Electron Microscopy of Herpes Simplex Virus DNA Molecules Isolated from Infected Cells by Centrifugation in CsCl Density Gradients

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SUMMARY

Herpes simplex virus (HSV) DNA molecules were isolated from infected BSC 1 cells and centrifuged in CsCl-ethidium bromide density gradients. Both newly labelled and mature virus DNA molecules were found to have a linear conformation. The morphology of virus DNA molecules at different stages of the virus growth cycle in BSC 1 cells, was studied by electron microscopy after separation of virus DNA from cellular DNA by centrifugation in CsCl gradients. In each sample, about 200 virus DNA molecules were photographed and the different morphological forms were studied. Four classes of virus DNA molecules were observed: (a) mature linear DNA molecules, $52.4 \pm 3.3 \mu m$ in length, (b) DNA molecules that contain a replicative loop or are Y-shaped, resembling replicative intermediates, (c) virus DNA molecules having one or more single-stranded filaments attached to them and (d) molecules with collapsed regions or with branches. A few circular molecules as well as linear DNA molecules longer than unit length were also observed. The virus DNA molecules resembling replicative intermediates gradually increased in number and reached a maximal amount of about 5% of the virus DNA population at 12 h after infection. The other forms of virus DNA were found to persist after the number of replicating DNA molecules decreased.

INTRODUCTION

Studies on replicative intermediates of lambda DNA (Kiger & Sinsheimer, 1969), adeno-virus DNA (Sussenbach, Ellens & Jansz, 1973) φX174 DNA (Sedat, Kelly & Sinsheimer, 1967), parvovirus DNA (Tattersall, Crawford & Shatkin, 1973) and mammalian cell DNA (Scudiero & Strauss, 1974) demonstrated that single-stranded sequences can be detected in these molecules. Chromatography of mature and replicating DNA molecules on benzoylated-naphthoylated-DEAE(BND)-cellulose columns separates non-replicating DNA molecules from the replicative intermediates (Kiger & Sinsheimer, 1969).

Shlomai, Friedmann & Becker (1976a) and Shlomai et al. (1976b) demonstrated that HSV DNA molecules isolated from infected cells by centrifugation in CsCl gradients, elute from BND-cellulose columns with caffeine. These DNA molecules have single-stranded sequences, behave kinetically as precursors to mature virion DNA and band in CsCl gradients together with mature linear HSV DNA. The transition time for replicating to mature HSV DNA molecules was found to be approx. 20 min (Shlomai et al. 1976a). This conclusion was based on the properties of the virus DNA molecules when centrifuged in

[Note: The rest of the text continues with detailed descriptions of the morphological forms of the DNA molecules.]
sucrose gradients, their partial sensitivity to an enzyme which degrades single-stranded DNA sequences and their behaviour on BND-cellulose columns. Preliminary studies on the morphology of such HSV DNA molecules revealed virus DNA molecules with a replicative loop as well as Y-shaped molecules, each having non-replicated DNA segments of varying lengths (Shlomai et al. 1976a).

The present study was undertaken (a) to analyse the relative amounts of virus DNA molecules that have replicative loops or are Y-shaped, in the population of linear DNA molecules present in the nuclei of infected cells, (b) to accurately measure the virus DNA molecules resembling replicative intermediates, (c) to calculate the position on the virus DNA molecule where replication is initiated and (d) to describe the various forms of virus DNA found in infected cells.

