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An improved high pressure freezing and freeze substitution method to preserve the labile vaccinia virus nucleocapsid

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ABSTRACT

In recent years, high pressure freezing and freeze substitution have been widely used for electron microscopy to reveal viral and cellular structures that are difficult to preserve. Vaccinia virus, a member of the *Poxviridae* family, presents one of the most complex viral structures. The classical view of vaccinia virus structure consists of an envelope surrounding a biconcave core, with a lateral body in each concavity of the core. This classical view was challenged by Peters and Muller (1963), who demonstrated the presence of a folded tubular structure inside the virus core and stated the difficulty in visualizing this structure, possibly because it is labile and cannot be preserved by conventional sample preparation. Therefore, this tubular structure, now called the nucleocapsid, has been mostly neglected over the years. Earlier studies were able to preserve the nucleocapsid, but with low efficiency. In this study, we report the protocol (and troubleshooting) that resulted in preservation of the highest numbers of nucleocapsids in several independent preparations. Using this protocol, we were able to demonstrate an interdependence between the formation of the virus core wall and the nucleocapsid, leading to the hypothesis that an interaction exists between the major protein constituents of these compartments, A3 (core wall) and L4 (nucleocapsid). Our results show that high pressure freezing and freeze substitution can be used in more in-depth studies concerning the nucleocapsid structure and function.

1. Introduction

In recent years, high pressure freezing and freeze substitution have been widely used for electron microscopy to reveal viral and cellular structures that are difficult to preserve (Buser et al., 2007; Hawes et al., 2008; Monaghan et al., 2004). During freezing, structures are cryoimmobilized at a faster rate (scale of milliseconds) than when conventional methods of fixation using glutaraldehyde and osmium tetroxide are used (scale of several minutes or hours). During freeze substitution (done at sub-zero temperatures), the decreased movement of cell components results in reduced extraction of cell constituents and helps preserve the ultrastructure in an almost native state (McDonald, 2014). In virology, high pressure freezing and freeze substitution are helping to elucidate the structure of entire virus particles that cannot be preserved using conventional fixation techniques as well as solving controversies about viral substructures (Monaghan et al., 2004).

Vaccinia virus, a member of the *Poxviridae* family, presents one of the most complex viral structures. This complexity arises in part from the fact that poxviruses are DNA viruses that replicate in the cell cytoplasm and must, therefore, encapsidate a complete virus-coded transcription system. The classical view of vaccinia virus structure consists of an envelope surrounding a biconcave core, with a lateral body in each concavity of the core (Condit et al., 2006). The virus core is composed of a protein core wall surrounding an amorphous electron-dense material, supposed to contain the viral DNA and the transcription enzymes.

This classical view was challenged by Peters and Muller (1963), who demonstrated the presence of a folded tubular structure inside the virus core. However, this structure could only be observed if the pH of the stain used in the negative staining technique was shifted to basic values. Consistent with the need to change a classic and widely used technique, Peters and Muller stated that this internal core structure was the most difficult to visualize, possibly because it is labile and cannot be preserved by conventional sample preparation. Therefore, this tubular structure has been mostly neglected over the years. Even nowadays, with the availability of an array of microscopy techniques, the majority of studies concerning vaccinia virus structure fail to reveal this tubu-

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lar structure leaving the importance of the structure for the biology of vaccinia virus mostly unknown.

Using high pressure freezing/freeze substitution as an approach, we showed previously that the formation of the tubular structure requires packaging of the viral transcription apparatus. This apparatus is used during viral early transcription, which occurs inside the virus core after the virus enters the host cell. Therefore, we suggested a role for the tubular structure in early viral transcription (McFadden et al., 2012).

The correlation between transcription apparatus encapsidation and formation of the tubular structure led to the suggestion that this structure could be the equivalent of a nucleocapsid. The tubular structure, from now on called the nucleocapsid, would comprise a complex of the viral genome and transcription factors along with other nucleocapsid structural proteins. As an essential part of vaccinia virus structure, the nucleocapsid should be present in most, if not all infectious virus particles. However, in the study by McFadden and colleagues the number of viral particles containing a nucleocapsid varied between only 4–15% depending on the preparation (McFadden et al., 2012). Because of the high variation and the lower than expected number of viral particles containing a nucleocapsid, we were interested in refining the technique in order to image the nucleocapsid in a more consistent fashion, which is essential for future studies on the biological significance of the nucleocapsid.

In this paper, we report the protocol (and troubleshooting) that resulted in preservation of the highest numbers of nucleocapsids in several independent preparations. In these preparations, 40–60% (with an average of 50.8%) of the virus particles analyzed contained the nucleocapsid. Using this protocol, we were able to suggest an interdependence between the formation of the virus core wall and the nucleocapsid (Jesus et al., 2014 and present work), leading to the hypothesis that an interaction exists between the major protein constituents of these compartments, A3 (core wall) and L4 (nucleocapsid). Therefore, high pressure freezing and freeze substitution can be used in more in-depth studies concerning the nucleocapsid structure and function.

