Properties of Nucleocapsid Species Isolated from an In Vivo Herpesvirus Infection

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SUMMARY

Capsid types present in hepatocyte nuclei of Syrian hamsters infected with the animal strain of equine herpesvirus type 1 (EHV-1H) were studied and characterized. Three capsid species were isolated: L capsids (ρ = 1.23 g/ml) which are devoid of a core structure and appear to be empty shells, I capsids (ρ = 1.24 g/ml) which contain an electron-lucent, cross-shaped, immature core, and H capsids (ρ = 1.25 g/ml) which contain an electron-dense, mature core structure. All three capsids first appeared at approx. 6 h post inoculation and were present in a ratio of approx. 10:87:3 (L:I:H) at all times during infection. Analysis of the polypeptide and amino acid compositions of certain species indicated that these capsids are identical to L, I and H capsids isolated from cell cultures infected with the culture tissue strain of EHV-1. These findings support the model (O’Callaghan & Randall, 1976) that I capsids are a major precursor of mature capsids and play a major role in the maturation of this herpesvirus.

INTRODUCTION

For a number of years, we have investigated several biological and biochemical aspects of herpesvirus infection by studying equine herpesvirus type 1 (EHV-1) replication in cell culture systems and in the in vivo model of the Syrian hamster (see O’Callaghan & Randall, 1976; O’Callaghan, Allen & Randall, 1977; Allen, O’Callaghan & Randall, 1977). The features and uses of this in vivo model characterized by a fulminant, lethal hepatitis and pronounced viraemia have been reviewed (O’Callaghan et al. 1977). Purification of large quantities of extracellular virions from the tissue culture medium of infected cell cultures and from the serum of viraemic hamsters has allowed definitive analysis of the molecular anatomy of this herpesvirus. The polypeptide composition of virions of the tissue culture strain (EHV-1tc; Perdue et al. 1974; O’Callaghan & Randall, 1976) and hamster strain virus (EHV-1H; Kemp et al. 1974; O’Callaghan & Randall, 1976), as well as the amino acid composition of EHV-1H, have been reported (O’Callaghan, Rogers & Randall, 1972b).

Recently, we have isolated and characterized three major species of nucleocapsids from the nuclei of infected LM cells (Perdue et al. 1975) and have described the role of these capsids in the virus maturation process (Perdue et al. 1976; O’Callaghan & Randall, 1976; O’Callaghan et al. 1977). These capsid forms appear within the nucleus at 6 h post infection (p.i.) and differ in morphology, density, sedimentation properties, DNA content and structural protein composition. They are designated as ‘light’ (L), ‘intermediate’ (I) and ‘heavy’
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(H) on the basis of their densities in Renografin-76 gradients. H capsids are mature, core-containing capsids comprising six major proteins I, II, III, IV, IVa and V with mol. wt. of 148,000, 59,000, 46,000, 37,000, 30,000 and 18,000, respectively. I capsids comprise only five major proteins (I, II, III, IV and V) and contain an immature core that appears as an electron-lucent, cross-shaped structure. L capsids contain only four of the major proteins – I, II, IV and V – and have no internal core structure. Biochemical studies employing pulse-chase experiments to determine the fate of each capsid species and experiments analysing the time and rate of synthesis of each capsid type indicate that the I capsids serve as precursors to the mature H capsids which, in turn, are removed from the nucleus by participation in the maturation process; L capsids remain in the nucleus and appear to be defective products of virus assembly.

In this report, we show that L, I and H capsids species are present during *in vivo* EHV-I infection and can be isolated from hepatocyte nuclei of infected Syrian hamsters. In the hamster liver, I capsids are the predominant nucleocapsid species and are identical in size, ultrastructure, and polypeptide and amino acid composition to I capsids isolated from cells infected with EHV-1tc. The observation that L capsids are present in only very small amounts in the *in vivo* infection supports the hypothesis that these empty shells are defective by-products that do not play a major role in herpesvirus maturation.

