Modifications of the Nuclear Envelope of BHK Cells After Infection with Herpes Simplex Virus Type 1

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SUMMARY

Numerous discrete lesions, which we have termed blebs, appeared in the nucleus of BHK cells 10 to 15 h after infection with herpes simplex virus type 1 (HSV-1). They were formed in the inner portion of the nuclear envelope by the apposition of two thickened lamellae overlying a vacuole. As demonstrated by electron microscopic studies, blebs were regular and associated with the peripheral lamina in the nucleus, averaging 3.5 blebs per μm². They appeared to be associated with an enrichment of the 155K major capsid protein in the nuclear membrane subfractions as compared with the protein composition of nuclei and plasma membrane fractions. We propose that blebs represent the site of assembly of capsid proteins before DNA insertion and eventual envelopment.

INTRODUCTION

The morphological aspects of herpes simplex virus (HSV) replication in permissive cells have been described previously (Morgan et al., 1959; Nii et al., 1968; Schwartz & Roizman, 1969; Schaffer et al., 1974). The virus nucleocapsids are assembled in the nucleus and they seem to acquire their envelope mostly by budding through the nuclear envelope (Darlington & Moss, 1969; Nii et al., 1968). The HSV envelope contains five glycoproteins located outside the lipid bilayer (Keller et al., 1970; Spear et al., 1970; Spear, 1976; Baucke & Spear, 1979), and 15 non-glycosylated virus proteins probably located between the capsid and the lipid layer and forming an amorphous material called tegument (Roizman & Furlong, 1974).

Since the virion capsid and envelope proteins are predominantly virus-specified (Spear & Roizman, 1972), virus products must in some way influence the composition and organization of the cell nuclear envelope where specific recognition and association occur between the various virion molecules. This interaction may only be temporary and serve to align virus components at the budding sites. One can postulate a three-step process in herpes virogenesis: (i) synthesis and assembly of virion proteins at the nuclear envelope level; (ii) exclusion of host proteins from these sites; (iii) correct association of the nucleocapsid and envelopment beneath those areas of the nuclear membrane.

Morphological studies have shown that nuclear membranes of infected cells undergo considerable rearrangements characterized by proliferation, duplication and fusion, thus resulting in the formation of concentric lamellae that may project inwards into the nucleus or outwards into the cytoplasm. Previous reports from this laboratory have described the sequence of events from discrete to complex lesions in correlation with virus DNA synthesis (Bibor-Hardy & Simard, 1980). Indeed, the presence of both α-DNA polymerase and herpesvirus-specific DNA polymerase in the inner nuclear membrane fraction gives credence to the hypothesis for an involvement of the nuclear envelope in virus DNA replication (Herzberg et al., 1981). However, there is also a possibility that the discrete lesions found in the nuclear envelope could be related to the encapsidation process and/or the envelopment process and therefore be mostly composed of virus-specified proteins.
We have studied in parallel the ultrastructural modifications and the biochemical protein composition of the nuclear envelope during infection with HSV-1. In the present paper, we report that the nuclear envelope lesions are associated with an enrichment in major capsid protein and virus glycoproteins within nuclear membrane subfractions as compared to total nucleus.

METHODS

Cell culture and labelling. BHK cells were grown at 37 °C in α-medium (Gibco) supplemented with 10% calf serum (Flow Laboratories, mycoplasma-free). A temperature-sensitive mutant of HSV-1, tsG8 strain KOS (from Dr P. Schaffer, Harvard Medical School and Sidney Farber Cancer Institute, Boston, Mass., U.S.A.), was propagated and assayed on BHK cells. Confluent monolayers of BHK cells were infected with 2 or 5 p.f.u./cell and incubated at 37 °C for 45 min. After infection, the cells were incubated at the permissive temperature of 31 °C for 15 h in α-medium containing 2% foetal calf serum and 1% kanamycin.

Virus-induced proteins were labelled with radioactive methionine by incubating the cells from 4 to 15 h post-infection with 20 to 30 µCi/ml [35S]-methionine (1000 Ci/mmol, Amersham) in α-medium containing half the usual concentration of methionine and 2% foetal calf serum. Glycoproteins were labelled by incubating the cells from 4 to 15 h after infection with 20 µCi/ml [14C]glucosamine (200 mCi/mm, Amersham) in α-medium containing 2% dialysed foetal calf serum.

