

## Short Communication

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# Parvoviral nuclear import: bypassing the host nuclear-transport machinery

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The parvovirus *Minute virus of mice* (MVM) is a small DNA virus that replicates in the nucleus of its host cells. However, very little is known about the mechanisms underlying parvovirus' nuclear import. Recently, it was found that microinjection of MVM into the cytoplasm of *Xenopus* oocytes causes damage to the nuclear envelope (NE), suggesting that the nuclear-import mechanism of MVM involves disruption of the NE and import through the resulting breaks. Here, fluorescence microscopy and electron microscopy were used to examine the effect of MVM on host-cell nuclear structure during infection of mouse fibroblast cells. It was found that MVM caused dramatic changes in nuclear shape and morphology, alterations of nuclear lamin immunostaining and breaks in the NE of infected cells. Thus, it seems that the unusual nuclear-import mechanism observed in *Xenopus* oocytes is in fact used by MVM during infection of host cells.

Parvoviruses are small, non-enveloped DNA viruses with properties that make them attractive as potential vectors for gene therapy. As part of their replication cycle, parvoviruses must enter the nucleus of their host cells. How this is accomplished remains unclear. Most viruses that enter the nucleus do so by hijacking the host nuclear-transport machinery. Many viruses bind soluble cytoplasmic-import receptors by using specific nuclear-localization signals (NLSs) on viral proteins; the receptor–cargo complex is then transported through the nuclear pore complex (NPC) to the nucleus (reviewed by Izaurralde *et al.*, 1999; Smith & Helenius, 2004; Whittaker *et al.*, 2000). Thus, it has been largely assumed that parvoviruses also enter the nucleus through the NPC.

We have recently shown that, after microinjection into *Xenopus* oocytes, the parvovirus *Minute virus of mice* (MVM) induces breaks in the nuclear envelope (NE) that support nuclear import of proteins in a manner that is independent of the NPC (Cohen & Panté, 2005). We have also shown that MVM damages the nuclear membranes of purified rat liver nuclei (Cohen & Panté, 2005). Based on these results, we proposed that MVM enters the nucleus by using a unique mechanism that is independent of the host nuclear-import machinery: instead of crossing the NPC, MVM disrupts the NE and enters the nucleus through the resulting breaks. Consistent with this mechanism, Hansen *et al.* (2001) previously found that another parvovirus,

adeno-associated virus (AAV), can enter purified intact nuclei in the absence of nuclear-import receptors and other cytoplasmic factors required for NPC-mediated import. In addition, blocking the NPCs with the lectin wheatgerm agglutinin had no effect on the uptake of AAV into purified nuclei. These two studies suggest strongly that parvoviruses can bypass the host nuclear-transport machinery during entry to the nucleus. However, both studies used experimental systems that are ideal for studying nuclear import (Beck *et al.*, 2004; Panté, 2006; Panté & Aebi, 1996; Panté & Kann, 2002), but are far removed from the situation during infection of host cells. One study that examined cells infected with MVM provided indirect evidence that MVM can disrupt the NE: by using biochemical fractionation, Nüesch *et al.* (2005) found that the NE distribution of the NPC proteins Nup62 and Nup88 is altered during MVM infection of mouse fibroblasts. We have now used fluorescence and electron microscopy to investigate directly the effect of MVM on host-cell nuclear structure during infection of mouse fibroblast cells, in order to determine whether our proposed mechanism of nuclear import is really used by parvoviruses during infection.

The effect of MVM on host cells during infection was studied by using double-immunolabelling fluorescence microscopy. To detect possible breaks in the NE by immunofluorescence microscopy, we performed immunolabelling of the nuclear lamina, a meshwork underlying the inner nuclear membrane made of the filamentous protein lamin. LA9 mouse fibroblast cells (Littlefield, 1964) grown on coverslips were mock-infected or infected with MVM [purified as described previously (Cohen & Panté, 2005)] at an m.o.i. of 4 (about 14 000 DNA-containing particles per cell). Cells were incubated at room temperature for 1 h to