2. Materials and methods

2.1. Cells and viruses

BSC-40 cells, an African green monkey cell line, were used throughout the study. BSC-40 cells were cultured in Dulbecco's Modified Eagle's medium (Life Technologies – cat 12100-061) containing 10% fetal bovine serum, 0.12 mg/mL penicillin (Sigma), 0.2 mg/mL streptomycin (Sigma) and 250 μg/mL fungizone (Sigma). Wild type vaccinia virus strains WR and IHDW have been previously described (Condit and Motyczka, 1981; Dales et al., 1978). Thermo sensitive vaccinia virus mutants Cts8 (A3L mutant), Cts52 (E6R mutant), Cts18 (I8R mutant) and Dts23 (E8L mutant) have been characterized in previous studies (Boyd et al., 2010; Gross and Shuman, 1996; Kato et al., 2007, 2004). The temperature used for wild type virus infections was 37 °C or 31 °C, depending on the experiment. The permissive temperature for the mutants was 31 °C and the non-permissive temperature was 39.5 °C.

2.2. Virus infection

All infections were done at an MOI of 10. BSC-40 monolayers in 100 mm dishes were infected with vaccinia virus and incubated for 30 min at 31 °C, 37 °C or 39.5 °C, depending on the experiment. The virus inoculum was removed and medium was added to the cells. Infected cells were incubated for 24 h at the designated temperature.

2.3. High pressure freezing

After 24 h of infection, cells were washed twice with versene (PBS-EDTA) then 1.5 mL of accutase (Invitrogen) was added to the dishes following incubation for 5 min at 37 °C. Medium was added to the cells and the cells were centrifuged at 500*g* for 10 min at 4 °C. The cell pellets were fixed with 1% glutaraldehyde and 4% formaldehyde in sodium cacodylate buffer (0.1 M sodium cacodylate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 43 mM NaCl; pH 7.2). This initial fixation was necessary as a biosafety measure; the procedure for high pressure freezing and freeze substitution was done at the University of Florida Electron Microscopy core, where live viruses are not permitted. It is important to note that chemical fixation before high pressure freezing does not decrease the quality of sample preservation and can actually result in superior preservation (Sosinsky et al., 2008). Cells were incubated at 4 °C for at least 30 min in the fixative. Although 30 min were sufficient to inactivate all the virus particles, most of the samples were fixed for longer times, without an impact on the final ultrastructure preservation. Samples were handled one at a time, while the others were kept in the fixative in order to not lose ultrastructure. To minimize the time the samples were in the fixative, no more than 5 samples were handled in the same experiment and all steps were optimized in order to minimize the amount of time required to freeze each sample.

Before freezing, cells were centrifuged at 1000*g* for 2 min at room temperature, resuspended in 0.1 M cacodylate buffer [pH 7.2] and re-centrifuged. This step was repeated once more in order to remove the excess of fixative from the cells. Cells were resuspended in 25 μL of cryoprotectant (20% [w/v] dextran (39,000 mw) prepared in serum free medium). The dextran-protected cell suspension was loaded into 6 mm aluminum Type A planchette (Leica Microsystems, Buffalo Groove, IL), which was capped using the flat side of a 6 mm aluminum Type B planchette. The cell-planchette assembly was frozen immediately using a high pressure freezing system (HPM 100, Leica Microsystems, Buffalo Grove, IL). After freezing, cells were kept under liquid nitrogen and transferred to cryotubes containing 20% epoxy in dry acetone. The epoxy solution was adapted from Matsko and Mueller (2005). The 100% stock solution of epoxy was composed of 49% epon/araldite stock (Electron Microscopy Sciences), 49% DDSA (Electron Microscopy Sciences) and 2% DMP-30 (Electron Microscopy Sciences). The epon/araldite stock was composed of 57% Araldite stock (47.6% Araldite 502 and 52.4% DDSA) and 43% epon 812. During the course of the protocol standardization, epon 812 was replaced with Embed 812 (Electron Microscopy Sciences), a less toxic and less viscous substitute of epon 812. Although a slightly better preservation was achieved with epon 812, Embed 812 resulted in essentially the same morphology and number of nucleocapsids compared with epon 812. The change was necessary as epon 812 is currently unavailable. The 100% stock solution was then diluted to 20% in dry acetone (Acros Organics) and kept in liquid nitrogen.