**METHODS**

*Virus and in vivo model.* The hamster strain of equine herpesvirus type 1 (Kentucky A strain) was passaged in 3-week-old, male golden Syrian hamsters (*Cricetus cricetus* LUG: LUG random bred, 20 to 50 g) by inoculating 1 ml of clarified (12,000 g; 15 min) liver homogenate (20 %) intraperitoneally (Randall & Bracken, 1957; O’Callaghan, Randall & Gentry, 1972a).

*Purification of nucleocapsid species.* Nucleocapsids were isolated and purified from both isolated hepatocyte nuclei (Kemp *et al.* 1975) and from clarified homogenates of livers removed from infected hamsters at 12 h post infection, by several cycles of banding in Renografin-76 density gradients (Kemp *et al.* 1974; Perdue *et al.* 1974). The nucleocapsids obtained in Renografin density gradients were collected by centrifugation (20,000 rev/min, 30 min, JA20 rotor, J-21B centrifuge, Beckman Instruments Co., Palo Alto, California) and subjected to rate-velocity sedimentation in linear (10 to 40 %, w/w) sucrose gradients in TE buffer (0.01 M-tris-HCl pH 7.4, 0.001 M-EDTA) as described by Perdue *et al.* (1975, 1976). The gradients were fractionated mechanically in an ISCO Model D fractionator (Instrumentation Specialities Company, Lincoln, Nebraska) with continuous monitoring at 254 nm. Fractions containing each of the nucleocapsid species were pooled and the nucleocapsids were concentrated by centrifugation as described above. This procedure has been shown to quantitatively remove cellular macromolecules and to yield nucleocapsid preparations that exhibit reproducible polypeptide composition (Perdue *et al.* 1975, 1976; O’Callaghan & Randall, 1976).

*Polyacrylamide gel electrophoresis.* The procedures for preparation of SDS-discontinuous polyacrylamide gels, solubilization of nucleocapsid proteins, conditions of electrophoresis, staining and destaining of gels, and methods used for mol. wt. determinations have been reported previously (Kemp *et al.* 1974; Perdue *et al.* 1974, 1975, 1976).

*Amino acid analysis.* Purified intermediate (I) nucleocapsids were collected by centrifugation and washed in sterile distilled water. The methods for preparation of samples, acid hydrolysis in 6 N-HCl and amino acid analysis in the Beckman/Spinco Model 121 (Beckman...
Fig. 1. Electron micrographs of thin sections of nuclei isolated and purified from livers of Syrian hamsters. The isolation and purification of nuclei and the preparation and examination of thin sections are described in Methods. (a) Thin section of a representative nucleus isolated from the liver of an uninfected animal. (b) Thin section of a representative nucleus isolated from the liver of EHV-1 infected animal at 12 h p.i. Note the margination of chromatin and presence of three capsid species: L capsids appear as empty shells; I capsids are those with an immature core that appears as an electron-lucent, cross-shaped structure; H capsids are those that contain a centrally located, electron-dense core.
Table 1. Relative amounts of nucleocapsid species in hepatocyte nuclei isolated from livers of EHV-1 infected hamsters*

<table>
<thead>
<tr>
<th>Hour p.i.</th>
<th>L capsids</th>
<th>I capsids</th>
<th>H capsids</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>NP†</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>85</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>86</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>85</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>82</td>
<td>7</td>
</tr>
</tbody>
</table>

* Electron micrographs of thin sections of nuclei isolated and purified from livers of infected Syrian hamsters were prepared as shown in Fig. 1. The relative amount of each capsid species was estimated by counting sections of entire nuclei; nuclei containing thousands of capsids from at least three livers collected at each time point were scored.
† NP, Not present; nucleocapsids were not detected until 6 h p.i.

Instruments Co.) amino acid analyser have been described previously (O’Callaghan et al. 1972b; Perdue et al. 1976).