**METHODS**

*Isolation and purification of virus DNA by centrifugation in CsCl density gradients.* BSC 1 cell monolayers were infected with the HF strain of herpes simplex virus (HSV) type 1, in Dulbecco’s modified Eagle’s medium (DMEM). The cell monolayers (2 × 10⁶ cells per milk bottle), infected with 10 p.f.u. of virus per cell were incubated at 37°C and at different times after infection, fresh medium containing 10 μCi/ml methyl-³H-thymidine (sp. act. 16:5 Ci/mmol, Nuclear Research Centre, Negev, Israel) was added. After further incubation at 37°C for 1 h, the cells were washed twice with 10 ml vol. of cold 1 × SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate) and scraped into 1 × SSC. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% (w/v). The viscous cell lysates were treated with 0.3 mg/ml Pronase (free of nucleases, 90000 Units/mg; Calbiochem, Switzerland) for 5 to 7 h at 37°C. CsCl (E. Merck, Darmstadt, Germany) dissolved in TE buffer (0.1 M-tris/HCl, pH 7.5, 0.001 M-EDTA) was added to the DNA solutions to bring the density to 1.71 g/ml, and the solutions were carefully transferred to polyallomer centrifuge tubes (8 ml per tube) and centrifuged in an R50 Ti rotor at 35000 rev/min for 48 h, at 20°C, in the Beckman preparative ultracentrifuge. The gradients were collected dropwise from the bottom of the tube and the trichloroacetic acid (TCA) precipitable radioactivity in each fraction was determined. The buoyant densities were determined by weighing 100 μl samples in a Mettler H51 high precision balance. Virus DNA was separated from cellular DNA according to their different buoyant densities (1.718 g/ml and 1.700 g/ml, respectively).

*Centrifugation of HSV DNA in CsCl-ethidium bromide density gradients.* Samples of DNA from infected and uninfected cells were treated with SDS and Pronase as described above. The DNA was then extracted with phenol and isoamylalcohol-chloroform (1:24) and mixed with CsCl in TE buffer to give a final density of 1.54 g/ml. Ethidium bromide (300 μg/ml; Sigma Chemical Co., St Louis, Mo) was added (Pearson, 1975) and the tubes were centrifuged at 35000 rev/min for 48 h at 20°C in the 50 Ti rotor of the Beckman ultracentrifuge. After removal from the rotor, the tubes were viewed with a UVSL-25 (Mineralight) ultraviolet lamp and the fluorescent bands were marked. The gradients were collected dropwise and the density and TCA precipitable radioactivity in each fraction was determined.

*Electron microscopy of virus DNA preparations.* A modified Kleinschmidt technique (Kleinschmidt et al. 1962, 1964) was used in which 10 μl of a DNA preparation (0.05 to 0.1 μg/ml) was gently added to 40 μl of a freshly prepared solution of 0.1% (w/v) cytochrome c; 3.0 M-ammonium acetate-0.01 M-tris/HCl buffer (pH 7.2), 50% (v/v) formamide and 0.001 M-EDTA. After gentle agitation, 5 μl was withdrawn and spread on a 1 ml hypophase consisting of 0.3 M-ammonium acetate, 0.001 M-tris/HCl buffer (pH 7.2); 10% (w/v) formamide and 0.001 M-EDTA in a paraffin-coated depression slide. A carbon-coated
**RESULTS**

**Isolation and characterization of HSV DNA**

Since the aim of the present study was to characterize HSV DNA by electron microscopy, it was essential to obtain pure virus DNA free of cellular DNA. To determine whether sedimentation in CsCl gradients provides a good separation between cellular and virus DNA, BSC 1 cells were labelled with 14C-thymidine for 48 h during their growth in culture to ensure labelling of all the cellular DNA. After removal of the 14C-thymidine, the cells were combined with HSV-infected BSC 1 cells that had been labelled with 3H-thymidine for 1 h at 12 h after infection. The DNA was extracted and centrifuged in a CsCl gradient. It may be seen (Fig. 1) that 14C-thymidine-labelled cellular DNA banded only at the mean...
Fig. 2. Sedimentation of HSV DNA in CsCl-ethidium bromide density gradients. (a) BSC 1 cells in a milk bottle (4 x 10⁶ cells) were infected with HSV and labelled with 1 mCi of ³H-thymidine per culture for 40 min at 13 h p.i. (b) Another BSC 1 culture was infected with HSV and labelled with 25 μCi of ³H-thymidine for 21 h. (c) An uninfected BSC 1 culture was similarly labelled for 21 h. In (a) and (c), the DNA was extracted from the cells as described in Methods. The virion DNA (b) was isolated in a sucrose gradient and extracted with SDS and Pronase as described by Shlomai et al. (1976a). CsCl (density 1.54 g/ml) was added to the DNA preparations as well as 300 μg/ml of ethidium bromide. The preparations were centrifuged at 35,000 rev/min for 48 h at 20°C in the 50 Ti Beckman rotor. After centrifuging, the fluorescent DNA bands were marked with the aid of a u.v. lamp (d). The gradients were collected dropwise and the density and TCA precipitable radioactivity were determined.
Table 1. Electron microscopic analysis of HSV-DNA molecules isolated from infected nuclei at different stages of the virus growth cycle

