Defining the Cytoplasmic and Nuclear Transport of CPV Capsids in Cells

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Canine parvovirus (CPV) is a non-enveloped virus that replicates in the nucleus during infection. While it is known that it uses the cell's microtubule network to traverse the cytoplasm, the route of nuclear entry is unclear. The nuclear pore complex was thought to be used by the virus due to the presence of nuclear localization sequences (NLS) in the VP1 protein. But recent studies in the *minute virus of mice* (MVM) have proposed that it induces breaks in the nuclear envelop before entering the nucleus via these breaks. In this study, Crandell Feline Kidney (CRFK) cells stably transfected with GFP-lamin A/C were micro-injected with CPV capsids whose distribution within the cells was then detected using fluorescent-labeled antibodies. The CPV capsids did not appear to induce breaks in the nuclear lamin structure of the cells that was observed for the MVM.

Introduction

In order to replicate and begin a successful infection in the host cell, viruses must cross the plasma membrane and enter the host cell. In the case of most DNA viruses, replication occurs in the nucleus where the virus is able to take advantage of cellular factors needed for transcription and replication of the viral genome and post-transcription processing of viral mRNA. Hence they must first pass through the cytoplasm in order to get to a perinuclear location, and then the genome must somehow cross the nuclear membrane to get into the nucleus.

The cytoplasm of eukaryotic cells is a viscous mixture containing organelles, the cytoskeleton and high concentrations of protein, and these combine to restrict free diffusion of particles the size of viruses. In fact, the transport rate by diffusion of a particle the size of parvovirus capsids in the cytoplasm is likely about 10μm per hour which is about 600 times slower than that in water (1). This rate would be too slow for efficient infection and replication. Hence it is likely that the cytoplasmic transport of viral particles in the host cell is an active process, rather than due to just passive diffusion.

The cytoskeleton of the cell is made up of microtubules, and the actin and intermediate filament networks. Microtubules are polarized hollow cylindrical filaments

that have a slow-growing minus end located at the microtubule organizing center (MTOC) and a fast-growing plus end that extends out into the periphery of the cell. The microtubules and their associated molecular motors, dynein and kinesin, are involved in the transport of cellular components around the cell (2).

Despite the fact that all viruses have to transport their genome and associated proteins through the cytoplasm during infection, the details of this movement have been largely uncharacterized. Microtubules have been reported to be involved in the cytoplasmic transport of many viruses, with the microtubule-associated protein dynein in particular playing a role for adenovirus (3) and herpes simplex virus type 1 (HSV-1) (4), and suggested to play a role for parvovirus capsids (16).

After traversing the cytoplasm to a perinuclear location, many viruses enter the nucleus by making use of the cell's nuclear transport machinery. In particular, they bind to soluble cytoplasmic import receptors using the specific nuclear localization signals (NLS) on their viral proteins. This complex of receptor and cargo is then transported into the nucleus through the nuclear pore complex (NPC) (5). But it has been recently reported that a parvovirus, the *minute virus of mice* (MVM), may bypass the NPC route by inducing breaks in the nuclear envelope and entering the nucleus via these breaks (6, 7). It has also been shown that the viral capsids can pass through the nuclear pore in more-or-less intact form in a process that involves the nuclear transport machinery and regions of basic amino acids which are found in the unique N-terminal sequence of the VP1 protein (8). These sequences are buried within the capsids of the viruses when they are first formed, but are thought to become revealed during the cell entry process (8).

The canine parvovirus (CPV) is a member of the *Parvoviridae* family that causes enteritis and myocarditis in canidae (9). It is a small (26nm diameter), icosahedral, non-enveloped DNA virus that contains a ~ 5,000 linear, single-stranded genome. When first formed, the virion contains two capsid proteins (VP1 and VP2) with molecular sizes of 83 and 67kDa respectively (10), and a third VP3 protein is formed in full (DNA containing) capsids by proteolytic removal of ~ 19 residues from the N-terminus of VP2 (11). The exact function of this cleavage is not known, but the VP2 N-termini of MVM capsids contain residues that can be phosphorylated to allow nuclear export of the newly formed capsids (12).

