The genome of HSV-1 translocates through the nuclear pore as a condensed rod-like structure

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Summary

Incoming herpes simplex virus type-1 (HSV-1) capsids are known to dock to the nuclear pore complex (NPC) and release their genome. It has remained elusive, however, how the huge viral DNA translocates through the comparatively small NPC channel. In the present study, the interaction of HSV-1 with NPCs was analyzed by atomic force microscopy. In addition to capsids, smaller subviral structures – most with a diameter of 35-40 nm and a length of 130-160 nm – were visualized at the cytoplasmic side of the NPC. These components differed from capsids in their conformational changes that allow their outer surface to interact with channel components (Greber and Fassati, 2003). Alternatively, they could undergo disassembly factors (i.e. histone H1, importin β, importin 7 and Hsc70), the capsid disassembles and the deoxyribonucleoprotein genome containing the viral 36-kb dsDNA genome and a small subset of viral proteins including the terminal protein and DNA-binding protein VII (Cullen, 2001) is imported into the nucleus. The nuclear import of the adenoviral genome appears to be a relatively slow process, peaking at 30 to 60 minutes after the onset of capsid disassembly (reviewed by Greber and Fassati, 2003). In the case of herpes simplex virus type 1 (HSV-1), the virus enters the cell by fusion of its envelope with the cytoplasmic membrane. Capsids still surrounded by a layer of tegument proteins are then transported along microtubules towards the nucleus where they bind to the NPC in an importin-β-dependent manner (Lycke et al., 1988; Ojala et al., 2000; Sodeik et al., 1997). In the presence of cytosol and energy, binding of the virus capsid to the NPC is thought to trigger uncoating of the capsid on a particular vertex (Ojala et al., 2000), which might be identical to the portal of DNA entry during capsid maturation and genome packaging (Newcomb et al., 2003). Electron microscopy (EM) analyses support a mechanism in which the HSV-1 genome is rapidly and

Introduction

Nuclear pore complexes (NPCs) (Pante and Aeby, 1993) form a key transport barrier between the cytosol and the nucleus. In nondividing cells, nucleocytoplasmic shuttling of macromolecules is tightly controlled by their selective translocation through the 30- to 50-nm-long (Stoffler et al., 1999a) and 9-nm-wide (Pante and Aeby, 1993) NPC central channel (Oberleithner et al., 2003). This channel was shown to be expandable, enabling the transport of macromolecules with diameters of up to ~39 nm (Pante and Kann, 2002). In order to replicate and maintain their genomes, most eukaryotic DNA viruses and some RNA viruses have developed highly efficient strategies to gain access to the nucleus of their host cell via the NPC central channel. Although many aspects of the nuclear import of viral genomes have remained elusive, the size and shape of incoming viral nucleocapsids appear to play a key role in determining the principal mechanisms by which viral genomes are translocated through the NPC central channel and delivered into the nucleus. As reviewed by Greber and Fassati (Greber and Fassati, 2003), the following different scenarios lead ultimately to the nuclear import of viral genomes into nondondiving cells. (1) Intact virions and/or capsids with a diameter smaller than the maximum diameter of the NPC channel such as hepatitis B virus can translocate through the NPC by recruiting appropriate nuclear import receptors (Greber and Fassati, 2003). Alternatively, they could undergo conformational changes that allow their outer surface to interact with channel components (Greber and Fassati, 2003). Genome release then takes place in the nucleus, which in the case of hepatitis B virus is within the nuclear basket of the NPC (Rabe et al., 2003). (2) By contrast, virions or subviral particles significantly larger than the maximum diameter of the NPC channel have to partially disassemble or uncoat themselves prior to translocation of their genomes into the nucleus (Izaurralde et al., 1999). Disassembly or uncoating may take place in the cytosol or after docking at the NPC.