Electron microscopy. The methods for the preparation and staining of thin sections of nucleocapsid preparations and of infected cells and nuclei have been described previously (Abodeely, Lawson & Randall, 1970; Perdue et al. 1974; 1975). Preparations were examined in an RCA EMU-3G electron microscope.
RESULTS

Nucleocapsid species present in EHV-1 in vivo infection

Electron microscopic studies revealed that capsid species similar in size (approx. 100 nm in diam.) and morphology to the L, I and H nucleocapsids present in the nuclei of LM cells infected with the tissue culture strain of EHV-1, were also present in hepatocyte nuclei of Syrian hamsters infected with the in vivo strain of EHV-1. Examination of thin sections of infected livers and of isolated hepatocyte nuclei at different times during infection indicated that the three capsid species were present in relative amounts – ratio of L:I:H – different from that observed in cell culture systems (Fig. 1). The predominant capsid species in the in vivo infection was a capsid form with an immature core structure that was identical in ultrastructure to I capsids of the cell culture infection. Only small amounts of empty capsids (L capsids) and mature capsids with an electron dense core structure (H capsids) were observed in hepatocyte nuclei. Although the total amount of nucleocapsids increased during infection, the relative amounts of each capsid species remained constant (Table I). In hepatocyte nuclei isolated at all times from 6 h p.i. (the time that all three nucleocapsids first appeared) to 13 h p.i. the time of animal death, the I capsid species was the predominant species. In contrast, empty capsids (L), capsids with an immature core structure (I), and capsids with a mature core (H) comprise approx. 45, 45 and 10%, respectively, of the total capsid population in infected LM cells (Perdue et al. 1975, 1976).

To quantify the relative amounts of each species and to prepare capsids for analytical studies, capsids were isolated from hepatic tissue or purified hepatocyte nuclei of EHV-1
infected hamsters as described in Methods. Nucleocapsids purified by three cycles of isopycnic banding in Renografin-76 density gradients were separated into classes by rate-velocity sedimentation in 10 to 40 % sucrose density gradients. Three bands – one major and two minor – were visually detected, and the absorbance profile of a typical preparation isolated at 12 h p.i. is shown in Fig. 2. The major species isolated from infected hamster liver exhibited a density of 1.24 g/ml, which is identical to that reported for I capsids isolated from infected LM cell nuclei (Perdue et al. 1975), and comprised 85 to 90 % of total capsid species. Electron microscopic studies confirmed that this species was the I capsid as the overwhelming proportion of these isolated capsids exhibited an immature core structure (Fig. 3). Some minimal contamination of the I capsids with the other species was apparent.

The top and bottom minor bands comprised empty capsids (L) and capsids (H) with a densely staining core, respectively. These bands had densities of 1.23 g/ml and 1.25 g/ml, respectively, which correspond to the values reported for L and H capsids isolated from infected LM cells (Perdue et al. 1975). L and H capsids comprised only approx. 8 to 12 % and 4 to 7 %, respectively, of the total capsid population isolated in sucrose gradients.