Electron microscopy. Cells or nuclear membrane preparations were fixed on ice for 30 min with 2.5% glutaraldehyde in 0.1 M-cacodylate buffer pH 7.2, and post-fixed with 2% osmium tetroxide buffered with 0.1 M-cacodylate for 30 min. Specimens were dehydrated in ethanol and embedded in Epon 812. Cut sections of approx. 70 nm in thickness (gold range) were further stained with uranyl acetate and lead citrate.

For cytochemistry, cells fixed in glutaraldehyde only were embedded in glycol methacrylate (GMA). Thin sections were either digested before staining with 0.5% pepsin in 1 M-HCl pH 1.5 for 5 min followed by another digestion with 0.1% ribonuclease in distilled water pH 6.8 during 1 h at 37 °C, again before staining (Leduc & Bernhard, 1967), or were processed directly for preferential staining of ribonucleoproteins according to Bernhard (1969).

Preparation of nuclei and nuclear membranes. The cells were scraped off with a rubber policeman in phosphate-buffered saline, centrifuged and resuspended in 1 ml RSB buffer (0.01 M-Tris-HCl pH 7-7, 0.01 M-KCl, 0.015 M-MgCl2, 0.4 mm-CaCl2, 0.001 M-dithiothreitol). After addition of 2 vol. RSB buffer diluted three times, the cells were allowed to swell for 10 min on ice and were disrupted with 10 to 25 strokes in a loose-fitting Potter homogenizer. Nuclei were pelleted at 400 g for 2.5 min and washed three times with 15 ml ice-cold RSB buffer to discard most of the cytoplasmic contaminants. The crude nuclear pellet was resuspended in 1.8 M-sucrose, RSB buffer and centrifuged at 50000 g for 60 min. The purified nuclei, which formed a pellet at the bottom of the tube were then resuspended in 1 ml TE buffer (0.02 M-Tris-HCl pH 8-6, 0.02 M-EDTA).

Nuclear membranes were purified by two methods. In method A, a nuclear membrane-chromatin complex was obtained according to the method of Infante et al. (1976). Purified nuclei were disrupted by three rapid freeze-thawings and a 10 s sonication. Nuclear lysate (0.5 ml) was layered on to a preformed CsCl gradient (2-2 ml 2 M-CsCl in TE buffer, 2-2 ml 6 M-CsCl in TE buffer). The tubes were inverted twice slowly to form the gradient and then centrifuged in an SW50.1 rotor at 35000 rev/min for 19 h at 15 °C (Bibor-Hardy & Simard, 1980). Further purification was done by solubilization of the chromatin with heparin (Bornens, 1977): nuclear membranes were resuspended in 100 µl 0.1 M-phosphate buffer pH 7.4, and 900 µl of heparin (10000 USP units/ml) were added. After 40 min incubation on ice, nuclear membranes were washed three times in 15 ml TE buffer.

In method B, nuclei were purified essentially as already described but using a 0.32 M-sucrose buffer pH 7.4, 3 mM-MgCl2 instead of RSB and TE buffers. Nuclear membranes were then isolated according to the method of Kay & Johnston (1977) for rat liver nuclear envelopes. The purified nuclei were washed in ice-cold 0.25 M-sucrose buffer pH 7.4, 1 mM-MgCl2 and lysed in 4 vol. 8 mM-Tris-HCl pH 8-5, 0.1 mM-MgCl2, 4 mM-β-mercaptoethanol, 8% (w/v) sucrose with 5 µg/ml DNase I (Boehringer-Mannheim) for 15 min at 22 °C. Digestion was terminated by the addition of an equal volume of ice-cold water. The lysate was centrifuged at 38000 g for 15 min at 4 °C. A second digestion was carried out in 5 vol. 10 mM-Tris-HCl pH 7.4, 0.1 mM-MgCl2, 5 mM-β-mercaptoethanol, 10% (w/v) sucrose with 1 µg/ml DNase I for 20 min at 22 °C. Digestion was terminated and lysate centrifuged as described previously. Nuclear membranes were further purified by an isopycnic centrifugation on a sucrose density gradient.