In the case of adenovirus 2, partially disassembled capsids are released from early endosomes after internalization of the virus, and bind via a phenylalanine-glycine (FxG) motif to the NPC filament protein Nup214/CAN. After recruitment of several disassembly factors (i.e. histone H1, importin β, importin 7 and Hsc70), the capsid disassembles and the deoxyribonucleoprotein genome containing the viral 36-kb dsDNA genome and a small subset of viral proteins including the terminal protein and DNA-binding protein VII (Cullen, 2001) is imported into the nucleus. The nuclear import of the adenoviral genome appears to be a relatively slow process, peaking at 30 to 60 minutes after the onset of capsid disassembly (reviewed by Greber and Fassati, 2003). In the case of herpes simplex virus type 1 (HSV-1), the virus enters the cell by fusion of its envelope with the cytoplasmic membrane. Capsids still surrounded by a layer of tegument proteins are then transported along microtubules towards the nucleus where they bind to the NPC in an importin-β-dependent manner (Lycke et al., 1988; Ojala et al., 2000; Sodeik et al., 1997). In the presence of cytosol and energy, binding of the virus capsid to the NPC is thought to trigger uncoating of the capsid on a particular vertex (Ojala et al., 2000), which might be identical to the portal of DNA entry during capsid maturation and genome packaging (Newcomb et al., 2003). Electron microscopy (EM) analyses support a mechanism in which the HSV-1 genome is rapidly and

Key words: Atomic force microscopy, Herpes simplex virus type-1, Viral genome, Nuclear import, Nuclear pore complex
efficiently ejected from capsids bound to the NPC, and empty capsids dissociate from the NPC after release of the genome (Batterson et al., 1983; Lycke et al., 1988; Sodeik et al., 1997). Little is known, however, about the factors triggering the release of the genome from the HSV-1 capsid and mediating its translocation through the NPC central channel. In the present study we analyzed interactions between HSV-1 capsids and NPCs using atomic force microscopy (AFM). This approach revealed that the HSV-1 genome is delivered to the nucleus through the NPC central channel as a highly condensed, rod-like structure, and that translocation of the viral genome is associated with a remarkable widening of the NPC central channel.

Results
Differentiating individual subviral structures by determining adhesion forces and estimating stiffness by AFM
The present study applied AFM as a nanoscale tool that physically interacts with the sample surface. Force-distance analysis was used to distinguish between distinct subviral components on the nuclear envelope surface. Fig. 1 shows how adhesive forces between the scanning AFM tip and the sample can be directly measured based on the force-distance curve. The figure also explains how the sample stiffness can be estimated from the extent of cantilever deflection, which in turn can be calculated directly from the force-distance curve. The AFM employs a diminutive tip mounted on the end of a flexible cantilever that raster the sample surface. Several forces manifest between the tip and sample surface before and after contact. These are measured by recording the cantilever deflection as the tip approaches, contacts and retracts from a surface, then plotting a force curve as a function of the approach-retract travel distance. Thus, this force-distance curve records the cantilever deflection as well as the adhesive and repulsive forces exerted on the tip as it approaches and retracts from a point on the sample surface. The extent of cantilever deflection generally increases with stiffer samples. Adhesion forces between the tip and the sample surface develop for numerous reasons. During scanning in air, capillary forces are believed to be the predominant origin of adhesion, which are assumed to be caused by a fluid layer on the sample surface (Jang et al., 2004; Sedin and Rowlen, 2000). Capillary forces however, are not the sole reason for tip adhesion. The extent of tip adhesion is strongly dependent on tip properties and various characteristic chemical and physical properties of a given sample surface such as the degrees of hydrophobicity and hydrophilicity (Shahin et al., 2005). Throughout the present study all the force-distance curves were derived using the same AFM tip in order to ensure uniformity of experimental conditions and to ensure that the extent of tip adhesion and cantilever deflection was only dependent on the characteristic surface properties of the samples.

Visualization of HSV-1 capsids as spherical structures
125 nm in diameter, attached to glass coverslips, using EM and AFM
Prior to their injection into oocytes, the prepared detergent-treated HSV-1 capsids were characterized structurally by EM and AFM. EM analysis of negatively stained capsid preparations showed that treatment of HSV-1 virions with 0.2% Triton X-100 rendered capsids free of envelopes (Fig. 2). AFM analysis of capsid preparations adsorbed onto a poly-L-lysine-coated surface revealed that the particles were spherical with a height distribution exhibiting a sharp maximum between 120 and 130 nm (123.4±0.64 nm, mean ± s.d.; n=200; Fig. 2). This finding is in close agreement with data from cryo-EM imaging showing that the diameter of the viral capsid shell is approximately 125 nm (Zhou et al., 2000) and confirmed that the capsid preparation used in the present study for injecting oocytes was essentially free of debris and degraded subviral structures.