Polypeptide composition of capsids isolated from in vivo EHV-1 infection

The purification of quantities of the two minor nucleocapsid species sufficient for polypeptide analysis proved difficult. This was especially true for the DNA-rich H capsids as they were present in small amounts, and DNA released from the capsids during the multistep purification procedure served to trap debris and make purification of this species impossible. The polypeptide composition of L and I capsids isolated from the in vivo infection and a comparison of the major capsid proteins of these capsids to those of L and I capsids isolated from LM cells are presented in Table 2. There is a remarkable similarity in the overall polypeptide composition of the capsids isolated from the in vivo and in vitro EHV-1 infections. L capsids of the hamster strain virus comprised four major proteins designated I, II, IV and V (Perdue et al. 1975, 1976) with average mol. wt. of 146 000, 58 000, 38 000 and 20 000, respectively. A trace amount of a 45 000 (III) mol. wt. protein was also present; this protein was not present in L capsids of the tissue culture virus and its presence probably reflects contamination with I capsids during collection. These findings confirm that the EHV-1 capsid shell comprises four major polypeptides.
Table 3. Amino acid composition of I capsids purified from liver nuclei of EHV-1 infected hamsters*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>Average value</th>
<th>Enveloped virions from infected hamsters</th>
<th>I capsids from LM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I capsids (hamster liver)</td>
<td>Enveloped virions from infected hamsters</td>
<td>I capsids from LM cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>3.2</td>
<td>3.0</td>
<td>3.0</td>
<td>3.1</td>
<td>3.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.6</td>
<td>2.8</td>
<td>2.6</td>
<td>2.7</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.4</td>
<td>6.6</td>
<td>6.5</td>
<td>6.5</td>
<td>6.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.5</td>
<td>10.5</td>
<td>9.9</td>
<td>10.0</td>
<td>9.1</td>
<td>10.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.0</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
<td>8.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Serine</td>
<td>6.0</td>
<td>6.2</td>
<td>6.0</td>
<td>6.0</td>
<td>7.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.1</td>
<td>11.9</td>
<td>11.4</td>
<td>11.8</td>
<td>9.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Proline</td>
<td>7.1</td>
<td>7.9</td>
<td>8.0</td>
<td>7.6</td>
<td>5.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.1</td>
<td>9.0</td>
<td>9.2</td>
<td>9.1</td>
<td>8.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.5</td>
<td>12.5</td>
<td>12.9</td>
<td>12.6</td>
<td>10.2</td>
<td>11.0</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.4</td>
<td>0.6</td>
<td>1.0</td>
<td>0.7</td>
<td>0.5</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Valine</td>
<td>8.8</td>
<td>8.6</td>
<td>8.2</td>
<td>8.5</td>
<td>8.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8</td>
<td>1.2</td>
<td>1.8</td>
<td>1.6</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.0</td>
<td>3.8</td>
<td>3.8</td>
<td>3.9</td>
<td>3.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>11.4</td>
<td>10.8</td>
<td>10.1</td>
<td>10.7</td>
<td>9.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.7</td>
<td>2.9</td>
<td>2.8</td>
<td>2.8</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.1</td>
<td>3.4</td>
<td>3.4</td>
<td>3.3</td>
<td>3.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Nucleocapsids were purified from hepatocyte nuclear extracts by three cycles of isopycnic banding in Renografin-76 density gradients followed by rate-velocity sedimentation in sucrose gradients as described in Methods.
† Time of acid hydrolysis.
‡ Taken from O'Callaghan et al. (1972b).
§ Taken from Perdue et al. (1976).
|| ND, not determined.

I capsids isolated from animals comprised these four major proteins as well as protein III which is a major component of this capsid species. This observation confirms the finding that capsid protein III is a major component of the immature core structure (Perdue et al. 1975, 1976; O'Callaghan & Randall, 1976). I capsids contained trace amounts of protein IVa (30000) which has been shown to be a major protein of the mature core structure of EHV-1 (Perdue et al. 1975, 1976). Thus, each of these two nucleocapsid species isolated from two equine herpesvirus type 1-cell systems (EHV-1H infected hamsters and EHV-1tc infected LM cells) are identical in polypeptide composition and ultrastructure.

Amino acid composition of I capsids

Recently, we reported the amino acid composition of the three capsid species isolated from EHV-1tc infected LM cell nuclei. To confirm these studies and to demonstrate similarities between I capsids of the two EHV-1 strains, the amino acid composition of purified I capsids isolated from hamster liver was determined (Table 3). The overall composition of the I capsids isolated from in vivo infection was similar to that reported for I capsids of the cell culture system. I capsid protein contained more acidic amino acid residues than basic ones; the acidic to basic ratios were 1.7 and 1.6 for I capsids of the hamster and LM cell systems, respectively, in contrast to a ratio of 1.4 for the virion. The high percentage (~ 42%) of
amino acids which are 'non-α helix formers', the significant content of proline (7.6%) which is an α helix disrupter, the few sulphur-containing residues and the relatively high percentage of non-polar residues were features that were identical in the amino acid composition of I capsids of both systems. The most significant finding was the high content of glutamic acid; this discovery, considered in light of the finding that small amounts of poly-peptide IVa (mol. wt. 30,000) are present in I capsid preparations, confirms the earlier observation that this core-associated polypeptide is rich in glutamic acid. This observation is significant because poly (glutamic acid) and highly acidic internal proteins may play a role in the collapse of virus DNA and its packaging into the capsid structure (see Perdue et al. 1976; O’Callaghan & Randall, 1976).