2.4. Freeze substitution

The cryotubes containing the frozen cells and the fixative were transferred to a freeze substitution unit (EM AFSII, Leica Microsystems, Buffalo Grove, IL). Freeze substitution was performed using the following program: −90 °C for 12–48 h followed by slow warming from −90 °C to −70 °C for 16 h, from −70 °C to −45 °C for 12 h, from −45 °C to −20 °C for 2 h, from −20 °C to 4 °C for 2 h. After removing the cells from the freeze substitution unit, samples were kept at 4 °C (on ice). Cells were separated from the planchettes and washed 3 times for 10 min with ice-cold 100% dry acetone. After the washes, cells were incubated in ice-cold 1% tannic acid in dry acetone for 1 h. Cells were then washed 3 times for 10 min with ice-cold 100% dry acetone and incubated

for 1 h in ice-cold 1% osmium tetroxide in 100% dry acetone. The incubations with tannic acid and osmium tetroxide were required for better viral envelope preservation and contrast, which was not achieved with only 20% epoxy. We strongly recommend the use of dry acetone bottles that have not been opened too many times. Acetone is highly hygroscopic and the use of dry acetone that has been exposed to humidity results in blackening of the osmium solution. This in turn, results in cells that are jet black and virus particles that, although contain the nucleocapsid, present an inverted contrast in relation to the surrounding cytoplasm (the cytoplasm appears dark, while the particles are light, almost white). We achieved better results by buying the dry acetone in 100 mL bottles (ACROS organics). Cells were washed 3 times of 10 min with ice-cold 100% dry acetone and infiltrated with Embed or Spur's resin.

2.5. Resin infiltration

Samples were incubated in increasing concentrations of Embed or Spur's resin diluted in acetone (30%, 50%, 70%, 3 incubations with 100%). The last incubation in 100% resin was done over a period of 16–20 h at room temperature, with constant rocking. Infiltration was done using a Pelco BioWave laboratory microwave, ColdSpot and EM Pro Vacuum chamber (Ted Pella, Redding, CA), with the setting of 20 Hg vacuum, 220 W, 3 min, followed by a 5 min incubation at room temperature. Specimens were polymerized over a period of 2 days at 60 °C. Ultrathin sections (70–80 nm) were post-stained with 2% uranyl acetate for 2 min and lead citrate for 1 min and examined with an H-7000 TEM (Hitachi High Technologies America, Inc. Schaumburg, IL) operated at 100 kV. Digital images were acquired with a Veleta camera and iTEM software (Olympus Soft-Imaging Solutions Corp, Lakewood, CO).

3. Results and discussion

3.1. Epoxy as fixative

The interactions between biological material and different fixatives in a solvent environment are still mostly unknown. Therefore, choosing a suitable fixative for freeze substitution is mostly empirical. After testing a wide array of fixatives (Table 1 and Fig. 1), we concluded that the use of 20% epoxy in acetone resulted in the preservation of the highest number of viral particles containing the nucleocapsid (Fig. 2A and B). The quantification of ten independent preparations showed a 40–60% of viral particles containing the nucleocapsid, with an average of 50.8%, which is much higher than the 4–15% observed in the earlier study by McFadden and colleagues (2012).

Earlier observations of the nucleocapsid show that there are three possible views of the structure, depending on which plane the viral particles are sectioned. In our previous work, we designated these different views as transverse (reveals a linear arrangement of the three disk shaped structures), sagittal (reveals the length of one segment of the nucleocapsid) and coronal (reveals the folded structure of the nucleocapsid) (Jesus et al., 2014; McFadden et al., 2012; Peters and Muller, 1963). Our preparations recapitulate these earlier observations (Figs. 2 and 3).

It is unknown why epoxy resin is a good fixative for preserving the nucleocapsid. However, previous studies have suggested that the interactions of different amino acids with both epoxy and anhydride groups present in the resin are responsible for efficient preservation of proteins (Matsko and Mueller, 2005; Mittal and Matsko, 2012). In addition, epoxy also interacts with nucleic acids (Causton, 1985). This explanation is in agreement with the proposed composition of vaccinia nucleocapsid, namely proteins and DNA, and might explain why 20% epoxy resin demonstrated superior preservation of the nucleocapsid compared to glutaraldehyde, generally known for its protein cross-linking properties (Fig. 1C, D and E) (Sung et al., 1996).

The efficient preservation of proteins achieved with epoxy, including membrane proteins, precludes the staining of these membranes by uranyl acetate and lead. Therefore, even though we could visualize the nucleocapsid in several viral particles, the virus membrane contrast was not optimal in these preparations (Fig. 2A and B). To overcome this issue, we initially added osmium tetroxide to the cocktail containing epoxy. Even though the nucleocapsid was present, the viral particles substructures were poorly defined and not clearly apparent (data not shown). The use of

Table 1
Different types of fixatives tested in the present study.

OsO_4 based cocktails
1% OsO_4
1% OsO_4 + 0.1% UA
1% OsO_4 + 0.1% UA + 5% H_2O
2% OsO_4
5% OsO_4
5% OsO_4 + 5% water
Glutaraldehyde based cocktails
2% GA
2% GA; wash with acetone; 1% OsO_4 + 0.1% UA
2% GA + 1% OsO_4 + 0.1% UA
2% GA + 0.1% tannic acid
0.1% GA + 0.1% UA
Epoxy based cocktails
20% epoxy resin
20% epoxy resin + 0.1% UA
20% epoxy + 0.1% tannic acid
20% epoxy resin + 0.5% OsO_4
20% epoxy resin + 1% OsO_4
20% epoxy resin + 2% OsO_4
20% epoxy resin + 2% OsO_4 + 5% water
20% epoxy resin + 0.1% K^+ permanganate
1% OsO_4; wash with acetone; 20% epoxy resin[a]
20% epoxy resin, wash with acetone; 1% OsO_4[a]

[a] The first fixative cocktail was changed 24 h after freeze substitution started. At −90 °C, the cells were washed 3 times of 10 min with 100% acetone. The cells were then resuspended in the second cocktail and incubated for an additional 48 h at −90 °C before increasing the temperature according to the program described in Section 2.