Capsid binding to the cytoplasmic NPC site is associated with the appearance of various subviral components of different sizes on the nuclear envelope surface
We imaged the cytoplasmic and nucleoplasmic faces of the nuclear envelope 30 minutes after injecting either solvent (NIM) or HSV-1 capsids into Xenopus laevis oocytes. To ensure efficient visualization of capsid NPC interactions, approximately 7×10^5 HSV-1 capsids were injected into each oocyte. Fig. 3 displays representative AFM images of both the cytoplasmic and nucleoplasmic faces of the nuclear envelope 30 minutes after injecting solvent into the oocytes. Solvent injection resulted in NPCs appearing as smooth rings with a diameter of 98.3±0.53 nm (n=200, Fig. 3) at the cytoplasmic face of the nuclear envelope. NPCs were predominantly free of cargo. The nucleoplasmic face of the nuclear envelope also revealed cargo-free NPCs, surrounded throughout by lamins, characterized by their prominent filamentous structure.

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**Fig. 1.** Idealized force-distance curve describing a single approach-retract cycle of the AFM tip (modified from Shahin et al., 2005). The AFM tip is approaching the sample surface (A). The initial contact between the tip and the surface is mediated by the attractive van der Waals forces contact (contact) that lead to an attraction of the tip toward the sample (B). Hence, the tip applies a constant and default force upon the sample surface that leads to sample indentation and cantilever deflection (C). Subsequently, the tip tries to retract and to break loose from the surface (D). Various adhesive forces between the sample and the AFM tip, however, hamper tip retraction. These adhesive forces can be taken directly from the force-distance curve (E). The tip withdraws and loses contact with the sample upon overcoming the adhesive forces (F).
Compared with solvent treatment, remarkable changes took place on both faces of the nuclear envelope 30 minutes after injecting HSV-1 capsids.

**Cytoplasmic face of the nuclear envelope 30 minutes after injecting HSV-1 capsids: release of viral genomes and NPC dilation**

As evident in Fig. 4, on local patches of the cytoplasmic face of the nuclear envelope, the NPC rim was surrounded by highly visible structures with heights between 20 and 130 nm, the latter probably representing intact capsids as seen in Fig. 2.

**Determination of the height of viral structures demonstrated**

that capsid-like structures with a mean diameter of 125 nm accounted for only 10-15% of all structures associated with the cytoplasmic face of the nuclear envelope. The remaining viral structures ranged in height from 20 to 120 nm, and therefore most likely represented material released from capsids after docking to the NPC and/or capsid shells in various stages of disassembly. A striking observation was that approximately 40% of all viral substructures seen at the cytoplasmic side of the nuclear envelope had a uniform, rod-like shape, with a diameter of 35-40 nm and an estimated length of 130-160 nm.

**Fig. 2.** Prepared detergent-treated herpes simplex virus type-1 (HSV-1) capsids. Samples were characterized structurally by electron microscopy (EM) (A) and atomic force microscopy (AFM) (B-D). EM analysis of negatively stained capsid preparations shows that treatment of HSV-1 virions with 0.2% Triton X-100 renders capsids (which are 125 nm in diameter) free of envelopes. AFM analysis of capsid preparations adsorbed onto a poly-L-lysine-coated surface reveals spherical particles (B) with a height distribution exhibiting a sharp maximum between 120 and 130 nm (C,D) (n~200).