DISCUSSION

Our present understanding of the nucleocapsid structure of herpesviruses has come from recent studies employing herpes simplex virus (HSV) and the equine herpesviruses. Roizman and co-workers showed that two major capsid forms are present in the nucleus of HSV infected cells: 'A' capsids which are devoid of a mature core structure, contain little DNA and comprise of four major proteins (of mol. wt. 150,000, 53,000, 33,000 and 25,000) and 'B' capsids which exhibit a core structure, contain 10 times more DNA than A capsids, and comprise these four major polypeptides and an additional protein of 44,000 mol. wt. (Gibson & Roizman, 1972, 1974; Roizman & Furlong, 1974).

In EHV-1 infected LM cells, empty (L capsids) and core-containing (H) capsid species have also been isolated (Table 2), and the polypeptides of these particles have been shown to be remarkably similar to those of A and B capsids, respectively, of HSV (Perdue et al. 1975; O’Callaghan & Randall, 1976); in addition, a capsid form (I capsid) with an immature core structure has been isolated from EHV-1tc infected nuclei. Recent biochemical studies indicated that I capsids play a major role in the development of the herpesvirion and serve as precursors to the mature nucleocapsid structure. These three capsid species have recently been shown to be present in the nucleus of EHV type 3 (EHV-3) infected cells (O’Callaghan et al. 1977) and to have a polypeptide composition remarkably similar to those of EHV-1 and HSV capsid species (Allen & Bryans, 1976; O’Callaghan & Randall, 1976).

In the present report, we have continued our study of herpesvirus capsids and describe, for the first time, the capsid types present in an in vivo herpesvirus infection. Electron microscopic and biochemical studies reveal that the three major capsid species – L, I and H – present in EHV-1tc infected cells are produced in hepatocyte nuclei of Syrian hamsters infected with the animal strain (EHV-1H) of this virus. Each of the capsids are identical in ultrastructure and polypeptide composition to its counterpart in the other equine herpesvirus systems. Interestingly, I capsids are the major intranuclear capsids in the in vivo infection and account for more than 85% of the total capsid population. Since these capsids are first detected at approx. 6 h p.i. and since virus maturation within the liver is so rapid that by 9 h p.i. titres in excess of 10^10 virions/ml plasma are observed in the viraemic animal (Darlington & Randall, 1963; O’Callaghan et al. 1977), it is apparent that I capsids play a major role in the virus maturation process. The very small amounts of L capsids present in hepatic tissue also support the hypothesis, based on data from the LM cell system, that L capsids do not play a major role in the maturation of this herpesvirus (O’Callaghan & Randall, 1976; Perdue et al. 1976).

It should be noted that in all equine herpesvirus systems – both cell culture and the hamster, both EHV-1 and EHV-3 – all three nucleocapsid species are first detected as a mixed population within the nucleus; at no time during infection can nuclei containing only
Herpesvirus nucleocapsid species

one or two of the three particle types be found (Table 1). Although investigations employing electron microscopy offer a limited approach to elucidate the events of virus maturation, electron microscopic studies have shown that only H capsids are enveloped and that large numbers of H capsids are associated with the intact nuclear membrane and with strands and convolutions of nuclear membrane that appear to proliferate and invaginate into the nucleoplasm during infection (see Darlington & Moss, 1968; O'Callaghan & Randall, 1976). These observations probably explain, in part, why only small amounts of H capsids can be isolated from clarified nuclear extracts of EHV-1 infected cells.

Studies now in progress show that in LM cells infected with defective interfering (DI) particles of EHV-1tc, the synthesis of certain major structural proteins is greatly reduced within the cell, yet a significant number of virus particles is produced and released from the cell (W. W. Newcomb & D. J. O'Callaghan, unpublished observations; Henry et al. 1977). In a DI infection in which the synthesis of major capsid protein I (140,000 mol. wt.) is greatly reduced, significant amounts of I capsid but greatly reduced amounts of L capsid are assembled within the cell. The finding that virus maturation can occur under conditions in which very limited amounts of L capsid are present gives additional support for the hypothesis that these empty capsids do not serve as a major precursor of the virus particle.

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