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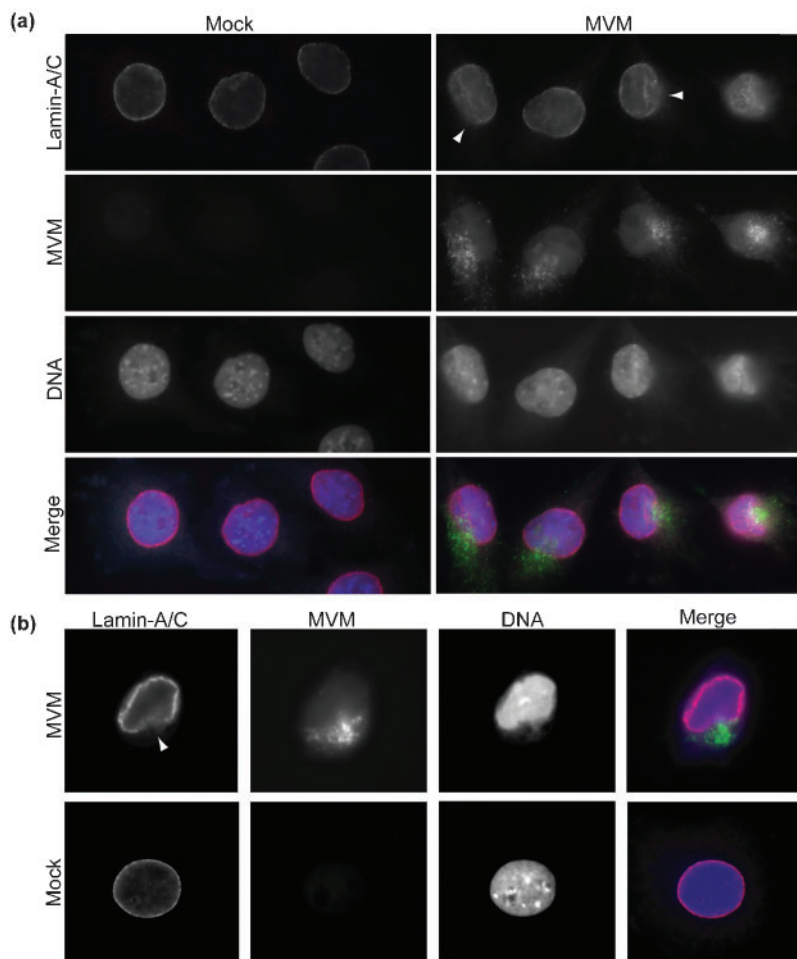
allow the virus to bind at the cell surface, followed by 1, 2 or 4 h at 37 °C. After incubation, cells were fixed (3% paraformaldehyde, 20 min), permeabilized (0.2% Triton X-100, 10 min) and labelled with primary antibodies for MVM (polyclonal; provided by Dr C. Astell, British Columbia Genome Science Centre) and for lamin-A/C (monoclonal; Covance), followed by appropriate fluorescently labelled secondary antibodies.

We observed that the nuclei of infected cells appeared shrivelled and irregular in shape compared with non-infected cells (Fig. 1a, b). In addition, we also observed alterations in the nuclear lamin-A/C immunostaining. These included invaginations or folding of the nuclear lamina in infected cells (Fig. 1a, top right; Fig. 1b, top left). Lastly, and consistent with our results in *Xenopus* oocytes, we found large, abnormal gaps in the lamin-A/C immunostaining of MVM-infected cells at 1, 2 and 4 h post-infection (p.i.) (Fig. 1a, b; indicated by arrowheads). Representative results are shown for mock- and MVM-infected cells at 2 h p.i. (Fig. 1). The gaps in the nuclear lamins of infected cells coincided with the location of the immunolabelling of MVM with the anti-MVM antibody, which was clustered at one pole of the nucleus. The asymmetrical perinuclear accumulation of MVM that we