**Fig. 3.** Representative AFM images of both the cytoplasmic and nucleoplasmic faces of the nuclear envelope 30 minutes after injecting solvent (nuclear isolation medium, NIM) into *Xenopus laevis* oocytes. Upon NIM injection, the cytoplasmic face of the nuclear envelope contains nuclear pore complexes (NPCs) appearing as cargo-free smooth rings exhibiting a diameter of 98.3±0.53 nm (n=200). The nucleoplasmic face of the nuclear envelope also reveals cargo-free NPCs, surrounded throughout by lamins, characterized by their prominent filamentous structure.
or more. A more reliable analysis of the length of this structure was not possible owing to convolution-related limitations of the tip geometry. We did not detect typical dsDNA strand-like structures on the surface of the nuclear envelope. In contrast to the NPC rim, the NPC central channel was found to be occupied exclusively by the rod-like structures with a diameter of 35-40 nm. When these structures were found plugging the NPCs central channel, the diameter of NPCs increased remarkably from 98.3±0.53 nm to 177.6±2.67 nm (Fig. 4).

Nucleoplasmic face of the nuclear envelope 30 minutes after injecting HSV-1 capsids: translocation of rod-like subviral structures into the nucleus
As seen in Fig. 5 and in contrast to the cytoplasmic face of the nuclear envelope (Fig. 4), only the rod-shaped subviral structures with a diameter of 35-40 nm were evident at the nucleoplasmic side, indicating that other subviral structures were excluded from the translocation. The rod-like structures were often observed within the NPC central channel, thereby suggesting that they left the central channel after translocation to the nucleoplasmic side (Fig. 5). Also, dsDNA strand-like structures were not detectable at the nucleoplasmic face of the NPC.

Analysis of force-distance curves to estimate the adhesion force and stiffness of subviral structures
The analysis of the force-distance curves also allowed differentiation between individual subviral components and the NPC. The force-distance curves exhibited consistent characteristics specific to capsids, the rod-like subviral components and NPCs (Fig. 6). The portion of the force-distance curves corresponding to retraction clearly indicates that the AFM tip was retracted more strongly by HSV-1 capsids (adhesion force: 1.3±0.03; n=12) than by single NPCs or the rod-like subviral structures (0.3±0.02; n=12). In addition, NPCs and rod-like subviral structures caused a markedly stronger cantilever deflection compared to capsids, as seen in the approach portion of the curves. This finding indicates that NPCs and rod-like subviral structures are stiffer than capsids.

Discussion
The aim of the present study was to use AFM to determine the elusive mechanism by which the bulky HSV-1 genome, which is far longer and larger than the NPC channel, overcomes the nuclear barrier. Over the past years AFM has proved to be an unimpeachable approach for visualizing conformational
Translocation of HSV-1 genome changes of the NPC as well as dynamic transport events at the NPC in three dimensions and at a nanoscale resolution (Oberleithner et al., 1994; Schäfer et al., 2002; Shahin et al., 2004; Shahin et al., 2005; Stoffler et al., 1999b). In addition, AFM has recently emerged as a powerful complement to other ultrastructural techniques such as X-ray crystallography and EM for the analysis of virus morphology and function (Kuznetsov et al., 2004; Malkin et al., 2003; Malkin et al., 2004; Plomp et al., 2002). AFM enables nonaveraged, nanoscale imaging of the surface topology of individual virions and of viral substructures (Day et al., 2001; Kuznetsov et al., 2005; Malkin et al., 2003; Plomp et al., 2002) in a hydrated state. Therefore, AFM qualifies as a suitable technique to elucidate dynamic processes associated with the life cycle of viruses in vitro (Malkin et al., 2004). All AFM images in the present study were obtained using the so-called contact mode, which is generally applied to visualize the surface topology of viruses. As the name implies, in the contact mode the AFM tip and sample remain in close contact during the scanning. This mode has proved to be the most suitable for visualizing the surface topology of biological membranes, particularly stiff ones, such as an isolated nuclear envelopes spread on a glass coverslip (Oberleithner et al., 2001; Perez-Terzic et al., 1997; Shahin et al., 2004; Shahin et al., 2005).