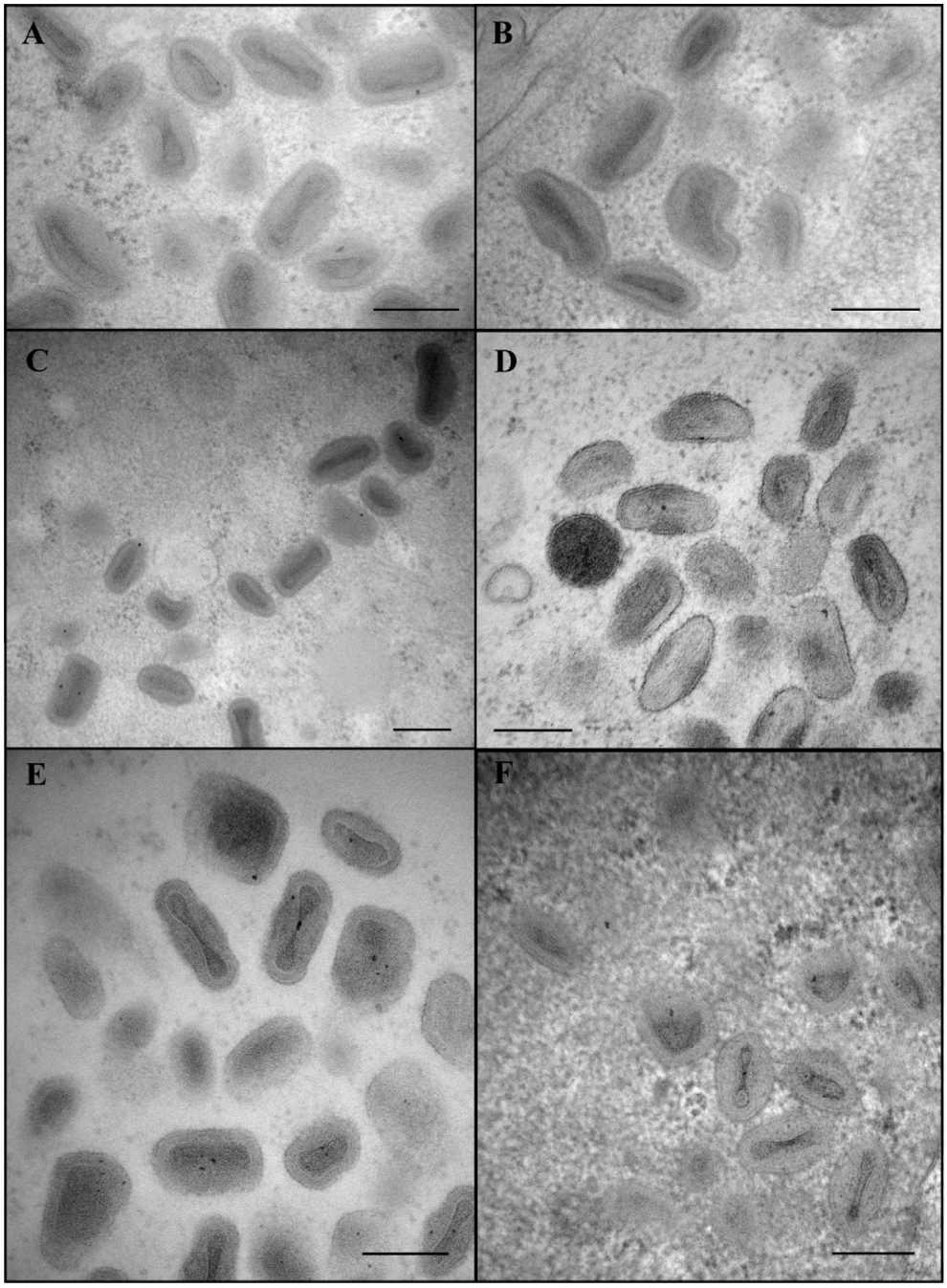

Fig. 1. Cells were infected with WR at an MOI of 10 and incubated at 37 °C for 24 h. Infected cells were processed for high pressure freezing and freeze substitution, as described in Section 2. These are representative images of selected fixatives tested: (A) 1% OsO_4, (B) 2% OsO_4, (C) 2% GA + 1% OsO_4 + 0.1% UA, (D) 0.1% GA + 0.1% UA, (E) 2% GA and (F) 5% OsO_4. Scale bars = 250 nm.

osmium tetroxide after the freeze substitution proved more effective. In addition, pairing osmium tetroxide with tannic acid, which functions as a mordant for the osmium, helped even further, resulting in both better membrane contrast and better contrast overall (Fig. 2C and D) (Jiménez et al., 2009). This allowed us to visualize the different viral substructures (membrane, core wall and nucleocapsid) more easily (Fig. 3).