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time p.i. (h)</th>
<th>Number of DNA molecules counted</th>
<th>Linear DNA molecules % (number)</th>
<th>Number of DNA molecules with a replicative loop % (number)</th>
<th>DNA molecules with attached filaments % (number)</th>
<th>Virus DNA molecules containing collapsed regions % (number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>250</td>
<td>94-0 (235)</td>
<td>0 (0)</td>
<td>4-8 (12)</td>
<td>1-2 (3)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>200</td>
<td>82-0 (164)</td>
<td>1-0 (2)</td>
<td>11-0 (22)</td>
<td>6-0 (12)</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>200</td>
<td>71-5 (143)</td>
<td>2-5 (5)</td>
<td>18-5 (37)</td>
<td>7-5 (15)</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>180</td>
<td>68-3 (123)</td>
<td>4-41 (8)</td>
<td>17-7 (32)</td>
<td>9-4 (17)</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>190</td>
<td>71-5 (136)</td>
<td>2-1 (4)</td>
<td>20-5 (39)</td>
<td>5-8 (11)</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>200</td>
<td>69-5 (139)</td>
<td>0-5 (1)</td>
<td>23-5 (47)</td>
<td>6-5 (13)</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>195</td>
<td>71-0 (142)</td>
<td>1-0 (2)</td>
<td>21-5 (43)</td>
<td>4-0 (8)</td>
</tr>
</tbody>
</table>

density of cellular DNA (1.700 g/ml) while at the density of virus DNA (1.718 g/ml) only ³H-thymidine-labelled HSV DNA was found. We assume that only virus DNA molecules were examined by electron microscopy.

The conformation of both mature virus DNA molecules and replicative intermediates which serve as templates for the synthesis of virus DNA was studied. The DNA was extracted from infected and uninfected cells and centrifuged in CsCl-ethidium bromide density gradients. Under these conditions, circular DNA molecules are denser than linear molecules (Hudson et al. 1969). Such an experiment (Fig. 2d) revealed two fluorescent DNA bands in infected cells. The upper band corresponded to cellular DNA from uninfected cells while the lower band corresponded to linear mature HSV DNA isolated from purified virions. No additional band was visible below the virus DNA band, at the position where circular DNA molecules could be expected to appear (Hudson et al. 1969; Adams & Lindahl, 1975). Analysis of infected cells labelled for 40 min at 13 h post infection (the peak of virus DNA synthesis; Becker & Asher, 1975), revealed two radioactive peaks in the gradient (Fig. 2a). The peak containing most of the radioactivity was situated in the same density region as virus DNA (Fig. 2b) while the second peak appeared in the region where cellular DNA from uninfected cells bands (Fig. 2c). A very small amount of radioactive DNA was found below the virus DNA at the expected density of circular DNA (Fig. 2a). The significance of these labelled DNA molecules is not known. Our results suggest that both mature and most of the nascent virus DNA molecules have a linear conformation. This conclusion is borne out by the electron microscopy analysis.

Analysis of HSV DNA molecules at different stages of the virus growth cycle

The molecular conformation of the virus DNA present in infected cells at different times after infection was studied. The infected cells were labelled for 60 min at 4, 6, 10 and 12 h after infection and the DNA molecules were isolated in CsCl gradients. The peak fraction in each virus DNA band (density 1.718 g/ml) was removed and prepared for electron microscopy as described in Methods.

