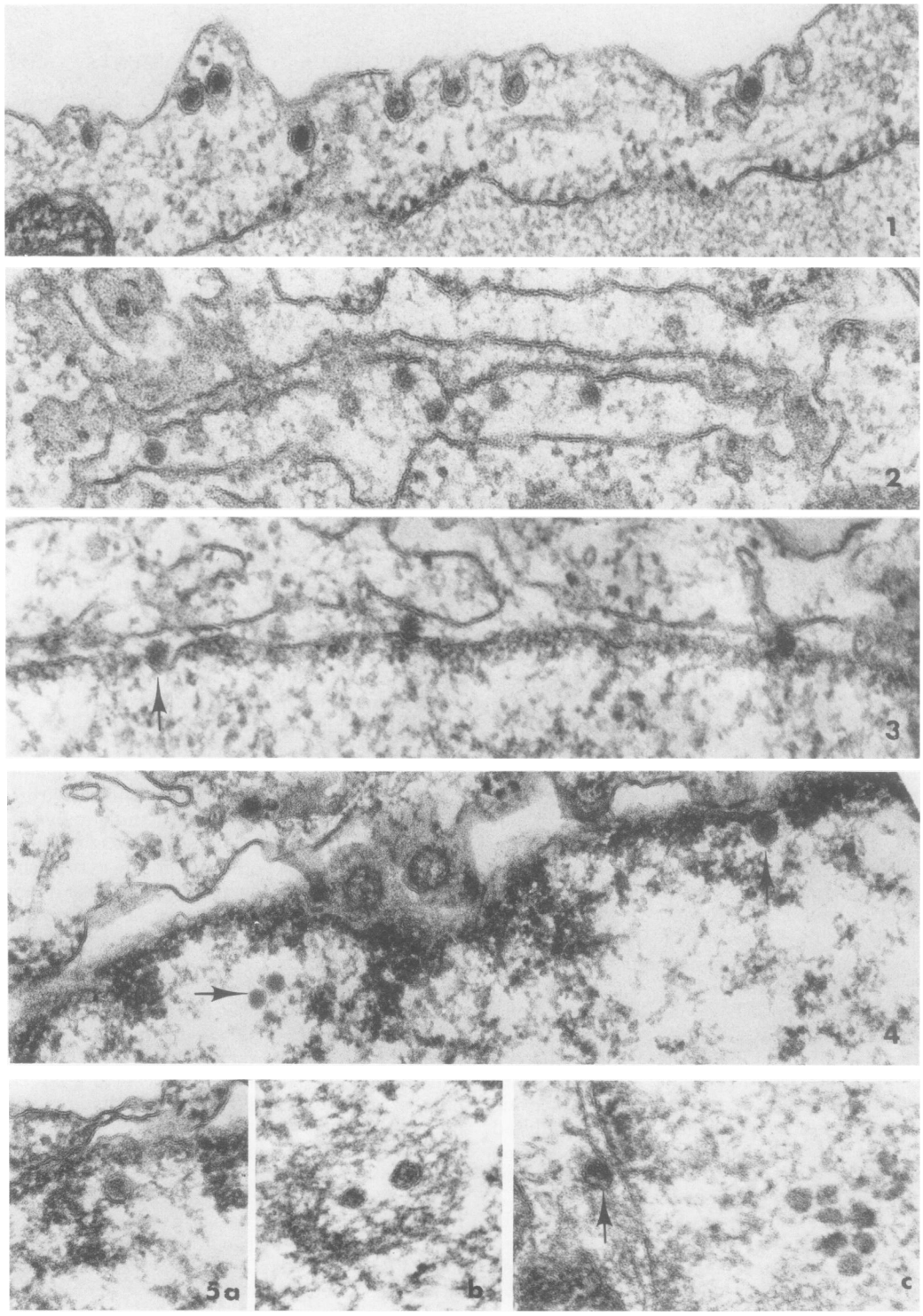


FIG. 1. *Cross-sectioned cultured mouse embryo cell 24 h after infection with SV40. There are no adsorbed virions; all are membrane enveloped and partially internalized. The RER is intact with ribosomes. ×100,000.*

