Analysis of Herpes Simplex Virus DNA Synthesized in Infected Nuclei by Chromatography on Benzoylated Naphthoylated DEAE Cellulose Columns

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SUMMARY

The nature of the DNA molecules synthesized in nuclei of herpes simplex virus (HSV)-infected cells in vivo and in vitro was studied by chromatography on BND-cellulose columns after shearing to DNA fragments of 10 to 20 × 10⁶ daltons. The incorporation of labelled precursors occurs in the DNA fragments containing single-stranded regions, presumably the replication forks. Prolongation of DNA synthesis leads to the accumulation of labelled DNA fragments that lack single-stranded sequences. Analysis of the isolated DNA fragments by density centrifugation in CsCl gradients revealed that most of the labelled DNA molecules are of virus specificity and the minority are cellular DNA fragments. Double-stranded virus DNA fragments and virus DNA fragments containing single-stranded sequences band in CsCl gradients at a density of 1.718 g/ml, the density of virion DNA. This suggests that the replicating HSV DNA molecules have the same density as the virion DNA and contain relatively little single-stranded DNA. The synthesis of HSV DNA molecules under in vitro conditions in isolated nuclei occurs by incorporation of the precursors into DNA fragments with single-stranded regions. The synthesis of cellular DNA in nuclei from hydroxyurea and cytosine arabinoside treated cells also occurs by elongation of nascent DNA chains.

INTRODUCTION

Analysis of HSV DNA synthesized in nuclei of herpes simplex virus (HSV)-infected cells by electrophoresis in polyacrylamide gels (Ben-Zeev, Weinberg & Becker, 1974) revealed the presence of at least two species of HSV DNA. One species resembled virion DNA and was capable of migration in the gel while the second species was retained at the top of the gel. It was suspected that the latter may be replicating virus DNA molecules that were retained by the gel because of their conformation. To study the nature of the HSV DNA molecules synthesized in lytically infected BSC-1 cells, we used benzoylated, naphthoylated DEAE-cellulose (BND-cellulose) columns to distinguish between double-stranded DNA molecules that elute with 1.0 M-NaCl and replicating DNA molecules, with single-stranded regions in the replicating forks, that elute with 2% (w/v) caffeine (Kiger & Sinsheimer, 1969; Sussenbach et al. 1972; Scudiero & Strauss, 1974). In the present study the DNA molecules synthesized in the nuclei of HSV infected BSC-1 cells in vivo and in isolated...
nuclei \textit{in vitro} (Becker & Asher, 1975) were analysed by chromatography on BND-cellulose columns in order to elucidate the following: (a) the properties of replicating DNA molecules synthesized \textit{in vivo} and (b) the properties of DNA molecules synthesized in nuclei isolated from infected and drug-treated cells. The results of the present study made it possible to characterize the mature and replicating DNA molecules.

\section*{Methods}

\textit{Virus and cells.} BSC-1 monolayers were infected with the HF strain of herpes simplex virus (HSV) type I, in Dulbecco's modified Eagle's medium. The cell monolayers (2 × 10^6 cells/ milk bottle) were infected with the virus at 10 p.f.u./cell and incubated at 37 °C for 18 h, at which time the virus growth cycle is complete.

\textit{Isolation and purification of HSV virions.} HSV infected monolayers incubated at 37 °C for 18 h were washed and scraped into cold tris-buffered saline (TBS: 0.2 M-tris-HCl, pH 7.3, 0.85% [w/v] NaCl). The cell suspensions were sonicated, layered on to linear sucrose gradients (12 to 52% [w/w] made in TBS), and centrifuged at 20000 rev/min for 55 min at 4 °C, in the SW 27 rotor of the Beckman ultracentrifuge. The virion bands were collected through the wall of the tube by means of a syringe.

\textit{Isolation and purification of DNA from mature herpes virions.} DNA was extracted from sucrose gradient-purified HSV virions, by two methods: (a) treatment with N-lauroyl sarcosine (Sigma, St Louis, Mo.; 4% [w/v] final concentration) and heating to 63 °C for 4 min, and (b) by treatment of the virus suspension in 1 × SSC (0.15 M-NaCl, 0.015 M-sodium citrate) with a final concentration of 0.5% (w/w) sodium lauryl sulphate and 0.3 mg/ml Pronase (free of nuclease 90000 units/mg, Calbiochem). When necessary further purification of virus DNA was done by sequential extractions with TE buffer (0.1 M-tris-HCl, pH 7.5, 0.001 M-EDTA)-saturated phenol and chloroform: isoamyl-alcohol (24:1) solutions. DNA solutions were precipitated with 2 vol. of absolute ethanol and the precipitate dissolved in TE buffer. CsCl (E. Merck, Darmstadt, Germany) was added to bring the density to 1.70 g/ml, and the preparations were centrifuged in the 50 Ti rotor of the Beckman preparative ultracentrifuge at 35000 rev/min for 48 h at 20 °C. 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 either by weighing 100 μl samples (in the Mettler H51 precision balance), or by determining the refractive index (in the Bausch and Lomb refractometer). Virus DNA was separated from contaminating cellular DNA according to their different buoyant densities (1.718 g/ml and 1.700 g/ml respectively).