About 200 molecules were surveyed in each preparation of virus DNA. According to their morphology, the molecules were divided into four classes (Table 1): (a) linear virus DNA molecules with an average size of 52 μm (ranging from 49 to 55 μm with a few molecules of about 60 μm), namely the virus genomes, constituted most of the HSV DNA (94%) at 4 h post infection (p.i.). At 6 h p.i., these molecules constituted 82% of the virus DNA population and subsequently dropped to about 70% from 10 h onwards; (b) DNA
molecules resembling replicative intermediates, namely DNA molecules containing a
replicative loop or having a Y-shape (Shlomai et al. 1976a), were not seen among the
molecules isolated at 4 h p.i. but the percentage of these molecules subsequently increased
reaching a maximum (4-4%) at 12 h p.i.; (c) linear (52 ± 3 μm) virus DNA molecules with
filaments were clearly seen at 4 h p.i. and constituted 4-8% of the total virus DNA popula-
tion. The percentage of virus DNA molecules showing this feature gradually increased
during the virus replicative cycle. At 6 h, 11% of the molecules had filaments attached to
them; at 10 h, 18.5% and after 14 h, about 20% or more of the virus DNA had this
feature. These virus DNA molecules have either one long filament or a number of shorter
filaments at different positions on the molecules. The nature of these DNA molecules and
their filaments is currently being investigated. The relative amounts of the replicative inter-
mediates decreased after 12 h when DNA synthesis in vivo declines (Becker & Asher, 1975);
(d) linear virus DNA species with collapsed regions in the molecule could be seen as early as
4 h p.i. The percentage of these molecules was maximal at 12 h (9.4%) and then remained
at a level of 6%.

The analysis presented in Table I provides information on the dynamic changes which
occur in the HSV DNA population at different stages of the virus developmental cycle.
It is of interest that the molecules which can be described as the replicative intermediates
of HSV DNA do not exceed 5% of the entire DNA population. This could explain the
difficulties encountered in the isolation of these molecules. Most of the HSV DNA consisted
of mature virus genomes and virus DNA molecules with filaments attached.

**Electron microscopy of HSV DNA molecules resembling replicative intermediates**

Two types of DNA molecules resembling replicative intermediates were demonstrated
in infected cells, namely, linear DNA molecules with a replicative loop (Fig. 3a) and mole-
cules which are Y-shaped (Fig. 3b). They can both be regarded as virus DNA molecules
undergoing semiconservative replication and it is most likely that the growing point on the
DNA chain is situated at the replication fork which advances along the template DNA
molecule (Dressier, 1975). About 60 DNA molecules having the features of replicative
intermediates were photographed and 19 of these molecules were accurately measured. A
detailed analysis is presented in Tables 2 and 3.

**HSV DNA molecules containing a replicative loop**

Fourteen DNA molecules, each having a replicative loop (Fig. 4a, b and c) are presented
in Table 2. Of these, seven DNA molecules have a small replicative loop with each arm
measuring from 0.18 to 0.8 μm and two molecules have a replicative loop with each arm
measuring from 2.0 to 3.0 μm. Most of the virus DNA molecules were found to have small
replicative loops with a total molecular length ranging from 6.6 to 60 μm (Table 2A).
However, two DNA molecules containing replicative loops with a total length of 45.5 μm
and 50.0 μm, respectively were also seen (Table 2B). Assuming that the initiation site for
DNA replication is in the centre of each arm of the replicative loop, we concluded that in
these nine DNA molecules, the initiation site is located about 10 μm (ranging from 8.1 to
12.09 μm) from one end of the molecule (End-1). End-1 is defined as the end nearest to the
proposed site for initiation of DNA synthesis, while End-2 is the molecular end distal to the
initiation site. These are arbitrary designations since it is not possible to determine from the
electron micrographs which end belongs to the S or L component of the molecule (Shel-
drick & Berthelot, 1975). The linear DNA molecules with a small replicative loop (Fig. 1a,
Fig. 2) at about 10 μm from End-1 (Table 2A) could be regarded as DNA molecules on
which DNA replication has been initiated. The molecular length of these nine molecules ranges from 52.6 to 57.8 μm and some are thus larger than the longest virus DNA molecules (55.7 μm) isolated from purified virions (average length 52.4 ± 3.3 μm). The molecules numbering 10 to 14 (Table 2B) constitute another subgroup of linear DNA molecules which resemble the replicative intermediates of HSV DNA. Two of these DNA molecules (numbers 13 and 14) have a large replicative loop with the two arms in each measuring.
Table 2. Position of the replicative loop on the replicative intermediates of HSV DNA