The enigmatic nuclear delivery of the HSV-1 genome through the NPC channel

The mechanisms by which genomes of large DNA viruses are translocated through the NPC are poorly understood. Upon release from the disassembling capsid, adenovirus 2 DNA may simply diffuse passively through the nuclear pore (Harel and Forbes, 2001). The uptake kinetics of individual dsDNA molecules into the nucleus of X. laevis oocytes has been studied in detail with DNA derived from bacteriophage lambda linked to a polystyrene bead, enabling real-time imaging, manipulation and length measurements of the DNA conjugate by optical tweezers (Salman et al., 2001). The nuclear uptake of the 50-kb dsDNA molecules requires no energy-consuming motor, but it can be blocked by wheat germ agglutinin. Although increasing the probability for contact of DNA molecules with the NPC, the presence of an NLS at one end of the DNA molecule had no effect on the kinetics of DNA translocation through the NPC. The initial linear rate of dsDNA uptake was found to be 28 nm/second, but was much slower for DNA strands shorter than 4μm. Mechanistically, the translocation process was characterized as a rather slow, passive ratchet driven by retention of the DNA segments already imported into the nucleus.

The linear 153-kb dsDNA genome of HSV is densely
packaged within the capsid cavity and free of bound histones or other proteins (Booy et al., 1991; Zhou et al., 1999). In a completely extended linear state, the length of the HSV genome is expected to be in excess of 50 μm. Thus, the nuclear import of the HSV genome as a linear DNA molecule by passive diffusion at a speed of 28 nm/second as described for the uptake of lambda DNA into X. laevis oocytes (Salman et al., 2001) would take over 30 minutes to complete. Furthermore, this type of DNA import implies unpackaging and unfolding of the viral genome at the cytoplasmic face of the NPC, thereby rendering the genome accessible to degradation. The data from the present study strongly suggest that HSV-1 uses an alternate mechanism to enable the efficient and rapid translocation of its genome, involving the nuclear import of the viral genome as a highly condensed rod-like structure and accompanied by widening of the NPC. Analysis of the interaction of HSV-1 capsids with NPCs of X. laevis oocytes allowed us to directly visualize numerous subviral components with a diameter of 35-40 nm and an estimated length of 130-160 nm or more at the cytoplasmic side of the nuclear envelope. The major finding of the present study is that this subviral component enters the central channel of the NPC at the cytoplasmic side and is translocated selectively towards the nucleoplasmic side.

Based on the following criteria, we conclude that this novel subviral component most likely represents the viral DNA genome extruded from capsids. (1) Although strictly missing in the capsid preparation used to inject oocytes, it is the predominant subviral component bound to the cytoplasmic face of the NPC. (2) It can be seen entering the central channel at the cytoplasmic side of the NPC. (3) It is the only subviral structure visualized by AFM that is translocated selectively through the central channel towards the nucleoplasmic face of the NPC. (4) Its surface properties as determined from force-distance curves clearly differ from those of capsids. Its markedly higher stiffness compared to capsids indicates that the rod-shaped subviral component includes a densely packed structure, probably DNA.

Capsid-like structures with a diameter of 125 nm account for only 10-15% of all viral structures detected by AFM at the cytoplasmic side of the nuclear envelope, and capsids captured exactly in the moment of genome release were not visualized by AFM. Thus, the genome appears to be released shortly after the capsids dock to the NPC, and capsids appear to dissociate rapidly from the NPC after release of the viral DNA. In contrast to empty capsids, the extruded genome may remain associated with the cytoplasmic side of the NPC for longer periods until nuclear translocation proceeds. The presence of pleomorphic viral substructures with intermediate diameters between 20 and 130 nm at the cytoplasmic side of the NPC may indicate that substantial capsid disassembly occurs during release of the genome.

Docking of HSV-1 capsids to the NPC has been visualized by EM and was also observed here by AFM (Ojala et al., 2000). Further EM analyses support a mechanism in which the viral genome is efficiently and rapidly ejected from the capsid bound to the NPC, followed by release of empty capsids from the NPC. The AFM data presented here extend this model. The
nuclear import of the HSV-1 genome as a highly condensed rod-like structure (as summarized in Fig. 7) may enable the virus to overcome common hindrances to the nuclear import of large linear DNA molecules by passive diffusion, and achieve rapid translocation of its genome, i.e. within 30 minutes of the injection of capsids into the cytoplasm. In AFM, rod-shaped genomes bound to the cytoplasmic and nucleoplasmic faces of the NPC were not observed to be regularly associated with fragments of the capsid shell or other prominent viral substructures. The significantly lower adhesive forces measured between rod-shaped genomes and the hydrophobic AFM tip (Shahin et al., 2005) as compared to intact capsids also argue for a highly hydrophilic surface structure essentially free of capsid substructures. It remains to be determined whether the import of the viral genome involves one or more of the proteins or tegument components known to be associated with capsids (Grunewald et al., 2003; Newcomb et al., 2001; Ojala et al., 2000; Saad et al., 1999; Zhou et al., 1999). Alternatively, yet to be defined HSV-1 ‘core’ proteins or exclusively cellular factors may direct the nuclear import of the genome.