\textit{In vivo labelling of virus and cellular DNA.} Three kinds of labelling experiments were performed. (a) ‘Pulse-labelling’ experiments in which HSV-infected monolayers, at different times after infection, were washed with medium (pre-warmed to 37 °C) and fresh medium containing 200 μCi/ml methyl-3H-thymidine (sp. act. 16.5 Ci/mmol, Nuclear Research Centre Negev, Israel) was added. The cell cultures were incubated at 37 °C for different periods of time, (b) ‘Pulse-chase’ experiments in which HSV infected monolayers, pulse labelled as above, were washed with medium (pre-warmed to 37 °C) containing thymidine (final concentration of 10 μg/ml) and incubated in this medium for different periods of time at 37 °C. (c) Labelling throughout the virus growth cycle with 10 μCi/ml methyl-3H-thymidine or 0.5 μCi/ml 2-14C-thymidine (sp. act. 50 mCi/mmol; Nuclear Research Centre Negev, Israel) added at 3 h post-infection (p.i.). This procedure was used for the preparation of DNA either from infected nuclei or from mature HSV virions.
The infected and labelled monolayers were washed twice with 10 vol. of 1 × SSC and the cells were scraped and resuspended in 0.1 ml of 1 × SSC. The cells were lysed by the addition of sodium lauryl sulphate to 0.2% (w/w) and by three cycles of rapid freezing and thawing. The lysates were then treated with 50 μg/ml of pancreatic RNase A plus 50 units of T1 RNase (Sigma, St Louis, Mo.) for 1 h at 37 °C, followed by 2 h of Pronase treatment (1–0 mg/ml) at 37 °C (according to Scudiero et al. 1975).

Isolation of nuclei from HSV infected cells. This was performed according to the method described by Becker & Asher (1975). HSV infected monolayers at 18 h p.i. were washed and scraped into reticulocyte standard buffer (RSB; 0.01 M-tris-HCl, pH 7.7, 0.01 M-KCl, 0.015 M-MgCl₂). After Dounce homogenization, the nuclei were washed in phosphate buffer (0.075 M-potassium phosphate, pH 7.4, containing 8% [w/v] sucrose), RNase free and centrifuged for 2 min at 800 rev/min in a PR-2 refrigerated centrifuge. The nuclear pellet was washed in the same phosphate buffer and resuspended at a concentration of 10⁶ nuclei/100 μl in the phosphate buffer to which 10⁻³ M-mercaptoethanol had been added.

Conditions for in vitro synthesis of DNA in the HSV infected nuclei. Each 200 μl of the reaction mixture contained 10⁶ nuclei in 75 mm-potassium phosphate buffer, pH 7.4, 8% sucrose (RNase free), 6 mm-MgCl₂, 3 mm-mercaptoethanol, 40 μmol each of dATP, dCTP, dGTP and 50 μCi methyl-³H-thymidine triphosphate (15 Ci/mmol, Radiochemical Centre, Amersham, England). The reaction mixture was incubated at 37 °C for periods of 10 or 30 min. Twenty × SSC was added to a final concentration of 1 × SSC and sodium lauryl sulphate was added to a final concentration of 0.2% (w/w). The mixture was frozen and thawed and treated with the RNase mixtures and Pronase as described for the in vivo system.

Inhibitors. Inhibitors of HSV DNA replication were used at concentrations and conditions that were previously found to be inhibitory for HSV in infected BSC-1 cells (Levitt & Becker, 1967; Rosenkranz & Becker, 1973) and also in the in vitro system of DNA synthesis (Becker & Asher, 1975). Hydroxyurea (HU) at a final concentration of 5 × 10⁻² M, and cytosine arabinoside (ara-C) at a final concentration of 50 μg/ml were added to the cells immediately after infection and were present in the infected cells only under in vivo conditions.

