Defective Herpes Simplex Virus DNA: Circular and Circular-linear Molecules Resembling Rolling Circles

By YECHIEL BECKER, YAEL ASHER
EYNAT WEINBERG-ZAHLERING AND SAMUEL RABKIN
Laboratory for Molecular Virology, Hebrew University-Hadassah Medical School, Jerusalem

ADAM FRIEDMANN AND ESTHER KESSLER
Department of Genetics, Hebrew University, Jerusalem, Israel

(Accepted 1 March 1978)

SUMMARY

The formation of defective herpes simplex virus (HSV) in BSC-1 cells and the synthesis of defective virus DNA was studied. The fourth consecutive passage of undiluted virus yielded defective DNA that was 0.008 g/ml more dense than wild type (w.t.) virus DNA. The amount of defective DNA increased at passage 6 concomitantly with the decrease in infectious virus progeny. The synthesis of defective DNA was always accompanied by w.t. virus DNA synthesis. Defective DNA from both infected nuclei and defective virions had a mol. wt. of 100 x 10^6 and was linear as determined by electron microscopy. Electron microscopy of defective virus DNA at passage 6 revealed circular molecules varying in size in addition to linear DNA molecules with the length of intact virion DNA. The circular DNA molecules had contour lengths of 10, 5, 2.5 and less than 2.5 μm. The smallest circular DNA molecules had a contour length of 0.3 μm, possibly one virus gene. In addition, circular-linear DNA molecules were observed in which both the circular and the linear components varied in length. Most of these DNA molecules had circular components of either 2.5 or 5.0 μm, and linear components varying in length from less than 1 to 50 μm. Based on the present study, it is proposed that the S component of w.t. virus DNA is fragmented into small circular molecules that serve as templates for DNA synthesis, possibly by the rolling circle mechanism.

INTRODUCTION

Defective virus particles arise as a result of passage of virus at high multiplicities of infection (reviewed by Huang, 1973). Such particles were found in cells infected with herpes simplex virus (HSV) (Bronson et al. 1973; Wagner et al. 1974; Murray et al. 1975; Frenkel et al. 1975; Schröder et al. 1975/6), pseudorabies virus (Ben-Porat et al. 1974; Rubenstein & Kaplan, 1975) and herpesvirus saimiri (Fleckenstein et al. 1975). DNA from defective HSV particles was found to have a higher density in CsCl gradients than wild type (w.t.) (passage 1) virus DNA (Wagner et al. 1974). In cells which produce defective virus DNA, a concomitant accumulation of the early virus polypeptide VP175 was noted (Murray et al. 1975). Defective DNA molecules from virions were found to be made up of a tandem arrangement of repeats derived from a sequence present in the S component of the normal
virus DNA (Frenkel et al. 1976). A similar arrangement was described for defective pseudo-
rabies virus (Rubenstein & Kaplan, 1975) and herpesvirus saimiri DNA (Fleckenstein
et al. 1975). The mechanism by which defective herpesvirus DNA is formed and replicated
is not yet completely understood.

In the present study on defective HSV DNA synthesis, we used the HF strain of HSV
which has DNA with a relatively low density (1.718 g/ml) and replicates more slowly
(a growth cycle of 18 h) in BSC-1 monolayers than in HeLa or BHK cells. The circular-
linear DNA molecules may be the replicative intermediates of defective HSV DNA,
possibly synthesized by a mechanism resembling the rolling circle model (Gilbert &

METHODS

Cells and virus. The HF strain of herpes simplex virus (HSV) type 1 was used to infect
BSC-1 cells (2 x 10⁶ cells/ml bottle) grown in Dulbecco's modified Eagle's medium with
10% foetal bovine serum (Grand Island Biological Co.). Stock virus preparations were
infected at a multiplicity of infection of 0.1 p.f.u./cell. For normal experimental procedures,
BSC-1 cells were infected with 10 p.f.u./cell.

To obtain defective virus, BSC-1 cells were infected with HSV at 100 p.f.u./cell. Undiluted
virus from the first passage was used in subsequent passages to re-infect BSC-1 cells up to
passage level 9. At every passage level, the virus progeny in samples of infected cells was
titrated on BSC-1 monolayers by the plaque assay (Levitt & Becker, 1967).