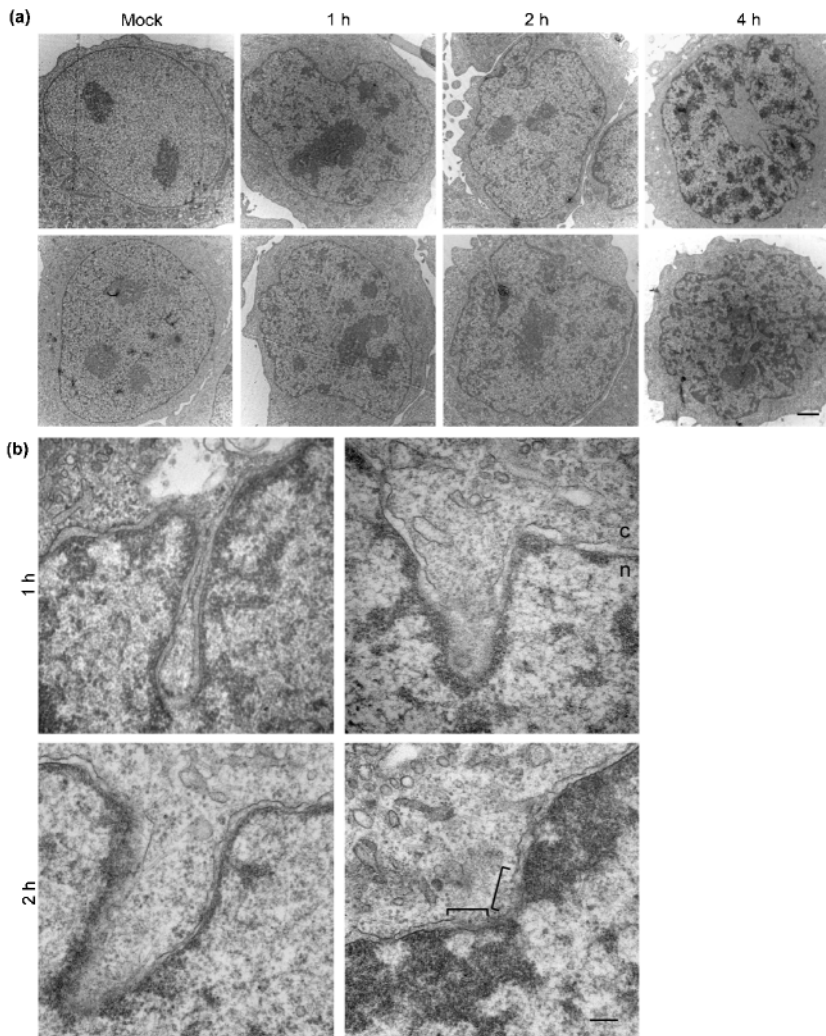
observed is similar to that reported previously for MVM and other parvoviruses (Bartlett *et al.*, 2000; Ros & Kempf, 2004; Vihinen-Ranta *et al.*, 1998). In contrast to the irregular lamin immunostaining of infected cells, mock-infected cells displayed normal continuous nuclear-rim staining of lamin-A/C (Fig. 1a, b). Whilst none of the mock-infected cells exhibited gaps in lamin-A/C immunostaining ( $n=56$ ), approximately 20% of MVM-infected cells showed gaps in lamin-A/C that were detectable in our immunofluorescence images at 1, 2 and 4 h p.i. (1 h,  $20 \pm 6\%$ ,  $n=51$ ; 2 h,  $25 \pm 6\%$ ,  $n=61$ ; 4 h,  $22 \pm 6\%$ ,  $n=50$ ).

To characterize further these nuclear perturbations at the ultrastructural level, MVM-infected cells were also analysed by electron microscopy (EM). Monolayers of LA9 cells were grown on Aclar film (Pelco) and infected as above. After infection, cells were fixed and embedded for EM as described by Fricker *et al.* (1997) and 50 nm thin sections were obtained and visualized by transmission EM as described previously (Cohen & Panté, 2005).

EM analysis revealed that MVM infection was associated with dramatic alterations in nuclear morphology (Fig. 2a). Whilst the nuclei of mock-infected cells were round with smoothly delineated borders, the nuclei of MVM-infected



**Fig. 1.** MVM disrupts the NE of infected cells. (a, b) LA9 cells were mock-infected or infected with MVM at an m.o.i. of 4 and prepared for indirect immunofluorescence microscopy 2 h after infection. For immunofluorescence microscopy, the cells were labelled with anti-lamin-A/C (red) and anti-MVM (green) antibodies and with DAPI to detect DNA (blue). Arrowheads indicate disruptions in the nuclear lamin-A/C immunostaining.



**Fig. 2.** MVM causes dramatic alterations in nuclear shape and morphology. LA9 cells were mock-infected or infected with MVM at an m.o.i. of 4 and prepared for thin-section EM 1, 2 or 4 h after infection. (a) Cells infected with MVM exhibited dramatic alterations in nuclear shape. Bar, 2  $\mu\text{m}$ . (b) In addition, MVM caused invaginations of the NE, distension of the outer and inner nuclear membranes and breaks in the outer nuclear membrane (ONM) (indicated by brackets). Bar, 200 nm. c, Cytoplasm; n, nucleus.