One remarkable characteristic of vaccinia virus structure is the resistance of the viral core wall to disruption by chemical agents, such as detergents and reducing agents (Moussatche and Condit, 2015). This resistance might also theoretically preclude fixatives from entering the virus core in a timely manner, resulting in loss of the nucleocapsid architecture during the preparation process. However, freezing the viral particles likely stabilizes the substructures, allowing more time for the fixative to penetrate the core wall and reach the nucleocapsid. In addition, the nucleocapsid structure may be intrinsically labile and not easily preserved with any type of fixative. We have developed a new protocol that is able to preserve the vaccinia virus nucleocapsid. The technique helped to illuminate the general structure of the viral particle and would be interesting to use with other viruses that are also difficult to preserve. Using this protocol with different viruses might uncover previously undetected features of viral particles.

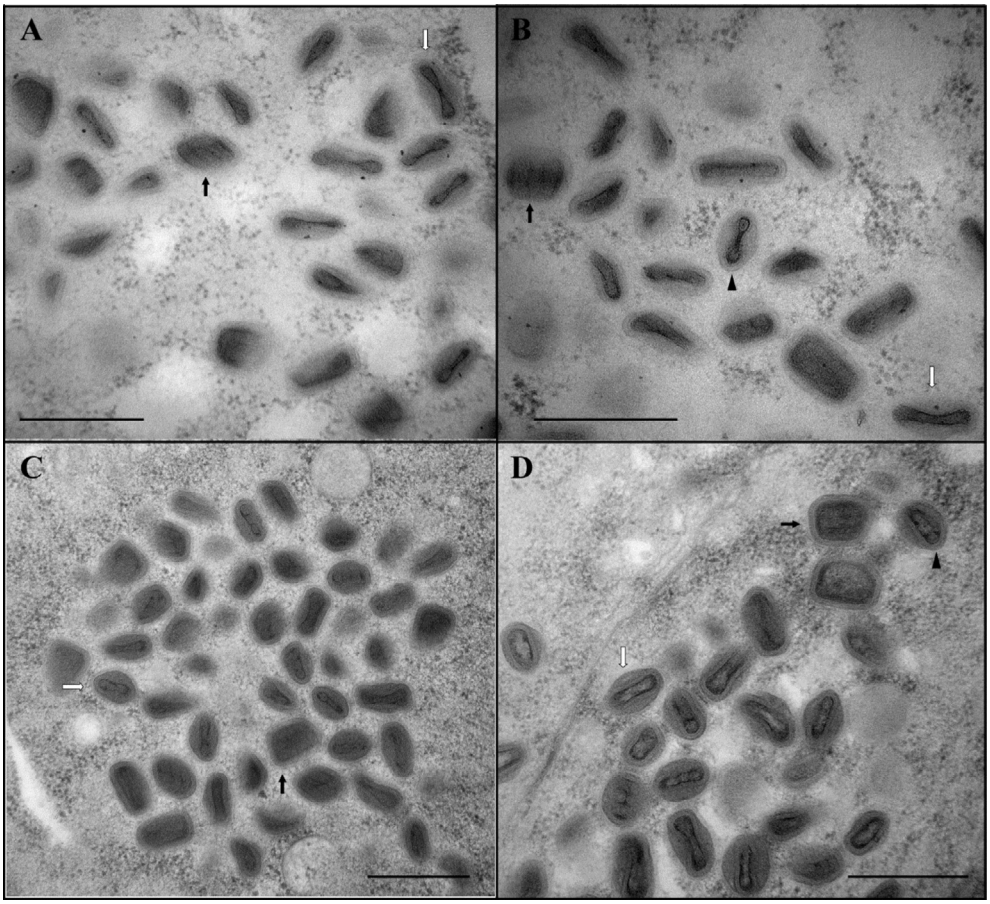

Fig. 2. Cells were infected with WR at an MOI of 10 and incubated at 37 °C for 24 h. Infected cells were processed for high pressure freezing and freeze substitution, as described in Section 2. (A) and (B) The cocktail used for freeze substitution was 20% epoxy in acetone. No further treatment was done before embedding. (C) and (D) The cocktail used for freeze substitution was 20% epoxy in acetone. After freeze substitution, cells were incubated with 1% tannic acid in acetone, followed by incubation with 1% osmium tetroxide and embedding. The different arrows point to different views of the nucleocapsid (open arrow: sagittal plane; closed arrow: coronal plane; arrowhead: transverse plane). Scale bars = 500 nm.