FIG. 2. *SV40 is contained in RER without ribosomes (cultured mouse embryo cell). ×100,000.*

FIG. 3. *A cross-sectioned nuclear envelope containing SV40. Arrow points to a particle partially enveloped by the inner nuclear membrane at a site devoid of heterochromatin. ×100,000.*

FIG. 4. *Membrane-enveloped and free SV40 within the nucleus of a preimplantation mouse embryo. ×100,000.*

FIG. 5. *(a) Membrane-enveloped SV40 in the nucleus of a preimplantation embryo. (b) Membrane-enveloped and free SV40 in the nucleus of a cultured mouse embryo cell. (c) Membrane-enveloped SV40 attaching to the outer nuclear membrane and free particles in the nucleus of CV1 cells 6 h after infection. ×100,000.*

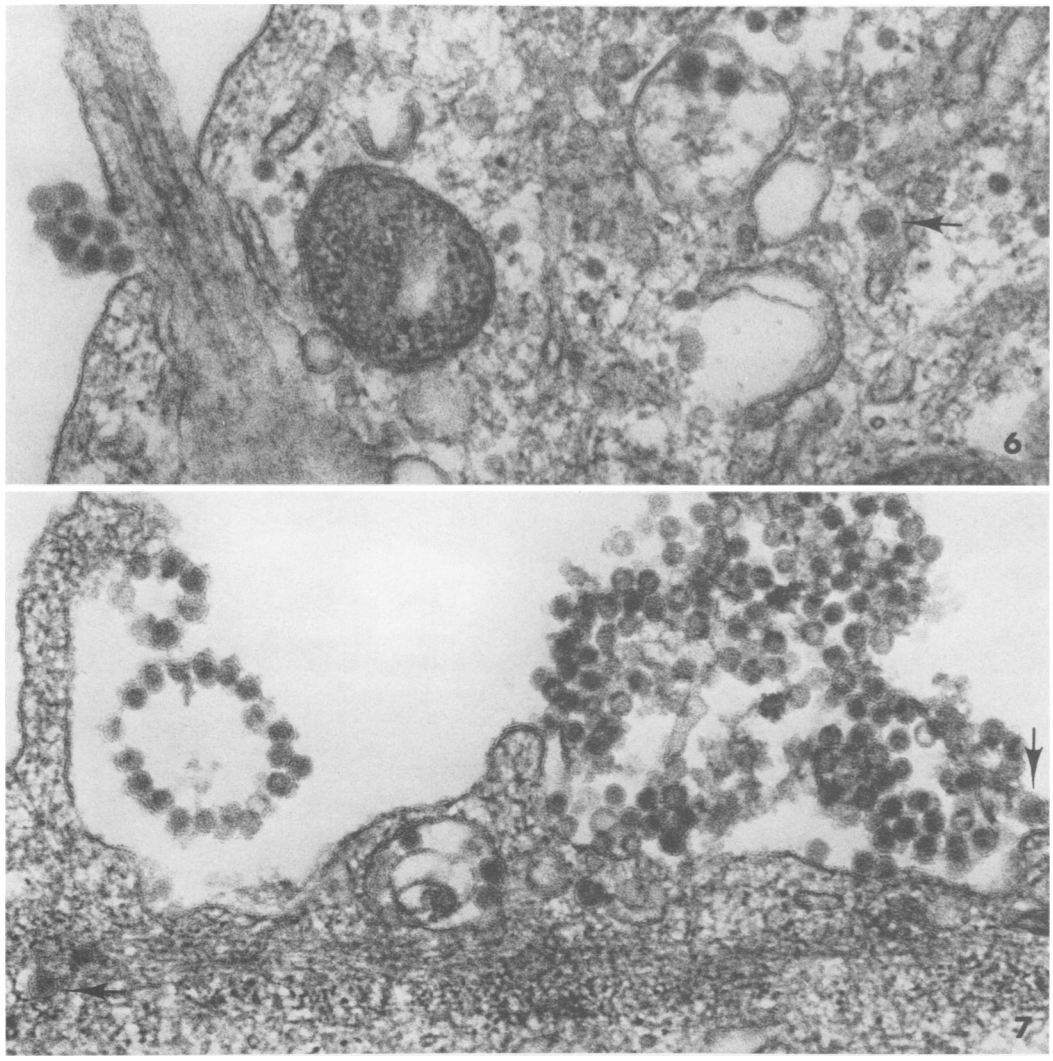
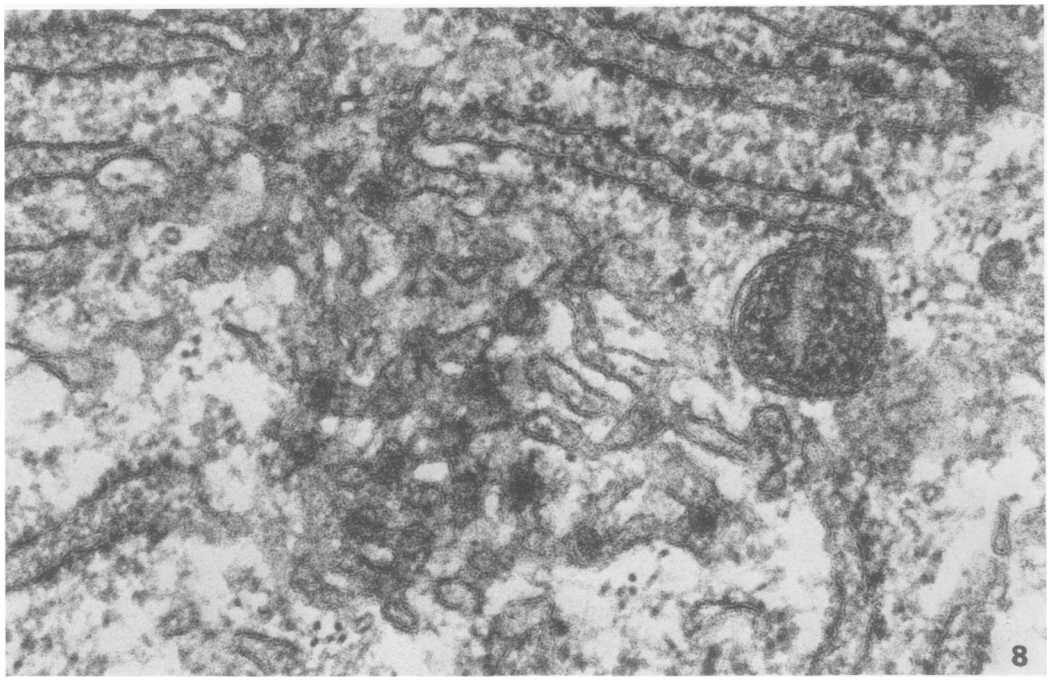