Purification of plasma membrane. Plasma membranes were isolated according to the method of Harshman & Conlin (1978). The supernatants obtained after the homogenization of the cells were centrifuged at 100000 g. The pellets were resuspended in 2 ml TE buffer, layered on a stepwise gradient (4 ml 10% sucrose; 13% dextran T-500, 3 ml 25% sucrose, 3 ml 64% sucrose) and centrifuged in an SW41 rotor at 35000 rev/min for 60 min at 4 °C. Plasma membranes were collected just below the interface with the dextran T-500 fraction.
Preparation of nuclear matrix. BHK nuclear matrices were isolated according to the procedure of Pardoll et al. (1980), slightly modified by Bibor-Hardy et al. (1982). Purified nuclei from infected BHK cells were incubated in 10 vol. low salt buffer (10 mM-Tris-HCl pH 7.4, 0.2 mM-MgCl₂) for 10 min on ice and centrifuged at 300 g for 3 min. After a second low salt extraction, the pellet was carefully resuspended in the same volume of low-salt buffer and DNase I (Worthington) was added at a final concentration of 20 μg/ml, digestion was carried out for 10 min at 22 °C. After centrifugation at 300 g for 3 min, the pellet was resuspended in 5 vol. low-salt buffer, brought to a final concentration of 2 M by slow addition of 4 M-NaCl, incubated for 10 min on ice and centrifuged. After a second treatment of 10 min in high-salt buffer, the nuclear matrices were centrifuged at 1000 g for 5 min and washed once in high-salt buffer and once in 10 mM-Tris-HCl buffer. 1 mM-α-phenylmethylsulphonyl fluoride was present in all extraction buffers.

Protein analysis. Pellets of cells, nuclei or nuclear membranes were dissolved in sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 1.5 mm-thick slab gel with a 7 to 14% gradient separating gel and a 4% stacking gel (Marsden et al., 1976). Gels were either stained with Coomassie Brilliant Blue R-250 or fluorographed on X-Omat RP-X-ray film at -70 °C. The HSV-induced polypeptides were denominated according to mol. wt. markers (from Bio-Rad and Pharmacia) ranging from 200K (myosin) to 14.4K (α-lactalbumin).

RESULTS

Changes in the nuclear envelope morphology

The earliest lesions observed in the nuclear envelope of BHK cells infected with HSV-1 are shown as discrete areas (blebs) where the internal nuclear membrane is duplicated to two closely associated and thickened lamellae overlying a vacuole (Fig. 1 a) (Bibor-Hardy & Simard, 1980). Blebs were seen 8 to 10 h after infection and therefore preceded the more important rearrangements of the nuclear envelope which are late events; 15 h after infection, they were numerous in most infected cells. In glutaraldehyde-fixed and GMA-embedded infected cells, the two thickened lamellae and the vacuolar material appear heavily contrasted, whereas the nuclear membranes appear in negative contrast (Fig. 1 b). The preferential stain for ribonucleoproteins (RNP) is consistently positive for the lamellae and occasionally positive for the vacuole (Fig. 1 c). We have isolated a nuclear matrix subfraction in BHK-infected cells where only structures resistant to high salt, detergents and nuclease extraction are preserved; these structures consist of pore–lamina complexes, residual nucleoli and a fibrogranular interconnecting network. A large number of virus particles with a core of low electron density remained attached to the fibrogranular network (Fig. 1 d). Although the lipid bilayers of the nuclear membranes were extracted, the two thickened lamellae of the bleb are still visible and associated with the peripheral lamina (Fig. 1 d) of the nuclear matrix.

Serial sections were made in order to determine the structure, size and relative number of the blebs; they can be described as roughly circular, similar to one another, with a diam. averaging 230 nm. By comparison, the size of the HSV capsid is approx. 100 nm and that of the enveloped virion 130 to 140 nm. It should be noted that the length of the two thickened lamellae corresponds to the circumference of the capsid (300 to 315 nm). Using serial sections, we calculated that in Fig. 2 there could be as many as 3.5 blebs per μm² and a total of about 400 per nucleus; however, these figures can vary considerably from one cell to another.