<table>
<thead>
<tr>
<th>Molecule number</th>
<th>Distance of replication fork from End-I of molecule*</th>
<th>Distance of initiation site from End-I†</th>
<th>Length of each arm of the replicative loop</th>
<th>Total length of DNA in the replicative loop μm</th>
<th>Total length of replicative DNA molecule μm</th>
<th>Length of original molecule†</th>
<th>Number of molecular ends</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Initiation site at approximately 10 μm from End-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8-8</td>
<td>9-9</td>
<td>0-2</td>
<td>0-4</td>
<td>58-0</td>
<td>56-0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>8-0</td>
<td>8-1</td>
<td>0-2</td>
<td>0-4</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>8-3</td>
<td>9-8</td>
<td>3-0</td>
<td>6-0</td>
<td>58-3</td>
<td>55-3</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>9-8</td>
<td>9-9</td>
<td>0-2</td>
<td>0-4</td>
<td>58-0</td>
<td>57-8</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>10-0</td>
<td>10-15</td>
<td>0-3</td>
<td>1-0</td>
<td>57-0</td>
<td>56-7</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>10-3</td>
<td>10-70</td>
<td>0-8</td>
<td>1-6</td>
<td>53-0</td>
<td>52-6</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>11-0</td>
<td>11-15</td>
<td>0-3</td>
<td>0-6</td>
<td>54-3</td>
<td>53-7</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>11-8</td>
<td>11-95</td>
<td>0-3</td>
<td>0-6</td>
<td>53-3</td>
<td>52-7</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>12-0</td>
<td>12-09</td>
<td>0-18</td>
<td>0-35</td>
<td>54-5</td>
<td>54-67</td>
<td>2</td>
</tr>
<tr>
<td>B. Initiation site at 16 to 20 μm from End-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16-0</td>
<td>16-2</td>
<td>0-4</td>
<td>0-8</td>
<td>60-4</td>
<td>60-0</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>17-6</td>
<td>18-20</td>
<td>1-2</td>
<td>2-4</td>
<td>55-0</td>
<td>54-4</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>19-0</td>
<td>19-45</td>
<td>0-9</td>
<td>1-8</td>
<td>56-2</td>
<td>55-8</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>7-8</td>
<td>20-30</td>
<td>2-5</td>
<td>5-0</td>
<td>80-4</td>
<td>55-4</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>4-6</td>
<td>15-85</td>
<td>22-5</td>
<td>45-5</td>
<td>71-8</td>
<td>49-3</td>
<td>2</td>
</tr>
</tbody>
</table>

* Each replicative loop has two replication forks. The distance between the replication fork closest to End-I, and End-I of the molecule was determined. End-I is the end of the DNA molecule closest to the replicative loop as marked in Fig. 3.
† The initiation site was calculated to be located at the centre of each arm within the replicative loop.
‡ The original length of the replicated virus DNA molecule was calculated to include one arm of the replicative loop and the non-replicated portions of the DNA molecule.
§ Not determined.