FIG. 6. *Some cells contained both enveloped and free SV40 in the cytoplasm (arrow). Membrane-enveloped SV40 was also found in small vacuoles, presumably lysosomes. Free SV40 was seldom found intact in lysosomes.* ×*80,000.*

FIG. 7. *Three hours after infection of CV1 cells the SV40 virion may be present in a membrane vesicle (left), free (the bulk), or individually enveloped in a membrane (arrow). Membrane-enveloped virions are also present in the cytoplasm of the cell.* ×*80,000.*

smaller and contained few viral particles.

SV40 in mitochondria. Mitochondria are not normally known to harbor SV40. Since membrane fusion of the enveloped SV40 seemed to be the mode of changing from one compartment to another, we searched for SV40 in mitochondria. Virions with and without membrane envelopes were found in mitochondria of mouse embryos infected in the morula stage for 14 h or longer. In Fig. 10 an enveloped SV40 particle has just completed fusion with the outer mitochondrial membrane. In the upper part of Fig. 10 the adsorption of an enveloped SV40 with a membrane vesicle can be seen. In Fig. 11 an enveloped SV40 particle is present in the matrix of the mitochondrion, and in Fig. 12 there are unenveloped SV40 particles present in the matrix. Large mitochondrial inclusions could be seen resembling unenveloped SV40. We have not been able to find clear evidence for the presence of virions between the inner and the outer mitochondrial membranes, but one would expect an enveloped particle to lose its membrane upon fusion with the outer membrane and