Purification of the nuclear membrane

In order to determine whether one or more virus polypeptides could be involved in the formation of blebs, we purified nuclear membranes according to two methods, one involving a CsCl gradient separation and treatment with heparin to remove chromatin (method A) and the other involving digestion with DNase and isopycnic centrifugation on a sucrose gradient (method B). Both methods provided fairly pure preparations of nuclear membranes (Fig. 3a, b), although it is clear that the pore–lamina complexes are also present in the preparations and that 'nuclear envelope' would be a more suitable term to qualify these subfractions. There is always some contamination with virus particles that remain trapped in the preparation during isolation procedures but this is unavoidable. Blebs can be recognized in the membrane preparation (method B) of infected cells: the two lamellae are preserved but the vacuole is empty (Fig. 3c).

To further assess the purity of nuclear membrane preparations, we compared uninfected
Fig. 1. BHK cells infected with HSV-1 for 10 h. In (a), the discrete lesion, which we have termed a bleb (arrows in a to d), is seen to consist of two thickened lamellae overlying a vacuole. In GMA-embedded cells (b) where membranes appear in negative contrast, the two lamellae and the vacuole are heavily contrasted. (c) Preferential RNP staining: the chromatin is bleached but the lamellae are contrasted together with a virus particle. (d) Nuclear matrix preparation: the lamellae remain associated with the peripheral lamina of the nuclear matrix. Bar marker represents 0.5 μm in (a) to (d).
Fig. 2. Serial sections of BHK cells infected with HSV-1 for 15 h. Four consecutive sections were mounted on top of one another by increasing the magnification consecutively by 10%. At least 11 different blebs (arrows) can be counted on these sections and only one is associated with a virus particle. Bar marker in (a) represents 1 μm and this measurement should be increased by 10%, 20% and 30% respectively, for (b), (c) and (d).
Fig. 3. Nuclear envelope preparations of BHK cells infected with HSV-1 for 10 h according to method A (a) and method B (b). Blebs can be identified in the membrane preparation (method B) of infected cells (c). Bar markers represent 1 μm in (a) to (c).

nuclei and nuclear membranes for the presence of polypeptides as analysed by SDS–PAGE and stained with Coomassie Brilliant Blue. There was a definitely different pattern of migration of the polypeptide bands between the two fractions. As many as 10 bands were missing in the nuclear membrane subfraction, especially the two major ones at 57K and 33K, whereas the 52K
Fig. 4. SDS–PAGE of nuclei (a, b) and nuclear membranes (c, d) purified from normal BHK cells (C) or from cells infected with HSV-1 for 15 h (i). An equal amount of protein was put on to each lane and, after electrophoresis, polypeptides were stained by Coomassie Brilliant Blue. Dots (●) to the right of lane (a) represent major polypeptide bands of nuclei that are significantly modified in the nuclear membrane subfraction. Dots to the right of lane (c) represent either polypeptide bands that are reduced or missing (○) or enhanced (●) in the nuclear membrane fraction. Numbers indicate apparent mol. wt. (× 10^{-3}) of polypeptides estimated from their mobilities relative to marker proteins of known mol. wt. (markers not shown).
Table 1. Quantification (%) of relative concentrations for HSV-1-specified polypeptides within nuclear, nuclear membrane and plasma membrane fractions

<table>
<thead>
<tr>
<th>HSV-1 polypeptides (mol. wt. x 10^-3)</th>
<th>Nuclear fraction</th>
<th>Nuclear membrane fraction</th>
<th>Plasma membrane fraction</th>
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<tbody>
<tr>
<td>155</td>
<td>5.1</td>
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<tr>
<td>145</td>
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</tr>
<tr>
<td>136</td>
<td>13.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>127/129</td>
<td>4.9</td>
<td>10.7</td>
<td>14.9</td>
</tr>
<tr>
<td>115</td>
<td>3.4</td>
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<tr>
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<tr>
<td>30</td>
<td>1.2</td>
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Protein was strongly enhanced (Fig. 4a, c). Fifteen h after infection, all polypeptides found in uninfected preparations were still present, with additional bands probably induced by the virus (Fig. 4b, d). A new prominent 155K protein band was evident in both fractions with an enrichment factor of at least four in the nuclear membrane preparation. These results were obtained by both methods of preparation but method B proved more reproducible and was used throughout the study.