Benzoylated naphthoylated DEAE-cellulose (BND-cellulose) chromatography. BND-cellulose chromatography was performed according to Scudiero et al. (1975). Approx. 0.75 g of BND-cellulose (Serva, Feinbiochemica, Heidelberg, Germany) was used for each analytical column. The resin (60 g) was suspended in 60 ml 0.3 M-NaCl, 10⁻³ M-EDTA, 0.01 M-tris-HCl, pH 8.1) and was washed sequentially on a Buchner funnel with 50 ml vol. each of distilled water, 0.3 M-NET buffer, 50% (v/v) formamide in 1.0 M-NET buffer (1.0 M-NaCl, 10⁻³ M-EDTA, 0.01 M-tris-HCl, pH 8.1) and again with 0.3 M-NET buffer (100 ml). The washed resin was suspended in 72 ml 0.3 M-NET buffer and 0.75 g of resin in 9.0 ml buffer were packed in each 5 ml plastic syringe of 1.1 cm diam., containing a glass filter at the bottom. The columns were washed again with 50 ml of 0.3 M-NET buffer and stored overnight at room temperature.

Cell lysates from in vivo experiments or lysates of nuclei from in vitro experiments were sheared by passing five times through a 20 G needle. The lysates in 0.3 M-NET buffer in a total vol. of 5 ml, were adsorbed by slow passage through the column.

Elution of DNA from the columns was carried out by two methods: (a) elution with a linear gradient of 0.3 to 1.0 M-NaCl in NET buffer, followed by a second gradient of 0 to 2% (w/v) caffeine in 1.0 M-NET buffer (for calibration of the BND-cellulose column) and (b) stepwise elution using solutions of 0.3 M-NET buffer, 1.0 M-NET buffer, 2% (w/v) caffeine in 1.0 M-NET buffer, and 50% (v/v) formamide solution in 1.0 M-NET buffer.
buffer. Fractions were collected in an LKB fraction collector, and the TCA precipitable radioactivity in each fraction was determined.

To determine the density of the labelled DNA, molecules from BND-cellulose samples eluted by 1.0 M-NET or 1.0 M-NET + 2 % (w/w) caffeine were transferred to a polyallomer centrifuge tube with 14C-DNA from herpes virions to serve as a marker. CsCl crystals were added to bring the density to 1.70 g/ml and the gradients were centrifuged as described above.

**Electron microscopy of DNA molecules.** The 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 % cytochrome c; 3.0 M-ammonium acetate-0.01 M-tris 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 buffer (pH 7.2); 10 % (v/v) formamide and 0.001 M-EDTA, in a paraffin-coated depression slide. A carbon-coated grid was used to pick up a small drop from the hypophase surface. The grid was dehydrated gently in 100 % ethyl alcohol and shadowed with palladium–platinum (20:80) at an angle of 5° under a vacuum of 5 x 10⁻² mmHg. A JEE-4B high vacuum evaporator with a rotatory table (60 rev/min) was employed in all experiments. To obtain information on the size of the DNA fragments isolated from CsCl or sucrose gradients and from BND-cellulose columns the DNA was spread directly without removing any CsCl, sucrose or buffer. Philips 300 and JEOL-7A electron microscopes with 40 µm foil objective apertures and 80 kV accelerating voltage were used. Magnification was calibrated with carbon grating replica at 28,000 line/inch (Ernest F. Fulla, Inc. Schenectady, New York). The length of the DNA fragments were measured with a map ruler directly on the electron microscope screen.

ϕX174 RF DNA circles (a generous gift from Dr Aharon Razin, Dept. Cellular Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem) were used as an internal standard with a mean length of 1.8 ± 0.3 µm.

**RESULTS**

**BND-cellulose chromatography of HSV DNA from purified virus particles**

To determine the conditions for the elution of native and denatured HSV DNA from BND-cellulose columns, tritiated DNA molecules were prepared from HSV particles isolated by centrifugation in sucrose gradients. One half of the DNA preparation was used in its native form while the second half was denatured with alkali (Gordin et al. 1973) prior to analysis. The two preparations were layered on to two BND-cellulose columns and eluted with a linear sodium chloride gradient (0.3 M to 1.0 M-NET buffer) followed by a gradient of 0 to 2 % (w/v) caffeine in 1.0 M-NET buffer. The recovery of labelled DNA was more than 90 %. Fig. 1(a) demonstrates that most (80 to 85 %) of the double-stranded virion DNA eluted with NET buffer at a concentration close to 1.0 M. All the denatured single-stranded (ss) virion DNA molecules were eluted with 1.0 M NET buffer containing caffeine at a concentration lower than 2 % (w/v; Fig. 1b). Some (15 to 20 %) of the native virion DNA molecules also eluted with caffeine. Their properties are currently being investigated. To elute the virus DNA in a stepwise fashion buffer (with 1.0 M-NaCl) lacking and containing 2 % caffeine was subsequently used to elute double-stranded (ds) and single-stranded (ss) DNA respectively.
BND-cellulose chromatography of HSV DNA