Materials and Methods
Cells, virus and infection
Vero and BHK-21 cells were grown in Dulbecco’s modified minimal essential medium containing 10% fetal calf serum. HSV-1 strain 17 was used for infection and capsid isolation. Titrations of the virus were performed in Vero cells.

Purification of virus particles and capsid isolation
HSV-1 penetrates into the host cell by fusion of its envelope with the cytoplasmic membrane. To obtain virus preparations efficiently binding to the nuclear pore complex, virus capsids were freed of their envelope by detergent treatment and purified as described (Ojala et al., 2000). Approximately $5 \times 10^9$ BHK-21 cells were infected at a multiplicity of 0.01. Three days later, the infected cells were harvested by centrifugation at 4000 g for 30 minutes. The supernatant was removed and centrifuged for a further 90 minutes at 4000 g in a Beckmann SW 28 rotor to pellet the extracellular virus. The resulting virus pellet was resuspended in MNT buffer (30 mM morpholineethanesulfonic acid, 100 mM NaCl, 20 mM Tris-HCl pH 7.4). The capsids were isolated from the envelope and tegument components by incubation in a lysis buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Triton X-100, 1 mM EDTA) for 30 minutes on ice in the presence of protease inhibitor (Complete, Roche). After sonication in a water bath (three times, 30 seconds each) the sample was layered onto a linear 20-40% sucrose gradient in MNT buffer (supplemented with 400 mM NaCl, 1 mM EDTA and 0.5 mM dithiothreitol) and centrifuged for 60 minutes at 4000 g in a Beckmann SW 28 rotor. Capsids were collected from the light-scattering zone of the gradient, and resuspended in nuclear isolation medium (NIM) (Shahin et al., 2001) and pelleted by centrifugation for 60 minutes at 4000 g in a Beckmann SW 28 rotor. The resulting capsid pellet was resuspended in NIM. For EM images, capsids were contrasted using tungsstophosphoric acid.

Injection of oocytes, isolation of nuclei and preparation of nuclear envelopes
Oocytes were collected as described previously (Shahin et al., 2004), and injected with 70 nl NIM containing $10^{10}$ HSV-1 capsids/ml. Thirty minutes later the nuclei were isolated as described previously (Shahin et al., 2004). Control experiments were performed by injecting the respective capsid-free solution (with NIM as the solvent). For nuclear isolation, oocytes were transferred into NIM and the nuclear envelope was removed and spread onto glass coverslips coated with Cell-Tak (Biosciences, Bedford, MA). For imaging the cytoplasmic and nucleoplasmic faces of the nuclear envelope, the nuclear envelope was spread on the coverslip with the nucleoplasmic and cytoplasmic sides facing downwards, respectively. The flattened nuclear envelope was rinsed gently with deionized water and air-dried. All procedures were performed at room temperature (~23°C).

AFM
We used a Bioscope atomic force microscope (NanoScope IIIa controller, Digital Instruments, Santa Barbara, CA) equipped with an optical microscope. V-shaped 282-μm-long silicon cantilevers (CSC21, Ultra-sharp, Anfatec, Oelsnitz, Germany) with a spring constant of 0.12 N/m and pyramidal tips with an estimated tip radius of 10 nm were used. The images were recorded with 512 scan lines per area, at constant force (height mode) in contact mode in air with a scan rate of 3.5 Hz. Data analysis was performed with commercially available software (NanoScope III software; Digital Instruments; Scanning Probe Image Processor, Image Metrology, Lyngby, Denmark).