CPV enters the host cell by binding to the transferrin receptor (TfR) on the cell surface, leading it into an infectious pathway that starts with clathrin-mediated microtubule-dependent endocytosis and the accumulation of viral capsids within perinuclear vesicles (13, 14). While the mechanism by which the capsids escape the endosomal vesicles are not known, the activity of a virally-associated phospholipase A2 in the N-terminal sequence of VP1 appears to be involved (15). Within the cytoplasm, the capsids move towards the nuclear membrane in a process mediated by microtubules, as seen from the blockage of nuclear localization in the presence of nocodazole that depolymerizes microtubules (16).

In some previous studies, it was seen that nuclear import of CPV capsids involved modifications of viral capsids that exposed sequences resembling classical NLS within the N-terminal unique region of the VP1 (17). In particular, the N-terminal sequence (PAKRARRGYK) between residues 4 and 13 mediated nuclear import of a heterologous protein, as seen when it was conjugated to bovine serum albumin (BSA) (18), and some

specific changes in this basic sequence reduced the relative infectivity of the capsids (17). Although the VP1 N-terminus is inaccessible in the native structure of the CPV capsid, it can be exposed *in vitro* without disassembly of the capsid (17).

In our experiments, we re-examined the cytoplasmic movement of the canine parvovirus capsids towards the nucleus using cells that have been stably transfected to express green fluorescent protein (GFP)-labeled lamin A/C (19). The nuclear lamin provides a network of intermediate filaments on which the nuclear envelope is erected upon. The objective was to define the effects of the capsids on the nuclear envelope integrity which might affect its ability to enter the nucleus, or to enter the nucleus by NPC-mediated transport mechanisms. These studies involved microinjecting purified viruses, or control proteins, into cells and then following the distribution of virus or protein in the cells. In some cases the viruses or proteins were linked to nuclear localization peptides. While some of these viruses or proteins were conjugated with fluorescent dyes, others were detected by staining using specific antibodies. In addition, we investigated whether the breaks in the nuclear envelope observed in the MVM system can be seen in the case of CPV.

Materials and Methods

Cells and viruses: Crandell Feline Kidney (CRFK) cells were grown and maintained in a 1:1 mixture of McCoy's 5A medium and Leibovitz L15 medium with 5% fetal calf serum at 37ºC with 5% CO_2. In addition, some CRFK cells were transfected with plasmids expressing GFP-lamin A/C to label the inner nuclear membrane using Lipofectamine (Invitrogen USA) before the transfected cells were selected with 400μg/ml of G418.

Prior to microinjection, the cells were grown at 1×10^4 per cm^2 on glass cover-slips in tissue culture dishes overnight.

CPV capsids were prepared by growth of the virus in Norden Laboratory feline kidney (NLFK) cells, and the capsids were then concentrated by precipitation with polyethylene glycol followed by sucrose gradient centrifugation. Before use in microinjection, the virus was washed in microinjection buffer (10mM Tris-HCl-120mM KCl pH7.4), and then concentrated to ~ 2μg/ml in a Microcon YM-30 concentrator (Millipore).

Antibodies and chemicals: In some cases the injected BSA was detected using chicken anti-BSA (Genetex) followed by goat anti-chicken conjugated to Cy 3.5 (Abcam) diluted in PBS containing 0.15% Triton X-100 and 0.01% sodium azide. Injected CPV was detected using a rabbit antibody against CPV capsids made in Cornell, followed by goat anti-rabbit conjugated to Texas Red (Jackson ImmunoResearch) diluted in PBS containing 0.15% Triton X-100 and 0.01% sodium azide.

Rhodamine-labeled BSA with SV-40 large T-antigen NLS was obtained from Sigma while unlabeled BSA (Fisher Scientific) was labeled with Alexa 594 dye (Invitrogen). Finally the nocodazole used to disrupt the microtubules in the cell was obtained from Sigma.

Microinjection, antibody and drug treatments: The CRFK cells were micro-injected with 0.1 – 0.5pl of full (DNA-containing) CPV capsids at ~ 2 mg/ml using Eppendorf 5171 Micromanipulator and 5246 Microinjector, with the microinjection needles being pulled from 1.0mm borosilicate glass capillaries (Harvard Apparatus, Kent UK) with Sutter P-97 Flaming/Brown Micropipette Puller. The viral capsids were concentrated to ~ 2mg/ml and dialyzed against microinjection buffer. The cells were then incubated at

37ºC for various time intervals between 0 and 4 hours before being fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature. For the directly labeled virus or protein, they were directly detected and observed in both live and fixed cells by fluorescent microscopy.