Fig. 1. Chromatography of double-stranded and single-stranded DNA on BND-cellulose. DNA was purified from N-thymidine-labelled herpes virions by sequential extractions with phenol and chloroform:isoamyl alcohol, and isopycnic centrifugation in CsCl gradients. 0.5 ml samples of virus DNA (density of 1.718 g/ml) were diluted and layered onto the BND-cellulose column either directly () or after alkaline denaturation (addition of KOH solution to a final concentration of 0.4 M and incubation at room temperature for 1 h) and neutralization with 1 N-HCl (O). Two-step elution of DNA from the column was carried out with (a) a linear gradient of 0 to 3 M to 1.0 M NaCl in NET buffer, and (b) a linear gradient of 0 to 2 % (w/v) caffeine in 1.0 M-NET buffer. The volume of each gradient was 60 ml.

Isolation of in vivo synthesized DNA molecules by chromatography on BND-cellulose

Analysis of the DNA molecules labelled during a 10 min pulse in the nuclei of HSV infected cells is presented in Fig. 2(a). The efficiency of DNA recovery and a quantitative analysis of the DNA species eluted from the BND-cellulose columns are summarized in Table 1. Most of the radioactivity (64 %) was eluted with caffeine, indicating the presence of single-stranded sequences in the labelled DNA molecules. About 31 % of the radioactivity chromatographed as double-stranded DNA. Conversion of DNA molecules with replicating regions to double-stranded DNA molecules is demonstrated in Fig. 2(b). Incubation of infected cells labelled for 10 min with N-thymidine, for a further 2.5 h after removal of the isotope, yielded a major fraction (70 %) of labelled DNA molecules with the properties of double-stranded fragments. Only 28 % of the radioactive DNA chromatographed as DNA containing single-stranded fragments (Table 1). It should be pointed out that the 2.5 h incubation (designated ‘chase’) is not really a chase since labelled precursors are continuously being incorporated into the newly synthesized DNA molecules. Similar results were obtained from the analysis of DNA molecules labelled throughout (7 to 16 h p.i.) the virus growth
Fig. 2. Characterization of DNA synthesized in vivo by BND cellulose-chromatography. Two BSC-1 cultures infected with HSV were washed and replenished with fresh medium (pre-warmed to 37 °C) containing 200 μCi/ml ³H-thymidine at 12 h p.i. The cultures were further incubated at 37 °C for 10 min. The cells in one culture (a) were then washed and lysed as described in Methods. Another culture (b) was washed with medium (pre-warmed to 37 °C) containing 10 μg/ml of thymidine and incubated for an additional 2-5 h before lysing the cells. A third infected culture (c) was labelled at 6 h p.i. for 11 h with ³H-thymidine (10 μCi/ml). The cell lysates were treated with RNase and Pronase, sheared and chromatographed on BND-cellulose columns as described in Methods.
Table 1. Characterization of DNA synthesized in vivo in HSV infected cells*

<table>
<thead>
<tr>
<th>Time</th>
<th>Used (ct/min)</th>
<th>Recovered (ct/min)</th>
<th>Elution with 1·0 M-NET plus 2 % (w/v) caffeine</th>
<th>Elution with 1·0 M-NET plus 50 % (v/v) formamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>2·2 × 10⁶</td>
<td>1·56 × 10⁶</td>
<td>48284</td>
<td>101995</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min + 2·5 h 'chase'</td>
<td>6·0 × 10⁶</td>
<td>3·65 × 10⁶</td>
<td>256106</td>
<td>102657</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-17 h</td>
<td>3·1 × 10⁶</td>
<td>2·16 × 10⁶</td>
<td>1463665</td>
<td>622854</td>
</tr>
</tbody>
</table>

* See the legend to Fig. 2.

Characterization of the in vivo synthesized DNA isolated by BND-cellulose chromatography

Previous studies (Ben-Zeev, et al. 1974; Becker, & Asher, 1975) showed that in infected nuclei about 90 % of the labelled DNA molecules are of virus specificity (density 1·718 g/ml) and the rest band in CsCl gradients at the position of cellular DNA (mean density 1·700 g/ml). The labelled DNA molecules eluted from BND-cellulose columns (marked a, b, c, d in Fig. 2) were characterized by centrifugation in a CsCl density gradient (Fig. 3). Most of the radioactive DNA molecules, labelled for 10 min with (Fig. 3 c) or without (Fig. 3 a) further incubation in vivo banded together with the HSV DNA marker. This indicates that in the analyses (Fig. 2) we are dealing mainly with HSV DNA molecules.