Table 3. Y-shaped replicative intermediates of HSV DNA

<table>
<thead>
<tr>
<th>Molecule number</th>
<th>Distance of replicative fork from End-2° of DNA molecules (μm)</th>
<th>Length of replicated arms (μm)</th>
<th>Total length of DNA molecule (μm)</th>
<th>Number of molecular ends</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15-0</td>
<td>32, 35</td>
<td>82-0</td>
<td>3</td>
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<tr>
<td>2</td>
<td>18-7</td>
<td>32-6, 37-8</td>
<td>89-1</td>
<td>3</td>
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<tr>
<td>3</td>
<td>20-6</td>
<td>32, 38</td>
<td>90-6</td>
<td>3</td>
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<tr>
<td>4</td>
<td>4-0</td>
<td>44, 47</td>
<td>95-0</td>
<td>3</td>
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<tr>
<td>5</td>
<td>2-2</td>
<td>49-3, 38-8</td>
<td>90-3</td>
<td>3</td>
</tr>
</tbody>
</table>

*As marked in Fig. 3.

22.5 and 25 μm, respectively. If the initiation site for DNA replication is in the middle of each arm, then the initiation site for DNA synthesis in molecules number 14, 10, 11 and 13 is at 15-85, 16-2, 19-45 and 20-4 μm, respectively. The calculated molecular size of the three original non-replicated DNA molecules (numbers 11, 12 and 13) was found to be 54-4, 55-8 and 55-4 μm respectively. The initiation site for DNA synthesis in these molecules was calculated to be at a distance of 18-2, 19-45 and 20-3 μm respectively from one of the molecular ends.

The two DNA molecules (numbers 10 and 14) which have an initiation site for DNA synthesis at 16 μm from End-I have original calculated lengths of 60 μm and 49 μm and are thus at the two extremes in the range of molecular lengths of the virus DNA molecules.
If the initiation site for DNA synthesis in molecule 14 is in the same position as in molecules 11, 12 and 13 (size 55 μm) then molecule 14 lacks a sequence of DNA of about 6 μm that contains End-1. Obviously, more HSV DNA resembling replicative intermediates with an original (non-replicated) length of 49 μm will have to be analysed to obtain a definite answer regarding the position of the initiation site for virus DNA replication. The second group of virus DNA molecules with an initiation site at 16 μm (molecule number 10, Table 2)
has a molecular length of 60 μm. The reason for the presence of virus DNA molecules longer than 55 μm is not yet known. The results in Table 2 thus reveal the presence of two types of HSV DNA resembling replicative intermediates with the calculated initiation site for DNA replication situated at either about 10 μm or about 16 to 19 μm from End-1.

Y-shaped HSV DNA molecules

Another type of HSV DNA molecule that resembles replicative intermediates has three molecular ends but lacks a replicative loop (Fig. 3b, 4d and Table 3). These Y-shaped molecules that have one replicative fork in the direction of End-2 differ from each other in the distance of the replication fork from End-2 of the DNA molecule. The five DNA molecules described in Table 3 have been arranged to show that as the non-replicated DNA becomes progressively shorter, the arms of the semi-conservatively replicated DNA become correspondingly longer. DNA molecules numbers 4 and 5 (Table 3) have only 4 and 2.2 μm of non-replicated DNA respectively. The mol. wt. of the entire molecule number 4 is close to 190 × 10^6. It is of interest that in each of the DNA molecules described in Table 3, one arm is longer than the other by 3 to 5 μm (except in molecule number 5).

Electron microscopy of the HSV DNA molecules with filaments

Virus DNA molecules with long filaments

The results presented in Table 1 indicate that these DNA molecules are easily detectable in virus DNA preparations isolated from infected cells at different times during the virus growth cycle. The double-stranded portion of the DNA molecule has the same size as mature virus DNA, while the DNA filaments vary in size (Fig. 5) and seem to be thinner than the double-stranded virus DNA. From the manner in which the filaments are attached to the virus DNA molecules, it is not easy to determine their properties or to perform length measurements, since the end of the filaments collapse into the form of a bush. However, they may be single-stranded DNA strands since treatment with micrococcal endonuclease (which digests single-stranded DNA only) resulted in removal of the filaments and the elution of all the virus DNA molecules from BND-cellulose columns as double-stranded DNA (Shlomai et al. 1976a, b). Treatment of the virus DNA molecules with RNase did not affect the filaments as judged by electron microscopy.