The viruses were then detected by incubating the cells in the respective antibodies as described above. After a final wash in 1X PBS, the coverslips were mounted on microscope slides with Immu-mount (Thermo, Pittsburgh PA).

In experiments involving nocodazole, the CRFK cells were pre-treated with 20μM of nocodazole for 1 hour before micro-injection. The drug treatment was maintained after micro-injection for 4 hours before the cells were fixed and stained as above.

In the control experiments, the BSA was either directly observed via its fluorescent label or stained using antibodies.

Microscopy and Imaging: The slides were examined using both regular fluorescent (Nikon Eclipse TE300 using Nikon Plan Fluor 40X or 60X objectives) and confocal fluorescent microscopes (Olympus IX70 with Olympus Fluoview using PlanApo 60X objective). Pictures of the cells were taken using the SimplePCI (Compix Inc., Imaging Systems) and Fluoview (Olympus) softwares, and assembled using Adobe Photoshop.

Results

Microtubule-dependent, nucleus directed transport of microinjected viral capsids.

In order to investigate the transport of cytoplasmic viral capsids to the nucleus, viral capsids were injected into cells in the presence and absence of nocodazole. In the absence of nocodazole, the full CPV viral capsids were distributed throughout the

cytoplasm of the cell in the form of numerous fluorescent dots (green in Figures 1A and 1B) that could represent either individual capsids or clusters of capsids immediately after microinjection (0hr). After 4 hours, there appeared to be localization of the viruses to the nuclear region in about 30% of the cells (Figure 1B).

By disrupting the microtubule network using nocodazole treatment that depolymerizes the microtubules, there was a reduction in nuclear accumulation of viruses (Figure 2), which remained distributed throughout the cytoplasm.

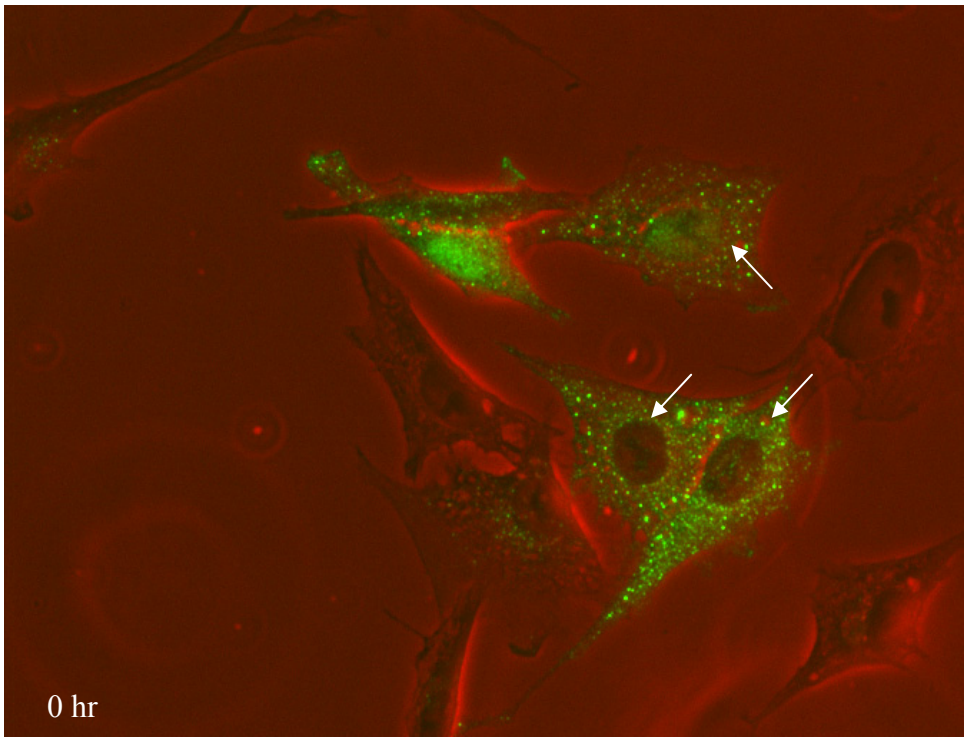

Figure 1A: Intracellular localization of CPV full capsids (green) after microinjection into the cytoplasm of CRFK cells (red) after 0 hr