3.2. Application of the technique

Electron microscopy has been largely used to understand the relationship between the ultrastructure and the functionality of organelles or protein complexes. A virus' structure is essential for success of the virus in the environment. Specific substructures allow viruses to bind to receptors on the host cell surface, to transfer their genetic material to the cell interior and to replicate their genomes. Investigating virus substructures is essential for understanding how these complex machines interact with the host cell.

One vaccinia virus substructure that has been linked to functionality is the virus core. The analysis of several vaccinia mutants suggests that an intact virus core is essential for virus transcription. Since the transcription enzymes are likely packaged in the nucleocapsid (McFadden et al., 2012), we hypothesized that the nucleocapsid structure is essential for the success of vaccinia virus early transcription. Studying the nucleocapsid structure might therefore provide insight into how the transcription of a 200 kbp genome occurs in the confinement of the virus core.

In this study, we used the method we formulated to analyze several transcription-defective vaccinia mutants in order to determine if the nucleocapsid was present in the mutant virus particles. Even though these mutants have been previously characterized, the analysis of the virus structure was done using conventional fixation and dehydration protocols; therefore, the nucleocapsid was not observed. We hypothesized that transcription-defective mutants would have a defective nucleocapsid, which could explain the block in transcription.

We analyzed thermo sensitive mutants in the genes A3L (Cts8), I8R (Cts18), E6R (Cts52) and E8R (Dts23) (Boyd et al., 2010; Gross and Shuman, 1996; Kato et al., 2007, 2004). Unfortunately, for Cts18, Cts52 and Dts23 we were not able to gather relevant data about the nucleocapsid structure. In all these cases, the percentage of viral particles containing the nucleocapsid was low even in the wild type control infections incubated at 39.5 °C. This precluded analysis of the presence of the nucleocapsid in the mutants grown under conditions where we could observe the mutant phenotype (39.5 °C). Although disappointing, these results are not surprising as, in our experience, vaccinia virus growth can be suboptimal when infections are done at high temperature. In addition, purified particles grown at 39.5 °C present a higher total particle/infectious particle ratio when compared to viruses grown at 31 or 37 °C. These observations indicate that some of the viral particles formed at high temperature are not infectious and might not indeed have nucleocapsids. Alternatively, the nucleocapsid of these particles may be more labile than normal because they were formed under conditions where proteins lose stability overall. Therefore, the use of thermosensitive mutants imposes a limitation in cases where the difference in phenotype between the wild type and the mutant is subtle. In these cases, drug inducible mutants would be of better value. Infections with inducible mutants are done at 37 °C, the temperature at which virus growth is optimal and the nucleocap-

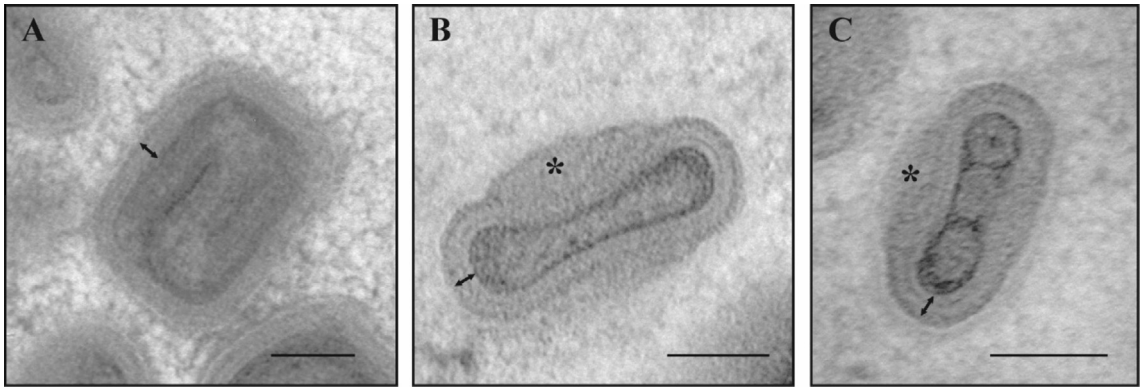

Fig. 3. Enlarged wild type viral particles showing details of the three different sectioning planes of the nucleocapsid. (A) Coronal section. (B) Sagittal section. (C) Transverse section. The double-sided arrow marks the core wall. The asterisk marks the lateral bodies. Scale bars = 100 nm.