Double-stranded DNA molecules, the size of mature virus DNA, which have a long filament attached (Fig. 5) may also be replicative intermediates of HSV DNA. Such a molecule can be formed when the initiation site for DNA replication is at the end of the molecule. If HSV DNA is replicated by a mechanism similar to that of adenovirus DNA (Sussenbach et al. 1973; Ellens, Sussenbach & Jansz, 1974) then HSV DNA molecules undergoing semi-conservative replication on one strand and displacement of the second non-replicated DNA strand, could give rise to the molecules described in Fig. 5.

Virus DNA molecules with short filaments

Numerous virus DNA molecules with filaments varying in size can be seen in each virus DNA preparation (Table 1). The filaments are situated randomly along the DNA molecules, with one filament per molecule in some and more than one in others. The role of the filaments is not known although they might be involved in recombination of virus DNA molecules (Holloman et al. 1975).
Fig. 5. HSV DNA molecule with a long filament. The virus DNA is 56 μm long with one single-stranded filament attached to it. The inset shows the portion of the DNA molecule with the single-stranded filament at a higher magnification. The length of the filament cannot be accurately measured.
Electron microscopy of virus DNA molecules with other molecular shapes

Virus DNA molecules with collapsed regions

A quantitative analysis of the virus DNA molecules (Table I) revealed that, depending on the time after infection (up to 9% at 12 h.p.i.), some of the DNA molecules with a molecular length of about 50 μm had collapsed regions even though the spreading solution contained formamide. Exact measurements were not possible because of the collapsed regions which appeared at different locations on various DNA molecules.

Branched virus DNA molecules

Some of the HSV DNA molecules display features similar to those seen in T4 phage DNA in the process of recombination (Broker, 1973).

Circular virus DNA molecules

A few virus DNA molecules having a circular conformation were also observed at different times after infection. Three of these molecules were found to have a molecular length shorter than the regular genome size, namely 43·2, 45·0 and 48·3 μm, respectively. The properties of these molecules and their relation to the replication of virus DNA are not yet known.

HSV DNA molecules longer than unit length

Four DNA molecules were found to have molecular lengths longer than unit size, namely 118, 130, 145 and 190 μm respectively. Whether these DNA molecules represent concatemers or aggregates is not known. They may be similar to the herpesvirus DNA molecules described by Ben-Porat et al. (1976).

DISCUSSION

The present study deals with the various forms of HSV DNA present in infected cells during the virus growth cycle and provides information on their molecular shape and length. CsCl density gradients efficiently separated virus DNA from cellular DNA and showed that both the mature and replicating virus DNA molecules have a linear conformation. Four different species of virus DNA were found to band in CsCl density gradients at the same density as virion DNA, in spite of differences in their structure and conformation.

The changes in the population of virus DNA molecules as a function of time after infection, were studied by electron microscopy. The number of DNA molecules resembling replicative intermediates gradually increased and reached a maximum amount at 12 h after infection (Table I), the time when the rate of DNA synthesis in the nuclei is maximal (Becker & Asher, 1975). The number of HSV DNA molecules having replicative loops or a Y-shape, subsequently decreased. In addition, virus DNA molecules with filaments attached to them were seen. These molecules did not decrease in amount after 12 h p.i., but remained at a level of 20% of the entire population of HSV DNA molecules. Their role in the life cycle of HSV is not known.

The HSV DNA molecules having one long filament attached may represent an additional class of replicative intermediates in which the initiation site for DNA replication is at one of the molecular ends. Semi-conservative DNA synthesis of one strand only and displacement of the second strand could give rise to DNA molecules with such filaments. In adenovirus, the initiation site for DNA replication is at one of the molecular ends and in some molecules,
semi-conservative DNA replication occurs on one strand only, while the second parental DNA strand is displaced and remains unreplicated (Ellens et al. 1974).