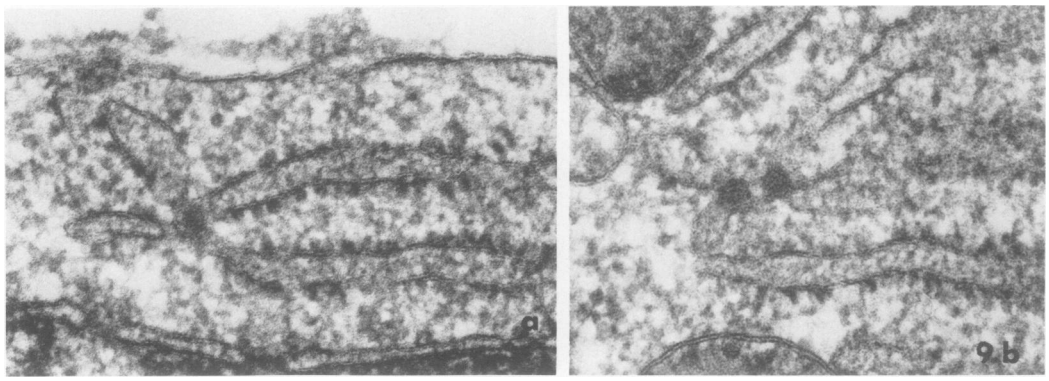


FIG. 8. *In CV1 cells the smaller tubular membrane specialization was more common 24 h after SV40 infection.* ×*100,000.*

FIG. 9. *(a, b) SV40 apparently agglutinating two RER cisternae.* ×*100,000.*

to gain a membrane by entering the matrix after envelopment by the inner membrane. Few mitochondria contained a single SV40; even fewer contained two or more. The same images have also been obtained with polyoma virus (results not shown).

DISCUSSION

It has been found that SV40 particles readily adsorb to membranes and are tightly enveloped before release into the cell (7, 8, 11; Fig. 1). The same sequence was reported for polyoma virus entry into the cell (10). After this process the viral particles are too large to penetrate the nuclear pores which are assumed to be passageways for nuclear-cytoplasmic exchange of macromolecules. Paine et al. (15) have shown that molecules of more than 70,000 daltons will not penetrate through the nuclear pores by diffusion, and Maul (11) has shown that SV40 cannot leave the nucleus via the pore structure since the virus is excluded from even approaching the pores by their associated fibrillar components. Sommers (16) showed that the 33-nm-wide tubular capsid of granulosis virus of *Trichoplusia ni* does not enter the ~80-nm-wide nuclear pore despite the fact that it lines up with the pore end on (see also Dales [5] for a review of virus

TABLE 1. *Distribution of SV40 particles in CV1 cells over a 48-h period*[a]

Determination	No. (%) of cells counted at h after infection:					
	Noninfected control	3	6	12	24	48
Location of SV40						
Nucleus	0 (0)	5 (6)	22 (47)	16 (32)	7 (27)	0 (0)
Cytoplasm	0 (0)	14 (17)	22 (47)	11 (22)	4 (15)	89 (66)
Cytoplasmic SV40 in membrane specialization	0 (0)	0 (0)	1 (2)	4 (8)	2 (8)	13 (10)
Cells producing SV40	0 (0)	0 (0)	0 (0)	0 (0)	5 (20)	94 (69)
Total cells counted	200 (100)	83 (100)	47 (100)	51 (100)	26 (100)	136 (100)

[a] Green monkey kidney cells (CV1) were infected with SV40 and, after fixation, observed under the electron microscope at a magnification of ×28,000. The different structures were then analyzed using the binoculars (total magnification ×280,000). Since the frequency of nuclear structures was being tabulated, only cells containing a nucleus were considered. This had the effect of underrepresenting the cytoplasm and reduced the number of membrane specializations observed. The cytoplasmic virus was usually abundant enough to be recognizable in relatively small areas of cytoplasm.

penetration into the nucleus). Our investigation of ultrastructural changes in permissive (CV1) and nonpermissive (preimplantation mouse embryos and cultured mouse embryo) cells for SV40 replication has yielded information about the transfer of viral particles into various cellular compartments, including not only RER, mitochondria, and lysosomes, but also and most importantly the nucleus. These findings have suggested that membrane interactions with the virus are essential in the entry of SV40 into different cellular compartments.