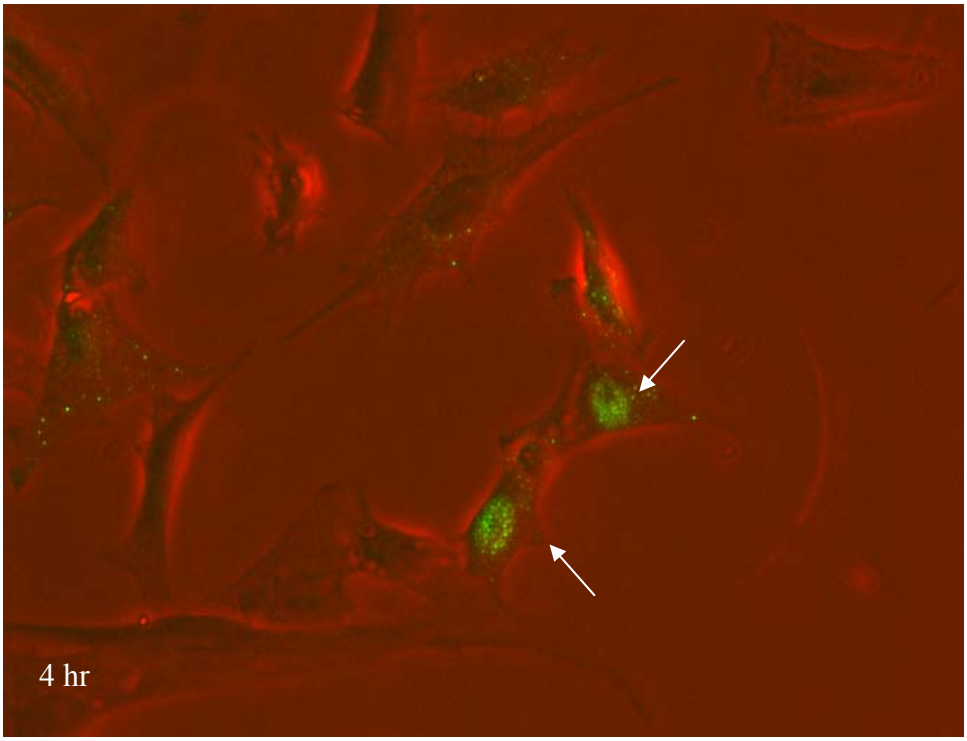

Figure 1B: Intracellular localization of CPV full capsids (green) after microinjection into the cytoplasm of CRFK cells (red) after 4 hr.