Structural analysis of HSV-1 capsids, viral particles, and NPC conformational states by AFM
The HSV-1 capsids were characterized structurally by thawing the frozen capsid suspension, and depositing it on a freshly prepared, poly-L-lysine-coated glass coverslip, which was imaged with AFM. Owing to the higher resolution of AFM in the z-axis, which is in the order of 1 nm for biological preparations, we always used height analysis to characterize viral structures. AFM analysis in the x- and z-axes may lead to severe overestimations of the dimensions of structures because of the convolution of the tip geometry with the sample surface. Additionally, and as described previously (Shahin et al., 2004), profile analysis was performed in order to determine the NPC diameter and the NPC opening diameter. The NPC diameter was obtained by measuring the diameter of the NPC ‘upper rim’, and the NPC opening diameter was determined by measuring the horizontal distance (within the NPC opening) at half-maximal height.

Statistical analyses
Data are presented as mean ± standard error of the mean (s.e.m.) values. Five nuclei were isolated in each experimental series.

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References
Batterson, W., Furlong, D. and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant
defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. 45, 397-407.
Booy, F. P., Newcomb, W. W., Trus, B. L., Brown, J. C., Baker, T. S. and Steven, A. C. (1991). Liquid-crystalline, phase-like packing of encapsidated DNA in herpes simplex virus. Cell 64, 1007-1015.
Cullen, B. R. (2001). Journey to the center of the cell. Cell 105, 697-700.
Day, J., Kuznetsov, Y. G., Larson, S. B., Greenwood, A. and McPherson, A. (2001). Biophysical studies on the RNA cores of satellite tobacco mosaic virus. Biophys. J. 80, 2366-2371.
Greber, U. F. and Fassati, A. (2003). Nuclear import of viral DNA genomes. Traffic 4, 136-143.
Grunewald, K., Desai, P., Winkler, D. C., Heymann, J. B., Belmont, D. M., Baumeister, W. and Steven, A. C. (2003). Three-dimensional structure of herpes simplex virus from cryo-electron tomography. Science 302, 1396-1398.
Harel, A. and Forbes, D. J. (2001). Welcome to the nucleus: CAN I take your coat? Nat. Cell Biol. 3, E267-E269.
Izaurralde, E., Kann, M., Pante, N., Sodeik, B. and Hohn, T. (1999). Viruses, microorganisms and scientists meet the nuclear pore. Leyssin, VG, Switzerland, February 26-March 1, 1998. EMBO J. 18, 289-296.
Jang, J., Schatz, G. C. and Ratner, M. A. (2004). Capillary force in atomic force microscopy. J. Chem. Phys. 120, 1157-1160.
Kuznetsov, Y. G., Victoria, J. G., Low, A., Robinson, W. E., Jr, Fan, H. and McPherson, A. (2004). Atomic force microscopy imaging of retroviruses: human immunodeficiency virus and murine leukemia virus. Scanning 26, 209-216.
Kuznetsov, Y. G., Dajiogou, S., Zhou, J., Semler, B. L. and McPherson, A. (2005). Atomic force microscopy analysis of icosahedral viral RNA. J. Mol. Biol. 347, 41-52.
Lycke, E., Hamark, B., Johansson, M., Krotoschwil, A., Lycke, J. and Svennerholm, B. (1988). Herpes simplex virus infection of the human sensory neuron. An electron microscopy study. Arch. Virol. 101, 87-104.
Malkin, A. J., McPherson, A. and Gershon, P. D. (2003). Structure of intracellular mature vaccinia virus visualized by in situ atomic force microscopy. J. Virol. 77, 6332-6340.
Malkin, A. J., Plomp, M. and McPherson, A. (2004). Unraveling the architecture of viruses by high-resolution atomic force microscopy. In DNA Viruses Methods and Protocols (ed. P. M. Liebermann), pp. 85-108. Totowa, New Jersey: Humana Press.