Another important finding is that the HSV DNA molecules which were eluted from the BND-cellulose columns by caffeine (Fig. 2a, b) banded at the density of herpes virion DNA, i.e. 1·718 g/ml (Fig. 3 b, d). ³¹C-HSV DNA was used as a density marker. Thus, the presence of single-stranded regions in nuclear HSV DNA, as indicated by BND-cellulose chromatography, does not change the buoyant density of the virus DNA molecules, implying a low proportion of single-stranded regions, in the virus DNA. Based on this conclusion, these virus DNA molecules were studied by electron microscopy.

The effect of micrococcal endonuclease on nuclear HSV DNA labelled in vivo for 1 h with ³¹H-thymidine revealed that 10 % of the labelled DNA was hydrolysed (Shlomai, Friedmann & Becker, 1976). The endonuclease treated DNA molecules chromatographed on BND-cellulose with 1·0 M-NaCl. Under the assay conditions, the micrococcal endonuclease degraded denatured DNA at least 30 times more effectively than double-stranded DNA. These results suggested that single-stranded sequences in the DNA are responsible for its binding to the column in a manner which permits elution with 2 % (w/v) caffeine.

Electron microscopy of the sheared DNA molecules

The size of the sheared DNA molecules eluted by 1·0 M-NET buffer or by 2 % (w/v) caffeine was determined by electron microscopy. The DNA molecules eluted by 1·0 M-NET buffer were found to be linear with an average length of 12 μm (ranging from 11 to 14 μm). It was not possible to differentiate between virus and cellular DNA molecules by this technique. The DNA molecules eluted by caffeine varied in length but single-stranded
regions were not easily detected. Further studies (Shlomai, Friedmann & Becker, 1976) dealing with unsheared HSV DNA isolated from infected cells, revealed the presence of a replicative loop in some of the virus DNA molecules.

**Time course of DNA synthesis**

To determine the relationship between molecules containing double-stranded and single-stranded DNA, infected cells were labelled with ³H-thymidine and samples of cells were removed at different time intervals. The cells were treated as described in Methods and the
Table 2. Time course of HSV DNA synthesis in infected nuclei in vivo

<table>
<thead>
<tr>
<th>Time</th>
<th>Total (ct/min)</th>
<th>0.3 M-NET</th>
<th>1.0 M-NET</th>
<th>1.0 M-NET+ 2 % (w/v) caffeine</th>
<th>1.0 M-NET+ 50 % (v/v) formamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 min</td>
<td>30099</td>
<td>502 1.7%</td>
<td>1594 5.4%</td>
<td>25314 84.4%</td>
<td>2599 8.7%</td>
</tr>
<tr>
<td>10 min</td>
<td>251788</td>
<td>1017 0.4%</td>
<td>26757 10.7</td>
<td>201744 80.1%</td>
<td>22270 8.8%</td>
</tr>
<tr>
<td>15 min</td>
<td>441788</td>
<td>1207 0.2%</td>
<td>46052 10.5</td>
<td>354682 80.3%</td>
<td>39766 9.0%</td>
</tr>
<tr>
<td>30 min</td>
<td>1334205</td>
<td>1765 1.2%</td>
<td>170556 12.8</td>
<td>1029767 77.1%</td>
<td>132117 9.9%</td>
</tr>
<tr>
<td>10 min+‘chase’</td>
<td>2316622</td>
<td>731 0.3%</td>
<td>694798 30</td>
<td>1478126 63.8</td>
<td>142967 6.2</td>
</tr>
</tbody>
</table>

* BSC-1 cultures infected with HSV were washed and replenished with fresh medium (pre-warmed to 37 °C) containing 200 μCi/ml ³H-thymidine at 13 h p.i. After further incubation for 3, 10, 15, 30 min and a 3 h ‘chase’ (see Methods) each culture was washed, lysed, treated with RNase and Pronase, and chromatographed on BND-cellulose as described in Methods.

DNA was analysed by chromatography on BND-cellulose columns. Table 2 shows that the amount of radioactivity incorporated into DNA molecules in infected nuclei in vivo gradually increased with time. However, the percentage of DNA molecules that elute with caffeine gradually decreased while the percentage of double-stranded DNA molecules gradually increased (Table 2). This indicates that most of the labelled thymidine is first incorporated into replicating DNA molecules, that have single-stranded regions and are eluted by caffeine. As DNA synthesis continues labelled DNA molecules are formed which elute from BND-cellulose with 1.0 M-NET buffer as double-stranded DNA. Unfortunately, the efficiency of the ‘chase’ with unlabelled thymidine differs in different experiments, most probably due to the state of the infected cells (Tables 1 and 2 ‘chase’).