Analysis of polypeptides induced by HSV-1 in the nuclear membrane

Virus-induced proteins pulse-labelled with [35S]methionine were analysed by SDS–PAGE, autoradiographed and compared with stained gels of infected nuclei (Fig. 5a) and nuclear membranes (Fig. 5c). Fifteen polypeptide bands were labelled in the nuclear fraction (Fig. 5b), and most of them were present in the nuclear membrane subfraction except for three at 136K, 57K and 45K (Fig. 5d). The absorbance of bands was measured with a microdensitometer and quantified by calculation of the area under each recorded peak with respect to the total surface. Table 1 shows that the relative proportion of the different proteins varied in each fraction. Polypeptides 155K, 127/129K and 115K were predominant in nuclear membranes, representing together 30% of the proteins synthesized after infection in the nuclear membrane, whereas they accounted for only 13% of the proteins in the nuclear fraction. On the other hand, the 66K protein represented 35% of labelled nuclear proteins, but only 10% of the nuclear membrane proteins.

The nuclear membrane or nuclear envelope fraction was further treated with 2 M-NaCl to remove all soluble proteins, and with 1% Triton X-100 to remove the lipid bilayer and its associated proteins. The remaining structure probably represents only the pore–lamina complex where we found, as described in Fig. 1(d), the two thickened lamellae of the bleb. Autoradiography of virus-induced proteins at the different steps of this procedure shows (Fig. 6) that most of the nuclear envelope-associated proteins were lost at the 2 M-NaCl step (Fig. 6e), except for the 155K polypeptide which was resistant to all treatments and remained clearly associated with the pore–lamina complex (Fig. 6g). A 110K polypeptide was also resistant to the high salt treatment, but part of it was extracted by Triton X-100 (Fig. 6h). If we look at other polypeptide bands with the same electrophoretic mobilities as the virus capsid proteins, we can see that the 41K and 38.5K polypeptides are lost at the 2 M-NaCl step (Fig. 6c, d); on the other hand the 51K and 32K polypeptides are associated with the pore–lamina complex (Fig. 6g) even if some are extracted by Triton X-100 (Fig. 6h).

Since the HSV envelope and HSV-infected cellular membranes contain several glycoproteins (Spear, 1976), we used [14C]glucosamine to label the proteins induced after HSV-1 infection.
Fig. 5. SDS-PAGE of nuclei (a, b) and nuclear membranes (c, d) purified from BHK cells 15 h after infection with HSV-1. The polypeptides were labelled from 4 to 15 h post-infection with [35S]-methionine, and an equal amount of the proteins was put on to each lane. Polypeptides for each fraction were detected either by Coomassie Brilliant Blue staining (1) or by autoradiography (2). Apparent mol. wt. (× 10^{-3}) is indicated.
Fig. 6. SDS-PAGE at different steps in the preparation of a pore-lamina complex. BHK cells were infected with HSV-1 and the polypeptides labelled from 4 to 15 h post-infection with [35S]methionine. Total BHK cells (a) were washed in 0.32 M sucrose with 0.2 mM-MgCl₂ (b), nuclei were purified (c) and a crude membrane pellet prepared by method B (d). This crude membrane pellet was treated with 2 M NaCl for 10 min (e) and the nuclear membrane purified on a sucrose gradient (f). The resulting nuclear membrane fraction was extracted with 1% Triton X-100 for 1 h and pelleted at 15000 g for 15 min. The supernatant (h) and the pellet (g) were analysed. Polypeptides in each fraction were detected by autoradiography.
Fig. 7. SDS–PAGE of nuclei (a, b) and nuclear membranes (c, d) purified from BHK cells 15 h after infection with HSV-1. The polypeptides were labelled from 4 to 15 h post-infection with [14C]-glucosamine, and an equal amount of protein was put on to each lane. Polypeptides for each fraction were detected either by Coomassie Brilliant Blue staining (1) or by autoradiography (2). Apparent mol. wt. (× 10^{-3}) is indicated.