The availability of two and possibly three classes of HSV DNA molecules representing replicative intermediates, with initiation sites at 8 to 10 μm, 16 to 19 μm and possibly at one
Fig. 7. Models of HSV DNA replication. (a) A model for HSV DNA replication in which the initiation site is situated 10 µm from one end of the molecule. (b) A model for HSV DNA replication in which the initiation site is situated 20 µm from one end of the molecule. (c) A model for HSV DNA replication in which the initiation site is at one of the molecular ends. The consecutive steps in the replication process are marked A-F, while ▲ indicates the initiation site.
of the ends, poses the question of the relationship between the sequences known to be present in HSV DNA (Sheldrick & Berthelot, 1975; Hayward et al. 1975) and the position of the initiation sites for DNA biosynthesis on the different virus DNA species. The model of Sheldrick & Berthelot (1975) for the structure of HSV DNA suggests the presence of a region of internally repeated sequences (IR) composed of two tandemly arranged subregions, the centre of which is about 10 μm away from the molecular end near the short (S) component of the virus DNA molecule. Inversions of the L and S components, relative to each other, could lead to the appearance of the four possible rearrangements of HSV DNA (Hayward et al. 1975). The possible positions of the initiation sites for DNA replication in the four classes of DNA molecules are presented in Fig. 6. If the initiation site is in the L component, it could be situated 10 μm from the TRs sequence (Fig. 6A, molecular forms I and III, Table 2A). When the L strand is inverted, the initiation site will appear closer to the S component, at about 20 μm from the TRs sequence (Fig. 6A, molecular forms II and IV, Table 2B). An alternate possibility is shown in Fig. 6B, assuming that the initiation site is in the repeat region (IR) of the S component. An initiation site at about 10 μm is possible in molecular forms 1 and 2 (Fig. 6B, Table 2A). A change in the orientation of the S component may lead to the presence of the initiation site in the TRs region, close to the end of the HSV DNA molecule (Fig. 5 and Fig. 6B, molecular forms 3 and 4). The analysis presented in Fig. 6 predicts that the initiation sites for DNA replication may be located (a) 10 μm from either end of the HSV DNA molecule inside the L component or in the IR region between the L and S components, (b) inside the L component at 20 μm from the molecular end close to the S component and (c) in the TRs sequence, close to the end of the HSV DNA molecule.

In the present study we have identified two possible initiation sites at about 10 μm and 20 μm from one end of the HSV DNA molecule. Bidirectional semi-conservative replication initiated at these positions could lead to the formation of the replicative loops and Y-shaped molecules described in this study. The third predicted initiation site at one of the ends of the HSV DNA molecule could lead to the formation of HSV DNA possibly by strand displacement as described for adenovirus DNA replication (Ellens et al. 1974). Models demonstrating three possible modes of replication for the eight molecular forms of HSV DNA shown in Fig. 6, are presented in Fig. 7.

Numerous virus DNA molecules with short filaments resistant to RNase were observed (Table 1) but the importance of these filaments is not known. The branched virus DNA molecules resemble the T4 phage DNA molecules that were considered to be intermediates in the process of recombination (Broker & Lehman, 1971; Broker, 1973). Recombination of HSV DNA is known to occur in infected nuclei (Wildy, 1955; Subak-Sharpe et al. 1974). However, the mode of HSV DNA recombination has not been elucidated and HSV DNA molecules in the process of recombination have not yet been described. A recent model for DNA recombination (Holloman et al. 1975) suggests that single-stranded filaments present in the DNA molecules are involved in the initiation of recombinational events. It is possible that HSV DNA molecules undergoing recombination are observed in the electron micrographs without being recognized as such. Further studies are needed to identify HSV DNA molecules in the process of recombination and to distinguish them from the replicative intermediates of HSV DNA.

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