Figure 13 shows hypothetical sequences of SV40 interaction with membranes that may result in the introduction of SV40 into the nucleus. The most complete morphological sequence documented is that depicted in Fig. 13B. All stages in this depiction were seen. According to this scheme, SV40 is enveloped at the cell membrane after adsorption. The enveloped virus is released and moves through the cytoplasmic matrix, and its membrane then fuses with that of the RER (Fig. 13C) or the outer nuclear membrane (Fig. 13B), which at present are assumed to be functionally the same. The SV40 is then released into the intracisternal space of the nuclear envelope. Free SV40 particles in the intracisternal space of the nucleus were also observed by Granboulan et al. (7) in CV1 cells after reinfection due to cytolysis of productively infected cells. We have observed the viral particle tightly adsorbed to the inner nuclear membrane, invaginated on the inner membrane, and present as a membrane-enveloped particle within the nucleus. How does the viral particle induce the inner nuclear membrane (which is covered and tightly bound to the dense lamina and heterochromatin) to invaginate? Is entry into the nucleus a regulated event, or do SV40 particles become newly enveloped randomly by either the inner or outer nuclear membrane? The fact that the heterochromatin underlying the inner nuclear membrane as well as the dense lamina (1) would have to be temporarily removed to allow invagination of the membrane and subsequent release of the membrane-enveloped viral particles argues for a sequence that favors the entry into the nucleus. Hypothetical mechanisms may involve virus receptor sites similar to those of the cell membrane since the attachment is again on the E face (3) of the nuclear membrane. (See also Morgan et al. [13] for a special reaction of the membrane-associated chromatin upon entry of adenovirus type 7 into the nucleus.) The membrane must then be removed from the SV40, an event not recognizable by electron microscopy.

Three other sequences can be imagined as indicated in Fig. 13A and J, but at present there is no evidence for an enveloping event at the outer nuclear membrane, for the presence of an enveloped particle in the intracisternal space of the nuclear envelope, or for fusion of an enveloped particle with the inner nuclear envelope. There is then also no morphological evidence for the reverse of the last-mentioned morphological sequence, i.e., release of viral particles from the nucleus.

Many of our observations were made on nonpermissive cells heavily infected over long periods of time and on permissive cells after reinfection had begun (48 h after the initial infection) due to lysis of productively infected cells. In addition, most structures were abundant in