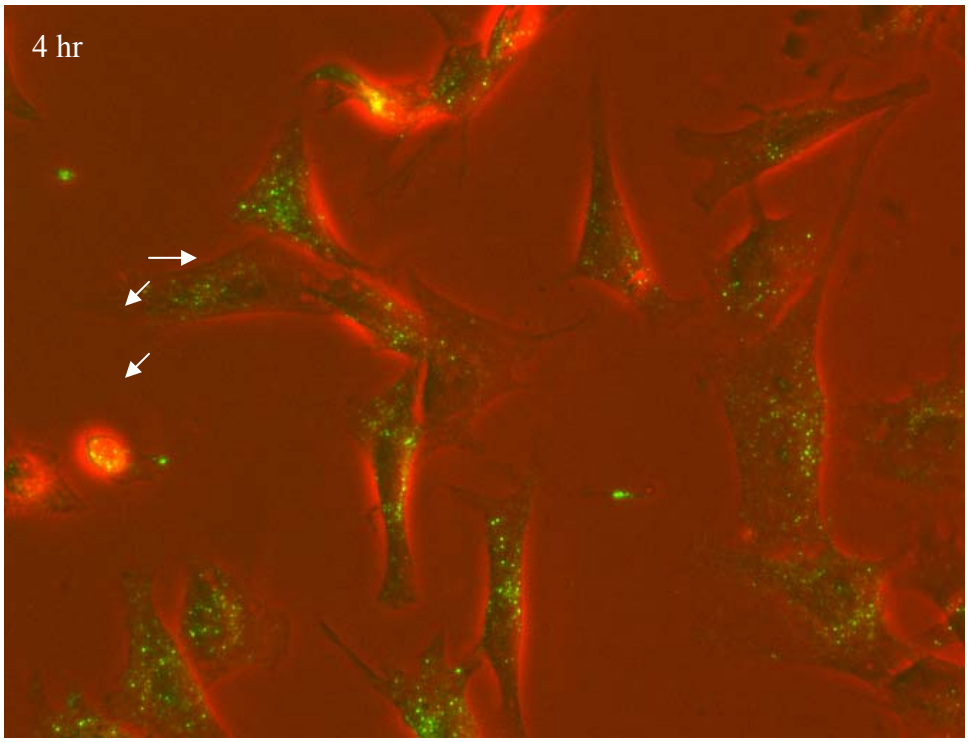

Figure 2: Intracellular localization of CPV full capsids (green) 4 hr after microinjection into the cytoplasm of CRFK cells (red) treated with nocodazole