DNA synthesis in untreated HSV infected nuclei in vitro

In a previous report (Becker & Asher, 1975) we demonstrated that nuclei from HSV infected BSC-1 cells continue to synthesize HSV DNA in vitro in the presence of the four deoxyribonucleoside triphosphates, 6 mM-Mg²⁺ and 8 % (w/v) sucrose.

The labelled DNA synthesized in nuclei from HSV-infected cells incubated in vitro for 10 and 60 min was analysed by chromatography on BND-cellulose columns and by centrifugation in CsCl gradients. The results presented in Fig. 4 and Table 3 show that 26 % of the labelled DNA molecules, synthesized in vitro during a 10 min period, eluted with 1.0 M-NET as double-stranded DNA. The rest of the labelled DNA (73.5 %) eluted with caffeine and formamide as DNA molecules containing single-stranded sequences (Fig. 4a, Table 3). The nature of the DNA molecules labelled within 10 min that eluted with formamide is not yet known. In other experiments (not shown) about 4 to 5 % of the labelled DNA eluted with formamide after a 10 min labelling period. After 60 min of DNA synthesis in vitro 40.5 % of the labelled molecules eluted as double-stranded DNA while 59.1 % eluted as DNA molecules with single-stranded sequences (Table 3). This demonstrates that a decrease in the amount of DNA molecules with single-stranded sequences is accompanied by an increase in the relative content of double-stranded sequences (Table 3).

The labelled DNA molecules synthesized in isolated nuclei during a 10 min period were chromatographed on BND-cellulose and centrifuged in CsCl density gradients. Most of the
Fig. 4. Characterization of HSV DNA synthesized in vitro in isolated nuclei by BND-cellulose chromatography. BND-cellulose chromatography of the reaction products synthesized for 10 min in vitro in the nuclei of BSC-I cells infected with HSV in the absence (a) and presence (b) of hydroxyurea (HU) at a final concentration of 5 x 10^{-2} M. The nuclei were isolated at 18 h p.i. and added to the in vitro DNA synthesizing system as described by Becker & Asher (1975). The in vitro reactions were allowed to continue for 10 min and then stopped. The nuclei were lysed, treated with RNase and Pronase and sheared prior to chromatography on BND-cellulose as described in Methods.

DNA molecules that eluted from the column as double-stranded DNA (Fig. 4a, region a) or as DNA molecules with single-stranded sequences (Fig. 4a, region b) banded in CsCl gradients at the density of virion DNA (1.718 g/ml; Fig. 5a and b, respectively). Some labelled cellular DNA (density 1.690 to 1.700 g/ml) was also detected in the two preparations. These results show that virus DNA molecules are mostly synthesized under in vitro conditions as was previously reported (Becker & Asher, 1975).
Table 3. Analysis of HSV DNA synthesized in isolated nuclei in vitro by chromatography on BND cellulose*

<table>
<thead>
<tr>
<th>Elution with</th>
<th>1·0 M-NET+ 2 % (w/v)</th>
<th>1·0 M-NET+ 50 % (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>caffeine</td>
<td>formamide</td>
</tr>
<tr>
<td>Used (ct/min)</td>
<td>Recovered (ct/min)</td>
<td>% (ct/min)</td>
</tr>
<tr>
<td>10 min</td>
<td>1·12 × 10⁶</td>
<td>9·8 × 10⁵</td>
</tr>
<tr>
<td>60 min†</td>
<td>8 × 10⁵</td>
<td>6·3 × 10⁴</td>
</tr>
<tr>
<td>10 min + HU</td>
<td>7·75 × 10⁵</td>
<td>5·86 × 10⁴</td>
</tr>
</tbody>
</table>

* See the legend to Fig. 4.
† In vitro synthesis for 60 min.