Isolation of labelled virus and cell DNA. Infected cells were labelled with 25/zCi/ml of
methyl-³H-thymidine (specific activity 26.5 Ci/m, Nuclear Research Centre, Negev, Israel)
for 21 h, starting at 3 h after infection. After labelling, the infected cells were rinsed with
1 x SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate), scraped into 1 x SSC and treated
with 500 µg/ml of pronase (Sigma, St Louis, Missouri; pre-incubated at 37 °C for 1 h) and
0.5% (w/v) sodium lauryl sulphate overnight at room temperature. Virus and cellular
DNA were separated by centrifugation in CsCl gradients (Shlomai et al. 1976).

Analysis of virion DNA. Virions from infected cells at the desired passage level were
purified by centrifugation of ultrasonically treated infected cells in sucrose gradients
(Shlomai et al. 1976). The isolated virus bands were suspended in 1 x SSC and treated with
pronase and sodium lauryl sulphate as described above. The purified virion DNA was
analysed in CsCl gradients.

Electron microscopy. The DNA preparations were spread and examined by electron
microscopy as described in detail by Friedmann et al. (1977b).

Hybridization of defective and normal HSV DNA. The hybridization technique used has
been described in detail by Cedar (1976).

RESULTS

Synthesis of defective HSV in BSC-1 cells infected at a high multiplicity of infection

Virus progeny was passaged in BSC-1 cells at an initial infection of 100 p.f.u./cell and
further passaged undiluted up to passage 9. Titration of the virus progeny revealed a marked
decrease in the virus titre in passages 6 and 7 followed by a subsequent rise at passages 8 and
9 (Table 1). These results are in agreement with the findings of Bronson et al. (1973).
Biosynthesis of defective HSV DNA

Table 1. Infectivity of HSV progeny from cells infected with subsequent passages of undiluted virus

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND*</td>
<td>3.5 × 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>2.25 × 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>4.6 × 10^6</td>
<td>2.25 × 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>6.5 × 10^6</td>
<td>7.5 × 10^6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>5.4 × 10^6</td>
<td>1.25 × 10^6</td>
<td>2 × 10^6</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>1.2 × 10^6</td>
<td>1.5 × 10^6</td>
<td>5 × 10^6</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>6.5 × 10^3</td>
<td>1.0 × 10^6</td>
<td>2.5 × 10^5</td>
<td>1.6 × 10^4</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.7 × 10^7</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.3 × 10^7</td>
</tr>
</tbody>
</table>

* ND = Not done.

Virus and cell DNA synthesis in cells infected at low and high passage levels

Centrifugation in CsCl gradients of ³H-thymidine-labelled DNA from infected cells at various passage levels revealed the following. (i) Cells infected at passages 2 and 3 (Fig. 1a and b) synthesized w.t. virus DNA. Virus DNA with a higher density started to be synthesized at passages 4 and 5 (Fig. 1c and d) and was most evident at passage 6 (Fig. 1e). The density of the new DNA species was 1.726 g/ml, namely 0.008 g/ml higher than virion DNA. At passage 7 the synthesis of this DNA species (designated defective virus DNA) markedly decreased (Fig. 1f). (ii) The synthesis of defective DNA (Fig. 1c, e and f) was always accompanied by the synthesis of w.t. DNA and (iii) when the synthesis of virus DNA (w.t. or defective) was maximal (Fig. 1a, b and e), there was a marked effect on the synthesis of cellular DNA. However, when the synthesis of virus DNA was low (Fig. 1c, d and f) the synthesis of cellular DNA was not affected.

The nature of the defective virus DNA species was characterized by hybridization to virion DNA (Cedar, 1976). All the denatured defective DNA (density 1.726 g/ml) was converted to a double-stranded form when hybridized with denatured herpes simplex virion DNA (data not shown). From all the above data it was concluded that the dense DNA is indeed defective virus DNA.

The relationship between the synthesis of defective and w.t. HSV DNA and cellular DNA is presented in Table 2. Although the three experiments differ slightly from each other, they have the same basic features, namely that the synthesis of defective DNA is accompanied by the synthesis of w.t. virus DNA.

Characterization of DNA in virions and in nuclei at the time of defective DNA synthesis

Infected BSC-1 cells at passage levels 4, 5 and 6 were incubated in the presence of ³H-thymidine as described in Methods. Half the cultures were used for the isolation of virion DNA and the rest for the isolation of nuclear DNA. Centrifugation of both preparations in CsCl density gradients revealed mainly defective DNA (Fig. 2a, b and c) although some w.t. DNA was also synthesized in the nuclei of these cells. Analysis of the DNA present in the virion preparations revealed both defective and w.t. DNA (Fig. 2a, b and c). Some cellular DNA was also present in virions from passage 5. It is not known whether the cellular DNA forms part of the virion or is due to contamination of the virus band. Thus at passages 4, 5 and 6 most of the virions contain defective DNA and this probably explains the decrease in the titre of infectious virus (Table 1).
Fig. 1. Characterization of HSV DNA synthesized in BSC-1 cells infected with subsequent passages (P-2 to P-7) of undiluted virus. The infected cells were labelled with $^3$H-thymidine (●—●) and the nuclear DNA was extracted and centrifuged in CsCl density gradients together with $^{14}$C-HSV DNA (○—○) as marker (see Methods).
Table 2. Virus and cellular DNA obtained from BSC-1 cells infected with undiluted virus*