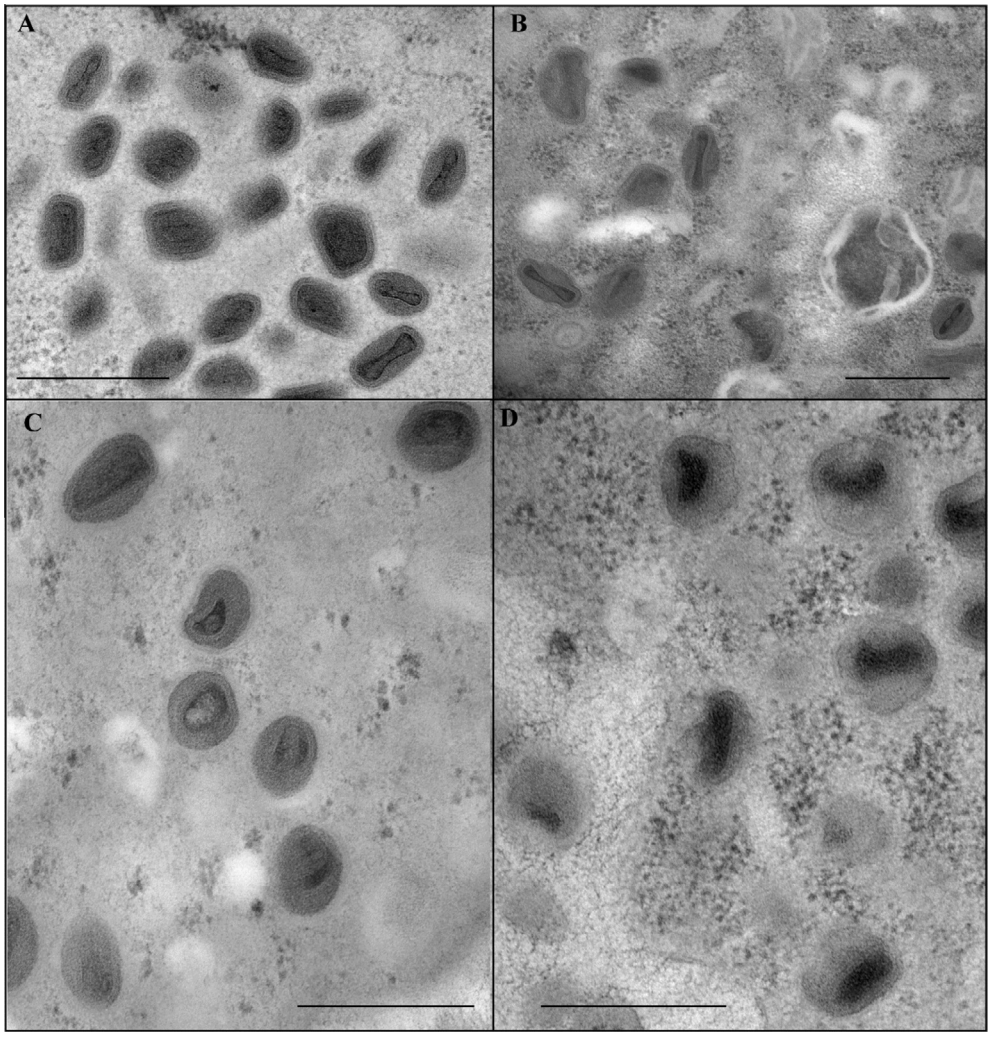

Fig. 4. Cells were infected at an MOI of 10 with WR or Cts8 and processed for high pressure freezing and freeze substitution. (A) WR at 31 °C. (B) WR at 39.5 °C. (C) Cts8 at 31 °C. (D) Cts8 at 39.5 °C. Scale bars = 500 nm.

sid is well preserved using the current protocol. On the other hand, when there is enough structural difference between the thermosensitive mutant and the wild type, our protocol can lead to interesting discoveries about vaccinia biology, as shown previously as well as in the present study, below (Jesus et al., 2014).

One of the viruses we analyzed was Cts8, a temperature sensitive mutant in the vaccinia A3 protein (Kato et al., 2004). A3 is an abundant structural protein and is part of the inner layer of the core wall (Moussatche and Condit, 2015). Cts8 presents an aberrant core structure when infection is done at high temperature

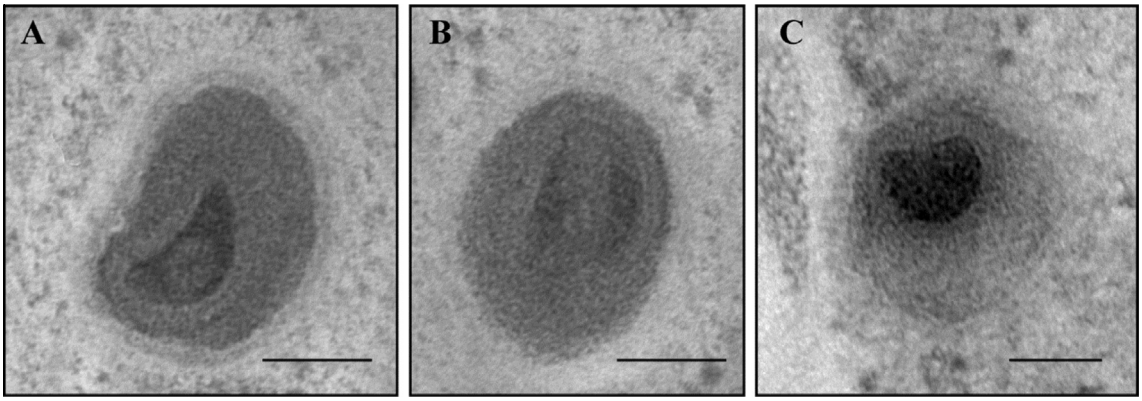

Fig. 5. Enlarged viral particles showing details of the nucleocapsid of Cts8 viral particles produced at 31 °C (A) and (B) and at 39.5 °C (C). (A) and (C) Transverse section. (B) Sagittal section. Scale bars = 100 nm.