Newcomb, W. W., Juhas, R. M., Thomsen, D. R., Homa, F. L., Burch, A. D., Weller, S. K. and Brown, J. C. (2001). The UL6 gene product forms the portal for entry of herpes simplex virus capsids and their role in formation of portal-containing capsids. J. Virol. 75, 9862-9871.
Oberleithner, H., Brinckmann, E., Schwab, A. and Krohne, G. (1994). Imaging nuclear pores of aldosterone sensitive kidney cells by atomic force microscopy. Proc. Natl. Acad. Sci. USA 91, 9784-9788.
Oberleithner, H., Schäfer, C., Shahin, V., Schlune, A., Schillers, H. and Reinhardt, J. (2001). Nuclear plug harvesting using atomic force microscopy. Single Mol. 2, 117.
Oberleithner, H., Schafer, C., Shahin, V. and Albermann, L. (2003). Route of steroid-activated macromolecules through nuclear pores imaged with atomic force microscopy. Biochem. Soc. Trans. 31, 71-75.
Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U. and Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. Mol. Cell Biol. 20, 4922-4931.
Pante, N. and Aebl, U. (1993). The nuclear pore complex. J. Cell Biol. 122, 977-984.
Pante, N. and Kann, M. (2002). Nuclear pore complex is able to transport macromolecules with diameters of ~39 nm. Mol. Biol. Cell 13, 425-434.
Perez-Terzic, C., Jaconi, M. and Clapham, D. E. (1997). Nuclear calcium and the regulation of the nuclear pore complex. BioEssays 19, 787-792.
Plomp, M., Rice, M. K., Wagner, E. K., McPherson, A. and Malkin, A. J. (2002). Rapid visualization at high resolution of pathogens by atomic force microscopy: structural studies of herpes simplex virus-1. Am. J. Pathol. 160, 1959-1966.
Rabe, B., Vlacou, A., Pante, N., Helenius, A. and Kann, M. (2003). Nuclear import of hepatitis B virus capsids and release of the viral genome. Proc. Natl. Acad. Sci. USA 100, 9849-9854.
Saad, A., Zhou, Z. H., Jakana, J., Chiu, W. and Rixon, F. J. (1999). Roles of triplex and scaffolding proteins in herpes simplex virus type 1 capsid formation suggested by structures of recombinant particles. J. Virol. 73, 6821-6830.
Salman, H., Zbaida, D., Rubin, Y., Chateman, D. and Elbaum, M. (2001). Kinetics and mechanism of DNA uptake into the cell nucleus. Proc. Natl. Acad. Sci. USA 98, 7247-7252.
Schäfer, C., Shahin, V., Albermann, L., Hug, M. J., Reinhardt, J., Schillers, H., Schneider, S. W. and Oberleithner, H. (2002). Aldosterone signaling pathway across the nuclear envelope. Proc. Natl. Acad. Sci. USA 99, 7154-7159.
Sedlin, D. L. and Rowlen, K. L. (2000). Adhesion forces measured by atomic force microscopy in humid air. Anal. Chem. 72, 2183-2189.
Shahin, V., Damker, T., Enos, K., Ossig, R. and Oberleithner, H. (2001). Evidence for Ca2+- and ATP-sensitive peripheral channels in nuclear pore complexes. FASEB J. 15, 1895-1901.
Shahin, V., Albermann, L., Schillers, H., Kastrup, L., Schafer, C., Ludvig, Y., Stock, C. and Oberleithner, H. (2004). Steroids dilate nuclear pores imaged with atomic force microscopy. J. Cell Physiol. 202, 591-601.
Shahin, V., Ludvig, Y., Schafer, C., Nikova, D. and Oberleithner, H. (2005). Glucocorticoids remodel nuclear envelope structure and permeability. J. Cell Sci. 118, 2881-2889.
Sodeik, B., Ebersold, M. W. and Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J. Cell Biol. 136, 1007-1021.
Stoffler, D., Fahrenkrog, B. and Aebl, U. (1999a). The nuclear pore complex: from molecular architecture to functional dynamics. Curr. Opin. Cell Biol. 11, 391-401.
Stoffler, D., Goldie, K. N., Feja, B. and Aebl, U. (1999b). Calcium-mediated structural changes of native nuclear pore complexes monitored by time-lapse atomic force microscopy. J. Mol. Biol. 287, 741-752.
Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. and Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. J. Virol. 73, 3210-3218.
Zhou, Z. H., Dougherty, M., Jakana, J., He, J., Rixon, F. J. and Chiu, W. (2000). Seeing the herpesvirus capsid at 8.5 A. Science 288, 877-880.