<table>
<thead>
<tr>
<th>Passage</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV DNA†</td>
<td>Cellular DNA†</td>
<td>HSV DNA</td>
</tr>
<tr>
<td>2</td>
<td>1920</td>
<td>0</td>
<td>700</td>
</tr>
<tr>
<td>3</td>
<td>630</td>
<td>348</td>
<td>1140</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>1000</td>
<td>80</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>1160</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>296</td>
<td>308</td>
<td>472</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>604</td>
<td>800</td>
</tr>
</tbody>
</table>

* Results are expressed as ct/min x 10⁻⁸.
† Defective, w.t. and cellular DNA were isolated in CsCl gradients as described in the legend to Fig. 1.
‡ ND = Not done.
Fig. 2. Characterization of nuclear DNA and purified virions from cells infected at passage levels 4, 5 and 6. BSC-1 cells were infected as above and labelled with $^3$H-thymidine. The DNA was extracted from the nuclei of infected cells and from purified virions. The nuclear DNA ($a$, $b$ and $c$) and the virion DNA ($a_1$, $b_1$, and $c_1$) was centrifuged in CsCl density gradients. Symbols as in Fig. 1.
Fig. 3. Mol. wt. of defective HSV DNA. Defective $^3$H-thymidine-labelled HSV DNA (●—●) from passages (a) 2, w.t. and (b) 6, defective, isolated in CsCl density gradients (Fig. 2), was centrifuged in sucrose gradients together with $^{14}$C-HSV DNA as marker (○——○). Wild type virus DNA from passage 6 was also analysed (c).
Molecular size of defective HSV DNA

The molecular size of the defective DNA molecules was determined either by centrifugation in sucrose gradients with linear virion DNA (100 × 10^6 daltons) as a marker or by electron microscopy. The results presented in Fig. 3(b) show that most of the defective HSV DNA co-sediments with the normal virion DNA marker and therefore can be regarded as unit length (100 × 10^6 daltons). W.t. DNA from passage 2 (Fig. 3a) and from passage 6 (Fig. 3c) also co-sedimented with the virion DNA. A small fraction of the DNA sedimented ahead of the marker (Fig. 3b and c) and a larger fraction sedimented more slowly than the intact DNA.

Electron microscopy of defective DNA from passages 5 and 6 revealed that most of the molecules are linear with a contour length of about 52 μm. Fig. 4 illustrates a linear DNA molecule with a contour length of 51.4 μm. [Linear molecules shorter than unit length were also seen (Fig. 10a and b). The nature of these DNA fragments is under study.] In addition to the linear DNA molecules of unit length, circular DNA molecules with a contour length of 4.6 μm (Fig. 4, arrowed) were also seen. Since the circular DNA molecules constitute a new species never detected in BSC-1 cells lytically infected with HSV (HF strain), the nature of these DNA molecules was studied.

Synthesis of defective DNA

Defective DNA synthesis was studied in infected cells at passage 6. The cells were labelled with 3H-thymidine (10 μCi/ml) for 3, 6 and 10 h starting at 2 h post-infection (p.i.). The DNA was extracted and centrifuged in CsCl density gradients. Fig. 5(a) shows that up to 5 h p.i., similar amounts of defective and w.t. virus DNA were synthesized. However, during the subsequent 3 h, more defective than w.t. DNA was synthesized (Fig. 5b) and at 12 h after infection most of the labelled DNA molecules were defective DNA (density 1.726 g/ml; Fig. 5c). This result is summarized in Fig. 5(d) which shows that more defective than w.t. DNA was synthesized in cells infected with defective virus. Linear virus DNA molecules were reported to require 20 min for replication by the semi-conservative mechanism (Shlomai et al. 1976; Shlomai & Becker, 1977). The rate of defective DNA synthesis is still under study.