DNA synthesis in vitro in nuclei from HSV-infected HU-treated cells

Treatment of HSV-infected cells with HU resulted in the inhibition of HSV DNA synthesis (Rosenkranz & Becker, 1973). Incubation of nuclei from HSV-infected HU-treated cells in vitro leads to the synthesis of ³H-TMP labelled DNA during the initial 10 min only and the analysis of the in vitro synthesized DNA revealed mainly cellular DNA molecules (Becker & Asher, 1975). To determine whether the incorporation of ³H-TMP into cellular DNA is due to semiconservative synthesis of the latter or due to repair synthesis, nuclei from HU-treated HSV-infected cells were incubated for 10 min under in vitro conditions, and the DNA was analysed by chromatography on BND-cellulose columns. If DNA repair synthesis rather than chain elongation were responsible for the incorporation, we would expect to see much of the label incorporated in the presence of HU into material eluting from BND-cellulose with 1·0 M-NET buffer (Scudiero et al. 1975). In addition, repair generates gaps so small they are not seen as single-stranded fragments on BND-cellulose. It appears (Higgins, Kato & Strauss, 1976) that unless the gaps can be filled in (e.g. there is repair capacity) the damage is not even excised. Fig. 4(b) shows that most (92 %, Table 3) of the radioactivity was present in DNA fragments that were eluted with caffeine (87 %) and with formamide (5 %). Only 8 % of the radioactivity was eluted with 1·0 M-NET buffer (Table 3). These results demonstrate that ³H-TMP is incorporated into DNA fragments which contain single-stranded sequences. This species (Fig. 4b, region c) was analysed by centrifugation in CsCl gradients (Fig. 5c). The DNA synthesized in vitro under such conditions was indeed found to be mainly cellular DNA (density 1·700 g/ml). The nature of the labelled DNA molecules that co-sediment with the virus DNA, are still to be studied.

BND-cellulose analysis of DNA molecules synthesized in vitro in nuclei isolated from HSV infected cells incubated in arginine deficient medium

Incubation of HSV-infected BSC-1 cells in an arginine-deficient medium results in inhibition of the synthesis of virus particles while the synthesis of HSV DNA is unaffected (Becker, Olshevsky & Levitt, 1967). Incubation of nuclei from HSV-infected arginine-deprived cells showed the presence of normal levels of DNA-dependent DNA polymerase activity and an unaffected synthesis of virus DNA in vitro (Becker & Asher, 1975).

Analysis of the DNA molecules synthesized in vitro in nuclei isolated from infected, untreated and arginine deprived cells on BND-cellulose columns is presented in Fig. 6(a) and (b). It can be seen that 11·6 % of the label was incorporated into double-stranded
Fig. 5. CsCl gradient analysis of the DNA isolated by chromatography on BND-cellulose. The DNA fractions eluted from the BND-cellulose columns by 1.0 M-NET (region a in Fig. 4a) and by 1.0 M-NET and 2.7% (w/v) caffeine (region b in Fig. 4a; region c in Fig. 4b) were further analysed by centrifugation in CsCl gradients (see legend to Fig. 3).
Fig. 6. Chromatography of DNA synthesized in vitro in nuclei from HSV-infected cells. Three HSV-infected BSC-1 cultures were used: (a) control with no treatment; (b) cells infected in medium without arginine and (c) cells infected in the presence of ara-C at a final concentration of 50 μg/ml. The nuclei were isolated at 18 h p.i. and placed in the in vitro DNA synthesizing system as described by Becker & Asher (1975). The in vitro reactions were allowed to proceed for 10 min (a, b, c) or 30 min (a₁, b₁, c₁) prior to lysis of the nuclei, treatment with RNase and Pronase and BND-cellulose chromatography of the products, as described in Methods.
DNA molecules after incubation for 10 min in vitro (Fig. 6a). After 30 min incubation 20.1% of the labelled DNA molecules chromatographed as double-stranded DNA fragments (Fig. 6a1). Fewer DNA fragments were eluted by caffeine after a 30 min incubation period as compared to 10 min (Fig. 6a and a1). Similar results were obtained with DNA molecules synthesized for 10 (Fig. 6b) and 30 min (Fig. 6b1) in nuclei from arginine deprived cells.

BND-cellulose analysis of in vitro synthesized DNA in nuclei from HSV-infected cells treated with ara-C

Analysis of the DNA synthesized in vitro in nuclei isolated from ara-C treated BSC-1 cells revealed that only cellular DNA molecules were synthesized (Becker & Asher, 1975). Chromatography on BND-cellulose showed that most (about 90%) of the labelled DNA fragments labelled in vitro for 10 min eluted with caffeine and formamide (Fig. 6c) and 9% eluted with 1.0 M-NET buffer. Incubation of ara-C treated nuclei for 30 min did not result in an increase in 3H-TMP incorporation into DNA and the chromatography on BND-cellulose showed that 12% of the DNA eluted with 1.0 M-NET buffer and 88% eluted with caffeine and formamide (Fig. 6c1). This indicates that 3H-TMP is incorporated into cellular DNA which has single-stranded sequences.