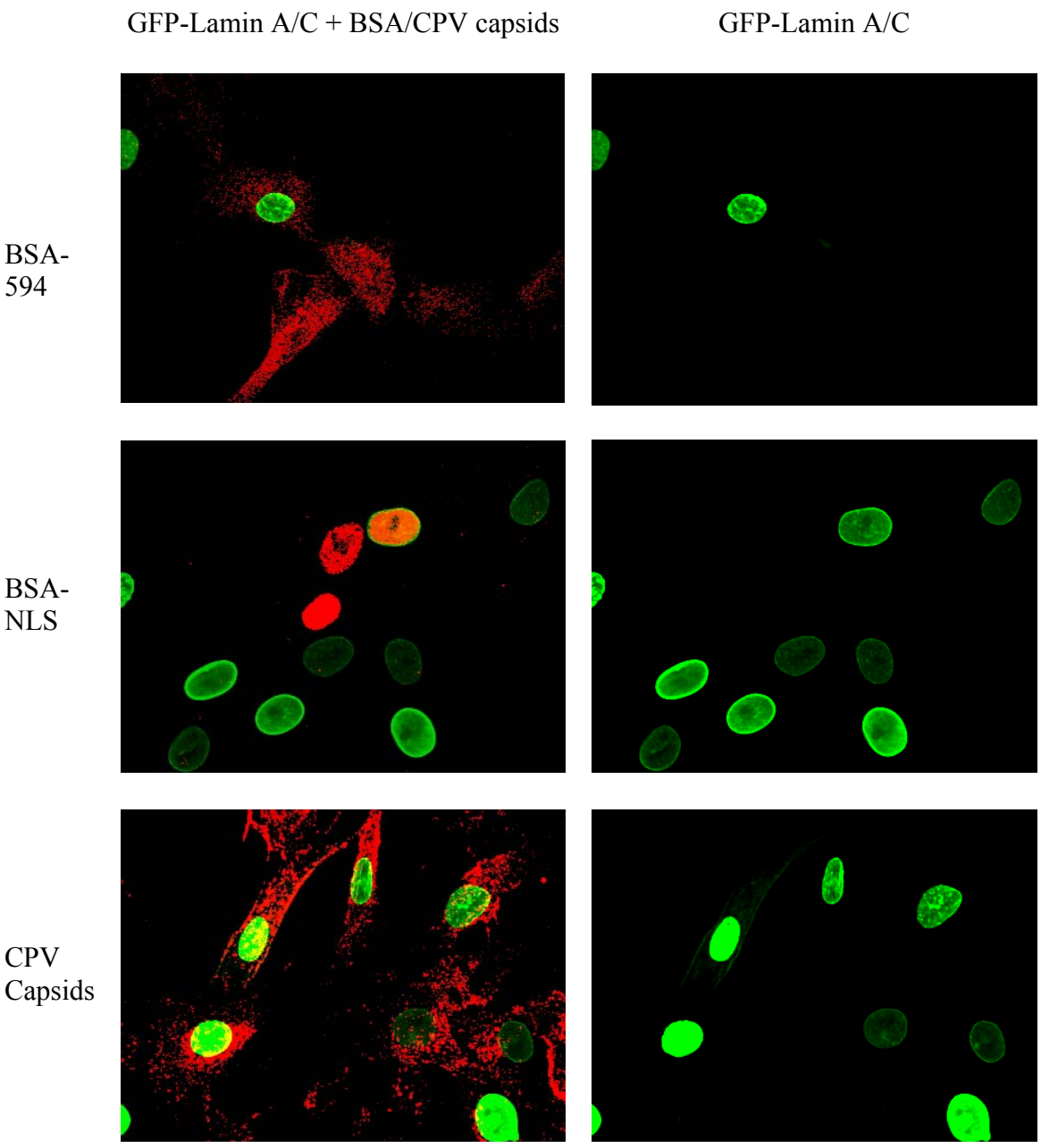

Figure 3: Intracellular localization of BSA-594, BSA-NLS and CPV capsids (red) 1 hour after microinjection into the cytoplasm of transfected cells, and structure of the nuclear envelope (GFP-Lamin A/C) in the cells (green)

Effect of microinjected viral capsids on nuclear envelope. Here I used cells expressing the nuclear envelope associated protein lamin A/C fused to GFP as a marker for the nuclear membrane. As controls, these GFP-lamin A/C-transfected cells were microinjected with BSA labeled with Alexa Fluor 594 Dye (BSA-594) or with

Rhodamine-labeled BSA with SV-40 large T-antigen NLS (BSA-NLS). The cells were then incubated at 37ºC for 1 hour before being fixed with 4% PFA. The nuclear envelope did not appear to have any breaks or changes in structure in both cases as observed from the complete oval outline of GFP-lamin in the nucleus of the microinjected cells (Figure 3). These transfected CRFK cells were also microinjected with purified full CPV capsids. After 1 hour, there was little to no localization of the virus inside the nucleus (Figure 3). In addition, there again appeared to be no changes in the lamin A/C structure of the nucleus (Figure 3).

Discussion

Passive diffusion of particles the size of viruses is limited by the characteristics of the cytoplasm, making it extremely unlikely that macromolecules of that size can move within the cell by just diffusion. Hence efficient transport of viruses in cells would require the hijacking of the host transport system. Previous studies have shown that intact microtubules are required during viral infection (14) and the role of these microtubules has been associated with transport of viral capsids through the cytoplasm to the nucleus using nocodazole treatment (16). The results presented here have re-affirmed the importance of microtubules to cytoplasmic transport of the viral capsids.

DNA viruses replicate in the nucleus to take advantage of the various host factors present there. To enter the nucleus, many of them have been found to use the nuclear pore complexes (NPC). In particular, parvoviruses are potentially small enough to pass through the NPC intact without capsid disassembly or deformation since they are only 18 – 24nm in diameter (20), well within the 28 – 38nm-diameter upper size limit of cargo

that has been found to be able to pass through the NPC (21). It has previously been shown that the MVM capsids can pass through the nuclear pore in more-or-less intact form by use of the nuclear transport machinery. In addition, there are regions of basic amino acids in the unique N-terminal sequence of the VP1 protein that play a role in the use of classical nuclear pore-dependent nuclear targeting (8). Although these sequences are buried within the viral capsids when they are initially produced, they are thought to become exposed during the cell entry process (8). But recent evidence has offered a novel alternative route taken by the MVM to enter the nucleus. It has been reported that the MVM may bypass the NPC route by causing breaks (~ 100 – 200nm) in the nuclear envelope, allowing viral particles to enter the nucleus via these breaks by some as yet undefined mechanisms (6, 7).

In the case of the CPV, similar sequences resembling classical NLS within the N-terminal unique region of the VP1 have been found. As with MVM, the VP1 N-terminus is inaccessible in the native structure of the CPV capsid, it can be exposed *in vitro* without disassembly of the capsid (17). The results presented here suggest that the CPV capsids do not induce the breaks or changes of structure in the nuclear envelope observed in the MVM (Figure 3).

The different results obtained here from those reported for the MVM capsids may reflect a difference in the pathways used by the viruses, or differences in the way in which experiments are conducted. In the MVM studies, the mammalian cells were permeabilized by treatment with Digitonin, which permeabilizes the plasma membrane but in general not the nuclear membrane. In those studies, the lamin B of the cells was detected with an antibody after fixing the cells and incubating them with further

detergents to permeabilize the nucleus. These treatments may therefore have made the cells more sensitive to nuclear disruption. To resolve these differences and further define the pathway of nuclear entry, we would need to use alternative approaches combining the different methods of exposing the nucleus to the virions, and to do these studies in the presence of other control reagents.

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