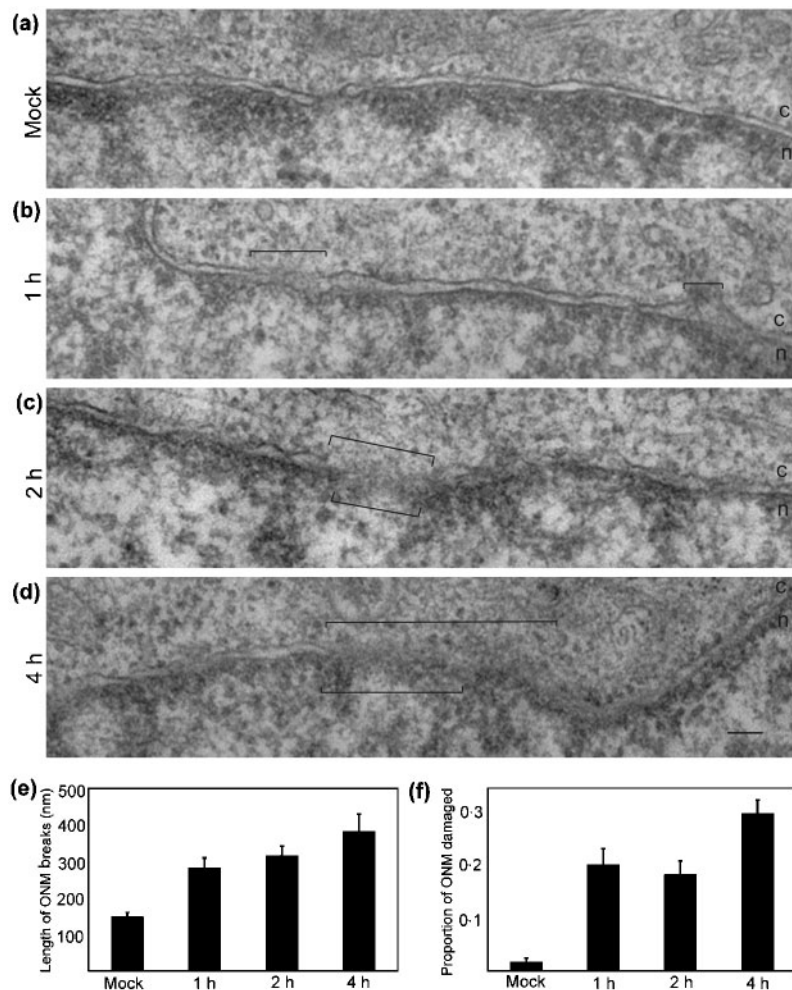
cells became increasingly irregular in shape with time p.i. Consistent with our lamin-A/C immunostaining, most micrographs of infected cells contained multiple amorphous invaginations of the NE. This irregularity was apparent as early as 1 h p.i., but became even more dramatic at 2 and 4 h p.i. In addition to changes in nuclear shape, we observed alterations in chromatin structure after infection with MVM. Whilst in mock-infected cells, densely staining chromatin was limited to a small area (the nucleolus), in MVM-infected cells, densely staining chromatin was more abundant and was present throughout the nucleus at 4 h p.i. (Fig. 2a).

High-magnification EM analysis showed ultrastructural changes in the NE of MVM-infected cells. At 1 and 2 h p.i., we observed numerous invaginations of the NE (Fig. 2b), and viral particles often seemed to be associated with the NE inside these invaginations. We also observed distension of the outer and inner nuclear membranes (Fig. 2b, top right) and breaks in the NE that were similar to those observed in *Xenopus* oocytes (Fig. 2b, bottom right). These breaks increased in size and frequency with time p.i. (Fig. 3). As in

microinjected *Xenopus* oocytes, NE breaks first appeared in the outer nuclear membrane (ONM) at 1 h p.i. (Fig. 3b) and later affected both the inner and outer nuclear membranes (Fig. 3c, d). In addition, we again noticed changes in chromatin organization at the NE in MVM-infected cells. In mock-infected cells, chromatin was packed densely at the nuclear face of the NE, except in proximity to NPCs (Fig. 3a). In MVM-infected cells, however, chromatin distribution at the NE was sparse and disrupted (Fig. 3b, d).