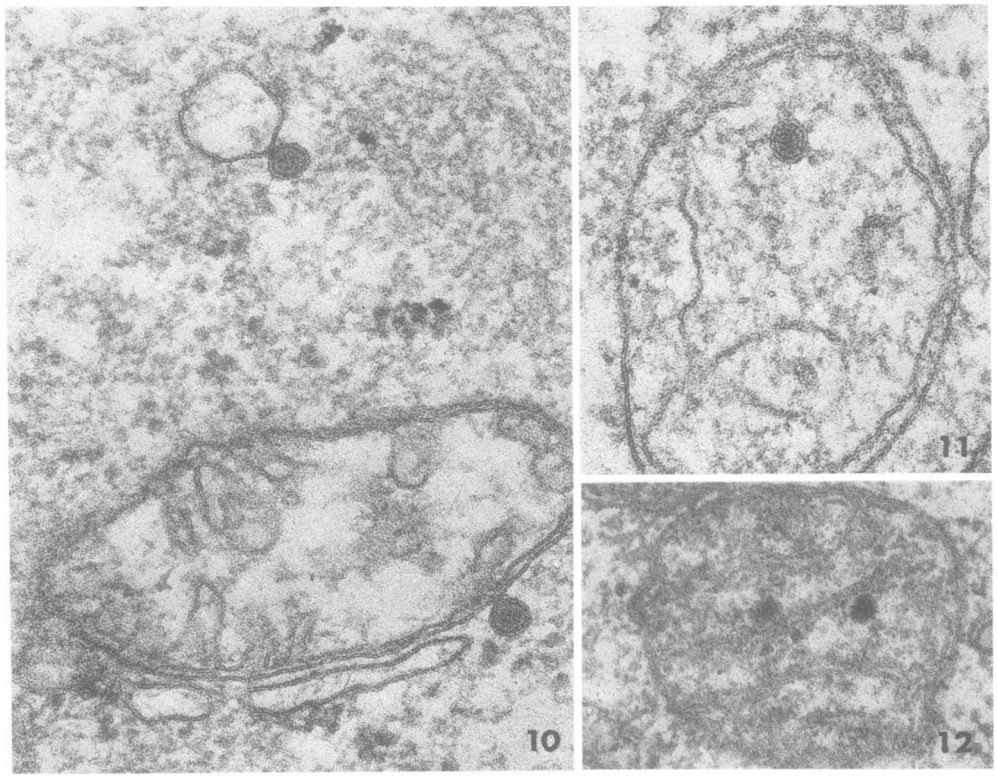


FIG. 10. *Enveloped SV40 fused with the outer mitochondrial membrane (14 h after beginning of infection of mouse preimplantation embryos). Another enveloped SV40 is apparently attached to a vesicle. ×100,000.*

FIG. 11. *Membrane-enveloped SV40 in the matrix of a mitochondrion of a mouse preimplantation embryo. ×100,000.*

FIG. 12. *Two unenveloped SV40 present in the matrix of a mitochondrion of a mouse preimplantation embryo. ×100,000.*

the cells in which they were found even when there were few such cells. SV40 was not often seen in the intracisternal space of the nuclear envelope, but in the nucleus represented in Fig. 3 there were five particles to every linear micrometer of nuclear envelope, which represented 0.1 μm^2 of nuclear envelope at a 100-μm section thickness. If the nucleus has a surface of approximately 300 μm^2 (12), there must have been 15,000 viral particles present in the intracisternal space of the nuclear envelope. Only this massive accumulation at certain stages of infection made the observation possible, and it may have come about because at different stages nonpermissive cells blocked further progress in the infectious process. Introduction of massive amounts of viral particles into the cytoplasm of nonpermissive cells has been shown to overcome this condition (7). In CV1 cells, however, adsorption and penetration with concomitant envelopment seemed a continuous process of which only the adsorption stage was seen frequently in the early stages of infection. This seems due to sampling problems.

Tubular membrane specializations were observed in CV1 cells, in trophoblastic cells of mouse preimplantation embryos, and in cultured mouse embryo cells. Although the appearance of these structures does not seem to be a function of virus replication, since in CV1 cells they were observed as early as 6 h after infection, they may represent the effect of accumulation and segregation of viral particles. Several lines of evidence could be constructed for this hypothesis. SV40 has a preference for the adsorbed state, as indicated by its attachment in large, densely packed sheets to membranous debris of dead cells. Figure 13E and F depicts two possible sequences of formation of tubular membrane specialization based on this preference. The membranes of the individually enveloped viral particles may either be removed (Fig. 13F) or remain intact (Fig. 13E). In either case they may be able to agglutinate RER. The expected ultrastructural pic-