(39.5 °C) and the viral particles are defective in virus transcription, therefore these virus particles are non-infectious. After an exhaustive analysis of Cts8 infections done at the non-permissive temperature using high pressure freezing/freeze substitution, we failed to observe any particles that contained a nucleocapsid (Fig. 4D), while in wild type infections done at 39.5 °C the nucleocapsid is present in some particles, even though the percentage was lower than in the wild type grown at 31 °C (Fig. 4B). These observations reinforce the hypothesis that the nucleocapsid structure correlates with viral transcription (McFadden et al., 2012). Besides the absence of the nucleocapsid, the overall structure of the mutant viral particles produced at 39.5 °C is disrupted. Instead of the normal brick-shape form, most mutant viral particles produced at high temperature are spherical with a non-biconcave shaped virus core dislocated to the particle periphery. In addition, the core wall is absent in viral particles formed under non-permissive conditions, consistent with the fact that A3 is one of the major core wall structural proteins (Figs. 4D and 5C). Interestingly, when the mutant was grown at the permissive temperature, the viral particles did not contain a nucleocapsid structure identical to the wild type virus (Figs. 4C, 5A and B). In the wild type virus, the transverse section of the nucleocapsid presents three circles, while in the coronal orientation three horizontal tubes are observed (Figs. 2 and 3). These three circles and three horizontal tubes are not observed in the Cts8 particles formed at the permissive temperature. Instead, only one of these circles/horizontal tubes is seen (Fig. 5A and B). It is important to emphasize that while the Cts8 virions produced at 31 °C differ in appearance from wild type virus they are nevertheless dramatically different in appearance from Cts8 particles produced at 39.5 °C. Specifically, under permissive conditions the particles retain structures resembling a core wall and nucleocapsid, while these structures are absent from Cts8 particles produced under non-permissive conditions (Fig. 5).

Nevertheless we are faced with an apparent conundrum: we hypothesize, based on previous experiments, that an intact nucleocapsid is required for virion transcription and yet the Cts8 virions produced under permissive temperatures display significant defects, in apparent contradiction of the hypothesis. We offer two solutions to this apparent conundrum. First, while the core and nucleocapsid of Cts8 particles at 31 °C differs from wild type virus, the structures that are present resemble the nucleocapsid and core wall and may retain sufficient functionality to maintain transcription and infectivity. It is important to understand that temperature sensitive mutants frequently retain some mutant character under non-permissive conditions. This appears to be the case with Cts8, and is likely be explained by the fact that the viral particles encapsidates a mutated version of A3, which might not have all the functionality of the wild type protein. Nevertheless, the mutant virus grown under permissive conditions is infectious, therefore at least some fraction of these viral particles must be transcriptionally active. Second, a caveat that must be considered in any imaging study of virus infections is that in a typical preparation of purified wild type vaccinia virus, only 1–2% of the particles are infectious, as determined by the ability to form viral plaques. Preparations of mutant particles may have an even higher particle to infectivity ratio even when grown at the permissive temperature. Therefore, the infectivity of a preparation of mutant virus under permissive conditions may represent the small fraction of virus particles that are morphologically normal. This fraction might be, in fact, too small to be reliably detected or quantified by electron microscopy in the case of virus mutants. Thus, while it is clear that Cts8 manifests a defect in virion morphology under permissive conditions, these abnormal particles need not necessarily be active to account for the infectivity of virus grown under permissive conditions. Based on our observations reported here and previous experiments, we favor the hypothesis that an intact nucleocapsid is required for faithful virion transcription, however clearly more work needs to be done to verify this hypothesis and detail the function of the nucleocapsid. Regardless of the transcriptional status of the Cts8 particles observed under permissive conditions, the complete absence of a nucleocapsid and core wall under non-permissive conditions reinforces our hypothesis, presented in previous studies, that the formation of nucleocapsid and core wall are interdependent (Jesus et al., 2015, 2014).

The analysis of two mutants of vaccinia virus (Ets85, presented in a previous study and Cts8, present work) using the protocol designed by our lab led to interesting findings about vaccinia biology: interdependence of core wall and nucleocapsid formation and a possible association between A3 (core wall) and L4 (nucleocapsid). The hypothesis states that the nucleocapsid structure is required for viral transcription and could be important for spatially organizing viral transcription, which occurs in the confinement of the virus core. However, the analysis of other mutants will be necessary for drawing mechanistic conclusions about the nucleocapsid function during vaccinia virus transcription.

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