In order to quantify membrane damage, micrographs of regions of approximately 2–3  $\mu\text{m}$  from 30 different cells were studied. For each region, the number of breaks on the ONM was counted and the length of these breaks was measured. The total length of the breaks was divided by the length of the NE on the micrographs (2–3  $\mu\text{m}$ ) to find the proportion of membrane damaged in each micrograph. Break length and proportion of membrane damaged increased with time, as indicated in Fig. 3(e, f). The breaks of the ONM observed in mock-infected cells were small and rare, and probably represent junctions where the NE is continuous with the endoplasmic reticulum, which





**Fig. 3.** NE damage induced by MVM is time-dependent. LA9 cells were mock-infected (a) or infected with MVM at an m.o.i. of 4 and prepared for thin-section EM 1 h (b), 2 h (c) or 4 h (d) after infection. Whilst mock-infected cells yielded intact nuclear membranes, MVM-infected cells showed breaks (indicated by brackets) in the NE, with dimensions that increased with time. c, Cytoplasm; n, nucleus. Bar, 100 nm. (e) Bar graph of the length of the ONM breaks measured from EM cross-sections of cells from experiments performed as indicated in (a–d). (f) Bar graph of the proportion of ONM damage found in our micrographs. This was calculated as the length of the ONM breaks divided by the length of the NE on micrographs (2–3  $\mu$ m) from EM cross-sections of cells from experiments performed as indicated in (a–d). Each bar graph (e, f) shows the mean value and SEM measured for 30 micrographs examined for each condition.

appear occasionally as breaks in thin-section EM. Consistent with this explanation, no breaks were observed in the inner nuclear membrane of mock-infected cells.

Based on these results, it seems that the mechanism of NE disruption that we observed previously in *Xenopus* oocytes injected with MVM and in isolated rat liver nuclei incubated with MVM is in fact used by MVM during infection of host cells. The molecular mechanism of this disruption remains unclear. It has been shown that parvoviruses, including MVM, exhibit phospholipase A2 activity (Farr *et al.*, 2005; Zádori *et al.*, 2001) and that this activity is important for escape from endosomes (Farr *et al.*, 2005). It is possible that MVM utilizes this viral phospholipase to chew through the NE. The fact that we observe NE disruption in infected cells, as well as in other experimental systems, reinforces the conclusion that parvoviruses do not require the NPC for nuclear import, but rather use a mode of viral entry to the nucleus that is different from those of any other group of viruses known to date.

An alternate mechanism of parvoviral entry to the nucleus has been proposed. It has been suggested that a putative NLS located at the N terminus of the VP1 capsid protein may

mediate nuclear import of incoming virions (Farr *et al.*, 2005; Vihinen-Ranta *et al.*, 1997, 2002). However, several studies have noted the slow nuclear entry of parvoviruses during infection. We observe MVM in the nucleus of host cells only 2–4 h p.i. (data not shown). Similarly, AAV is detected in the nucleus after 2 h p.i. (Bartlett *et al.*, 2000; Lux *et al.*, 2005). It has been suggested that escape from endosomes is the major rate-limiting step in parvovirus infection (Mani *et al.*, 2006). However, even when parvovirus is microinjected into cells, bypassing the endocytic route, nuclear uptake is slow: canine parvovirus (CPV) virions rapidly accumulate perinuclearly in microinjected cells, but enter the nucleus very slowly, 3–6 h p.i. (Vihinen-Ranta *et al.*, 2000). This indicates that nuclear import after endosomal escape is also a slow step in parvovirus infection, which is consistent with a mode of nuclear entry where parvoviruses must disrupt the NE to reach the nucleus. In contrast, classical NLS-mediated import is usually much more rapid (Ribbeck & Görlich, 2001).

In addition to breaks of the NE, we observed alterations to host nuclear shape and gaps in lamin-A/C immunostaining after infection of mouse fibroblast cells with MVM. We

observed virions associated with invaginations of the NE; similarly, AAV has been reported to associate with nuclear invaginations (Lux *et al.*, 2005). It is possible that MVM affects host nuclear morphology by disrupting the nuclear lamina directly, after entering the nucleus. MVM may also disrupt the nuclear lamina indirectly from outside the nucleus by interacting with NE proteins. Proteomics studies have indicated the existence of many novel proteins that are enriched in the NE (Schirmer & Gerace, 2005; Schirmer *et al.*, 2003); some of these integral membrane proteins link the nuclear lamina and cytoskeleton in ways that are just beginning to be elucidated (Crisp *et al.*, 2006). Thus, interaction of MVM with NE proteins could potentially result in disassembly of the nuclear lamina and disruption of NE morphology, as well as having other cell-wide effects.

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