DISCUSSION

The availability of BND-cellulose with the ability to bind double-stranded DNA molecules, makes it possible to analyse DNA molecules during their biosynthesis in virus-infected cells in vivo and in infected nuclei in vitro. Double-stranded DNA molecules that lack single-stranded regions elute from BND-cellulose with 1.0 M-NaCl. This leaves the replicating DNA molecules, that have single-stranded sequences in the fork regions of the growing DNA double helix, still bound to the column. This DNA species can be eluted with a solution of 1.0 M-NaCl containing 2% (w/v) caffeine (Kiger & Sinsheimer, 1969; Sussenbach et al. 1972; Scudiero & Strauss, 1974). This technique was utilized for the isolation of the replicating DNA molecules of adenovirus (Sussenbach et al. 1972) and φX174 (Kiger & Sinsheimer, 1969). Scudiero & Strauss (1974) also utilized this technique for the isolation of fragments of cellular DNA molecules that serve as the templates for DNA synthesis.

In the present study we used the BND-cellulose technique to study the DNA species synthesized in HSV-infected cells. Shearing of the DNA from the infected cells to fragments of about 20 x 10^6 daltons prior to chromatography on BND-cellulose columns, made it possible to distinguish between newly replicated virus DNA and fragments of virus DNA that contain the replication forks, the site of DNA synthesis on the virus DNA molecules.

We have demonstrated that labelled thymidine is incorporated in vivo into DNA fragments containing single-stranded regions. Only after a longer incubation period did the radioactive DNA fragments behave as newly replicated double-stranded molecules that lack single-stranded sequences. This happens in DNA molecules synthesized both in vivo and in isolated nuclei in vitro.

The DNA fragments that eluted from BND-cellulose as double-stranded molecules with 1.0 M-NET buffer and fragments containing single-stranded sequences that eluted with caffeine, were analysed by centrifugation in CsCl density gradients. Most of the DNA fragments that behaved as double-stranded DNA are of virus origin, although labelled cellular DNA fragments were also found. In addition, the labelled DNA fragments which have single-stranded sequences band at the density of virion DNA (1.718 g/ml). This
finding suggests that virus double-stranded DNA containing single-stranded sequences cannot be separated from mature virion DNA by centrifugation in CsCl density gradients. The analyses in CsCl gradients also revealed that labelled cellular DNA fragments with single-stranded sequences can be isolated from the infected cells. From previous studies it seems justified to conclude that the DNA fragments that elute from BND-cellulose with caffeine represent fragments of virus DNA and cellular DNA that contain replication forks and are the site for DNA chain elongation. The percentage of double-stranded fragments increased with the time of DNA synthesis (Table 2). This suggests that the synthesis of DNA occurs in the DNA fragments which have single-stranded sequences. As a result of chain elongation labelled double-stranded DNA fragments are subsequently formed. The mechanism of HSV DNA replication is currently being investigated.

Analysis of the DNA molecules synthesized in vitro in nuclei isolated from HSV-infected cells that were untreated or incubated in the absence of arginine or treated with metabolic inhibitors, demonstrated that the mechanism of HSV DNA synthesis in vitro and in vivo may be similar. The labelled \(^{3}\text{H}\)-TMP is incorporated into DNA fragments that have single-stranded DNA sequences. Thus, the synthesis of HSV DNA in isolated nuclei proceeds by the incorporation of the four nucleoside-triphosphates into the elongating DNA chains, a process mediated by a DNA polymerase specified by the virus (Weissbach et al. 1973; Becker & Asher, 1975). Labelled double-stranded DNA molecules are synthesized under in vitro conditions but it is not possible to conclude whether the synthesis of these DNA molecules can be completed in vitro. In a previous study (Becker & Asher, 1975) we demonstrated that only cellular DNA was synthesized in vitro in nuclei isolated from HSV-infected cells treated in vivo with HU and ara-C. The analysis of the DNA fragments labelled in vitro in nuclei from HU and ara-C treated cells revealed that cellular DNA synthesis occurs by incorporation of the deoxyribonucleoside precursors into DNA molecules which have single-stranded sequences. The reason why HU and ara-C completely inhibit the synthesis of virus DNA but not the synthesis of cellular DNA is not yet known.

The present study revealed the existence of single-stranded sequences in fragmented HSV DNA molecules, indicating that virus DNAs with single-stranded regions are probably involved in the DNA replicative process. Since HSV DNA fragments with single-stranded sequences were found to have the same buoyant density as double-stranded virus DNA, it was concluded that the replicative intermediates of HSV DNA would band in CsCl gradients together with virion DNA. Based on this conclusion, further experiments were done to isolate intact replicative intermediates of HSV DNA by chromatography on BND-cellulose (Shlomai et al. 1976).

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