Circular DNA molecules in the defective virus DNA band

Circular DNA molecules were found in defective DNA preparations isolated from BSC-1 cells at passage levels 5 and 6. Typical circular molecules from passage 6 are presented in Fig. 6. The DNA in Fig. 6(a) has a contour length of 0.65 μm while other circular DNA molecules have contour lengths of 2.4 (Fig. 6b), 2.9 (Fig. 6c), 3.4 (Fig. 6d), 3.9 (Fig. 6e), 5.3 (Fig. 6f), 9.7 (Fig. 6g and h) and 20 μm (Fig. 6i). In Fig. 6(h) two circular DNA molecules can be seen: the larger one has a contour length of 9.6 μm and the smaller one 3.5 μm. The small circular DNA molecule seems to be intertwined with the larger one. The circular DNA molecules found in defective DNA at passage 6 range from 0.65 to 10 μm (except one molecule of 20 μm). Most of the circular molecules had contour lengths of between 2.5 and 5 μm. The circular DNA molecules seen in defective DNA at passage 5 ranged from 5 μm (the longest) to 0.25 μm.
Biosynthesis of defective HSV DNA

Fig. 4. Electron microscopy of defective HSV DNA. Two molecules are shown: a linear DNA molecule (A indicates the molecular ends) with a contour length of 51.4 μm and a circular DNA molecule (→) with a contour length of 46 μm. Bar marker represents 1 μm.
Fig. 5. Time course of synthesis of defective and w.t. HSV DNA. BSC-1 cells were infected with undiluted virus at passage level 6 and labelled with ³H-thymidine starting at 2 h p.i. for 3 h (a), for 6 h (b) and for 10 h (c). The DNA was extracted and centrifuged in CsCl density gradients (●—●) together with ¹⁴C-HSV DNA (○—○). The total radioactivity obtained at 5 (a), 8 (b) and 12 (c) h from defective (●—●) and w.t. (○—○) DNA is plotted in (d).

Circular-linear DNA molecules in defective DNA

The circular-linear DNA molecules seen in defective DNA of passage 6 fall into two categories: molecules with a circular component up to 2·5 μm, or 5·0 μm long that have a linear component varying in length. Electron micrographs of circular-linear DNA molecules of the first type are presented in Fig. 7 and the second type in Fig. 8 and 9. The arrows in Fig. 8 and 9 show the point at which the linear component branches from the circular component.

A quantitative analysis of the linear, circular and circular-linear DNA molecules obtained at passage levels 5 and 6 is presented in the histogram in Fig. 10. The circular molecules (with or without a linear component) are shown on the left hand side and the linear molecules on the right hand side of the histogram. It can be seen that defective HSV DNA at passage 5 contains linear DNA molecules which range from the length of a complete genome to a length of 1 μm. The circular DNA molecules range from 0·25 to 5 μm and about half have a linear component not longer than 6·5 μm (Fig. 10a).

Defective DNA at passage 6 shows the two main subgroups having a circular component of either 2·5 or 5 μm (Fig. 10b). Circular DNA components larger than 5 μm and smaller than 2·5 μm were also seen. For example, one circular-linear DNA molecule has a circular component of 2 μm and a linear component of 49 μm which together constitute the length of almost a complete DNA genome. This suggests that the linear defective DNA molecules may be synthesized on the circular component by the rolling circle mechanism. Circular-linear DNA molecules with a linear component shorter than 51 μm may represent replicative intermediates in the synthesis of defective HSV DNA. The possible replication of
defective DNA by the rolling circle mechanism may explain the different rates of synthesis of normal and defective virus DNA (Fig. 5).

It may be seen in the histogram of defective circular DNA molecules at passage 6 (Fig. 10b) that two molecules differ from the rest. One molecule has a circular component of 41 μm and a linear component of 10 μm [another (not shown) had a circular component of 40 μm and a linear component of 8 μm] and the second is a circular DNA molecule with a contour length of 20 μm (Fig. 6i). The nature of the latter molecule is not known, but the first type resembles the circular-linear DNA molecules of genome size already described (Friedmann & Becker, 1977). It is proposed that the circular-linear DNA with a circular component of 40 μm may represent a DNA molecule in the process of internal recombination (Y. Becker, unpublished data).
Fig. 7. Circular-linear DNA molecules from defective DNA (passage 6). The contour lengths (\(\mu m\)) of the circular and linear components respectively are: (a) 2.0 and 4.6; (b) 1.9 and 0.8; (c) 1.4 and 2.1; (d) 2.0 and 1.3. Bar markers represent 1 \(\mu m\).