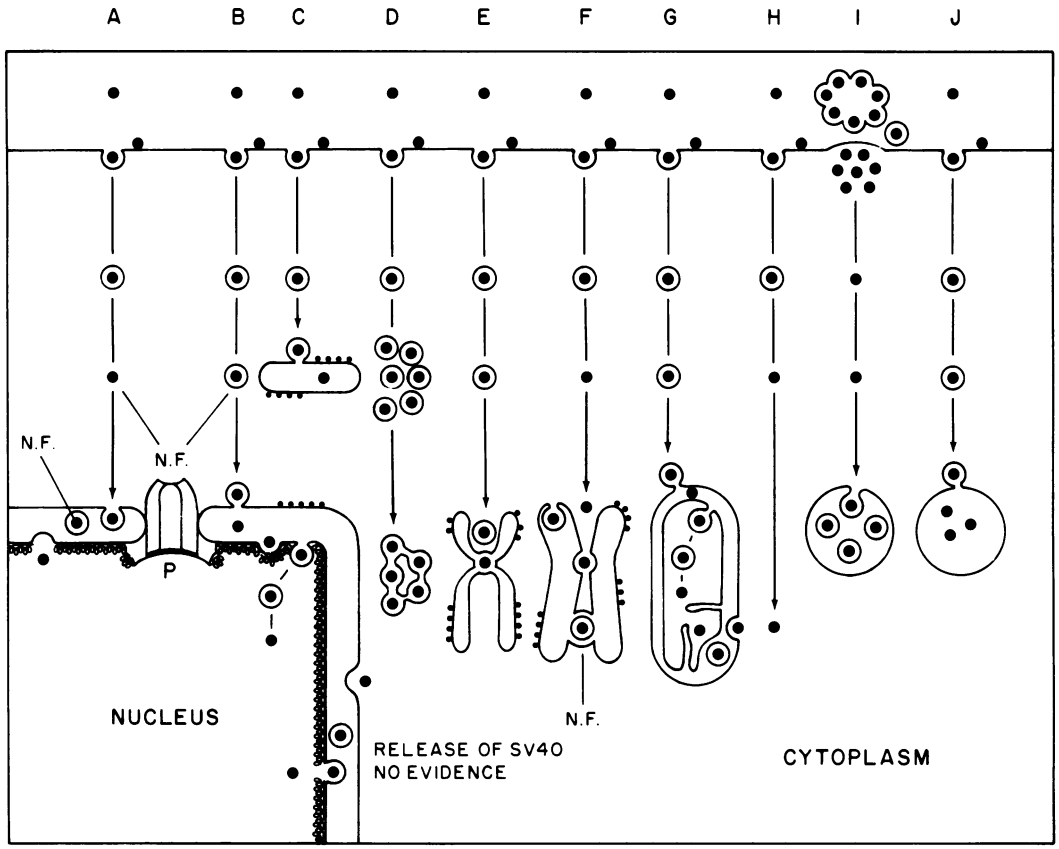


FIG. 13. *Schematic representation of possible entry of SV40 into the different compartments of the cell. All are based on a sequence of membrane fusion and fission. The sequences are more fully explained in the text. NF, Not found; P, nuclear pore.*

ture would be different: after fusing with two membranes the enveloped virus would appear in the cisternal space. The virus might still be tightly attached to the membrane, similar to the way in which it was adsorbed on the cell membrane. The membrane-free virus would become enveloped and released into the RER if both sheets of RER fused around it (Fig. 13F). No clear evidence has been found for this sequence. The mechanism of tubular membrane specialization formation shown in Fig. 13D, i.e., the selective fusion of membrane-enveloped SV40, seems to be the most likely sequence at present. This could explain why SV40 particles tend to aggregate in rather large tubular membrane specializations and not to be more equally distributed throughout the cytoplasm of cells that contain them.

The finding of enveloped SV40 in mitochondria was surprising. In Fig. 13G and H the two possible modes of SV40 entry into mitochondria are depicted. For both sequences the intermediate stage of the particle between the two mitochondrial membranes is missing. Also, no adsorption of an unenveloped particle at the outer mitochondrial membrane has as yet been encountered.

The general concept that can be formulated after finding SV40 in different spaces of the cell is that its entry into the different compartments is facilitated by its property to attach to and induce envelopment by the E face of a variety of membranes, notably the cell membrane and the nuclear membrane. In the nucleus the virion needs to be stripped of its membrane to be uncoated. Therefore, there must be an enzyme in the nucleus that is not in the cytoplasm. Enveloped and unenveloped SV40 have been found in the mitochondrion, which indicates successful infection of an environment conducive to DNA replication.

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