The autoradiographs were again compared with stained preparations of nuclear (Fig. 7a) and nuclear membrane (Fig. 7c) proteins. Glycoproteins 127/129K and 115K were present in the nuclei of infected cells as expected (Fig. 7b), but their amount was strongly increased in the nuclear membrane subfraction (Fig. 7d).
Comparison between two membrane fractions

Nuclear and plasma membrane proteins labelled with \(^{35}\)S\text{methionine} 4 to 15 h post-infection were co-analysed on SDS–polyacrylamide gels to determine whether there was a special constituent in the nuclear membrane fraction. Approximately 13 virus-induced polypeptides were detected in the plasma membrane fraction, with a profile similar to the one obtained by Heine et al. (1972) (Fig. 8). The two major polypeptides, 127/129K and 60/66K, correspond to the VP 7, 8 and VP 17, 18 described by these authors for their plasma membranes. Except for the 145K band, the same polypeptides are also components of the nuclear membrane. However, their relative proportion has been completely modified. For example, the 155K polypeptide appeared more as a contaminant of the plasma membrane fraction whereas it is a major component of the nuclear membranes (Table 1 and Fig. 8). The amount of 115K, 94K and 87K proteins was decreased in the plasma membrane fraction but the 60/66K and 45K proteins were slightly increased as compared with the nuclear membrane fraction.

Discussion

Although the modifications of the nuclear envelope in HSV-infected cells have been described extensively, little attention has been paid to the early morphological events. To our knowledge, the presence of numerous blebs in the nuclear envelope after 10 to 15 h post-infection has not been previously reported. The blebs appear to be formed in the peripheral matrix lamina, just underneath the inner nuclear membrane, either by accumulation of new material or reorganization of existing structures. Similar images have been observed in various types of cells infected with other strains of HSV-1 or HSV-2, but never in normal cultured cells, or in cells infected with adenoviruses or simian virus 40. Only rarely are they associated with a virus particle. The fact that they are positive for Bernhard’s regressive stain came as a surprise; as already pointed out (Bernhard, 1969), the EDTA regressive stain is not specific for but rather is preferential for RNP. In this context, it is interesting to note that both capsids and cores are also positive for the regressive stain (results not shown), a situation observed for the nucleoids of adenovirus 7 (Moyne et al., 1978). The serial sections indicate unambiguously that they could not be mistaken for budding images which are in any case late events.
Our hypothesis is that these blebs are actually specific regions of the nuclear envelope where protein reorganization takes place. Since host cellular protein synthesis is shut off, one may think that these proteins are virus-specified. Because of the size of the lamellae and their staining property, one may further speculate that they represent the site of assembly of capsid proteins before insertion of virus DNA.

We have purified a nuclear membrane subfraction where these blebs can be recognized (Fig. 3d). Previous studies on membranes of virus-infected cells have been done on pure preparations of smooth membrane (Spear et al., 1970) or plasma membranes (Heine et al., 1972) in order to avoid contamination from virions, ribosomes or other cellular components. Preparations of nuclear membranes probably include some degree of contamination with virions or ribosomes but their interest lies with their reported association with several events of herpes virogenesis, including the formation of blebs. To minimize errors due to contamination, two different techniques, method A and method B, were used to purify nuclear membranes but the results from each were similar.

Infected nuclear membranes contained about 14 proteins synthesized after infection. A significant enrichment in the 155K band, the major capsid protein, was consistently obtained. This protein was not removed by an extraction with high salt or by treatment with Triton X-100. Analysis of virus proteins in the nuclear matrix fraction also revealed an enrichment in the 155K protein (Bibor-Hardy et al., 1982), and therefore it seems logical to conclude that the peripheral lamina is included in our membrane preparation. Indeed, blebs which appeared to be associated with the matrix were also present in the nuclear membrane fraction. Comparison between the plasma and nuclear membrane fractions revealed a change in the relative protein composition. In plasma membranes the 155K protein appeared more as a contaminant, whereas the 145K band was absent.

The experimental data presented in this paper are an attempt to associate structural events in herpes virogenesis with assembly of virion proteins. They suggest that capsid proteins are associated at the nuclear envelope level and can be visualized in the form of numerous blebs just underneath the inner nuclear membrane, probably in association with the nuclear matrix lamina. Although virus DNA synthesis also occurs at the periphery of the nucleus, it is not clear whether the two processes are related or whether insertion of DNA occurs at these sites. In this respect, the comparison between various classes of mutants deficient for capsid production or with other defects in morphogenesis (Atkinson et al., 1978) should prove most helpful in understanding the various steps involved in herpesvirus assembly.

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