DISCUSSION

The synthesis and properties of defective HSV DNA in cells infected with undiluted virus were described by Bronson et al. (1973), Wagner et al. (1974) and Murray et al. (1975). It was demonstrated that the defective HSV DNA is made up of tandem repeats of a unique sequence derived from the S component of the virus DNA (Frenkel et al. 1976). The present study provides a novel explanation for the synthesis of defective DNA by revealing circular and circular-linear DNA molecules with features of DNA molecules replicating by the rolling circle mechanism described for \(\phi X\) phage DNA (Knippers et al. 1969) and ribosomal genes in \(Xenopus\) laevis (Hourcade et al. 1973).

The development of defective HSV (HF strain) in BSC-1 cells resembles that in other HSV-cell systems. In our system, as in that of Bronson et al. (1973), the appearance of defective virus particles was cyclic (Table 1). The defective HSV DNA synthesized in infected nuclei had a density in CsCl gradients higher than that of w.t. virus DNA as reported by Wagner et al. (1974) and Frenkel et al. (1975). The present study demonstrates that more defective HSV DNA is synthesized in infected cells than w.t. virus DNA and that circular and circular-linear DNA molecules are present in addition to the linear defective DNA genomes with a mean molecular length of 51 \(\mu m\). When aligned, the DNA molecules in defective DNA of passage 6 were found to fall into two classes, with circular components of 2.5 and 5 \(\mu m\) (Fig. 10b). The gradual increase in length of the linear components of the circular-linear DNA molecules (Fig. 10b) suggests that these may represent replicative intermediates of defective DNA molecules. The circular-linear DNA molecules probably replicate as rolling circles which leads to the formation of tandem repeats of the same sequence in the defective linear DNA genomes. This may be an explanation for the finding of Frenkel et al. (1976).
Fig. 8. Electron micrograph of circular-linear DNA molecules from defective DNA (passage 6). (a) Circular component 5.0 μm, linear component 16 μm. (b) Circular component 5.0 μm, linear component 7.1 μm. Bar markers represent 1 μm. The circular components in (a) and (b) are presented schematically in (a₁) and (b₁) respectively (the circles are marked with a thick line and the linear components with a thin line).
Fig. 9. Electron micrograph of a circular-linear DNA molecule (passage 6). The circular component of the DNA molecule has a contour length of 52 μm and the linear component, 31 μm. The arrow shows the branching point where the linear component is initiated. Bar marker represents 1 μm.
The presence of circular DNA molecules with the density of defective virus DNA at passage levels 5 and 6 indicated that the DNA is fragmented in cells infected with defective virions. It was demonstrated (Friedmann et al. 1977a) that the density of the repeat sequences of the virus DNA [described by Sheldrick & Berthelot (1974)] is similar to that of defective DNA, while the L region has a lower density than intact virus DNA. It is therefore possible that the circular DNA molecules are derived from the repeat sequences of the virus S region. Indeed, it was recently demonstrated that the terminal repeat sequence of the S region, tandemly arranged, constitutes the defective virus DNA (G. Vande Woude, personal communication). Further characterization of the circular DNA may provide an answer to the question of amplification of virus genes.

The differences in the size range of the circular DNA components from passages 5 and 6 (Fig. 10) suggest that cleavage of the virus DNA to fragments which become the circular DNA is done by a specific enzyme, possibly a restriction enzyme, and not by a known virus endonuclease. The nature of the DNA polymerase which may be involved in the replication of circular DNA molecules by the rolling circle mechanism is not known. Since w.t. virus DNA is synthesized concomitantly with defective DNA and virus structural proteins are used to coat the defective DNA molecules, it would appear that the w.t. virus DNA provides both the early and late virus gene products for the synthesis of defective virions.
The mechanisms which lead to specific cleavage of the terminal repeat sequences of the S region are not yet known. However, a model for intramolecular recombination (Y. Becker, unpublished data) may provide an explanation for this phenomenon. It was proposed that the molecular ends of a linear HSV DNA molecule integrate into the sequences between the internal repeat sequences, yielding 8-shaped DNA molecules. Cleavage of such a recombination intermediate by a specific restriction enzyme can yield fragments with single-stranded sequences at the ends leading to circularization. Further studies to elucidate the mechanism of the formation of small circular DNA molecules and their role in the synthesis of defective HSV DNA are under study.

The help of Dr H. Cedar in performing the hybridization experiments is acknowledged. Our thanks to Dr Julia Hadar for assistance in the preparation of the manuscript.

This research was supported in part by contract no. NOI-CP-3-3300 within the Virus Cancer Program of the National Cancer Institute, Bethesda, Md. and a grant from the Leukemia Research Foundation Inc., Chicago, Ill., U.S.A.

REFERENCES


(Received 15 July 1977)