The Structure of Herpes Simplex Virus Type 1 DNA as Probed by Micrococcal Nuclease Digestion

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SUMMARY

Micrococcal nuclease digestion was used to probe the structures in which herpes simplex virus type 1 (HSV-1) DNA is found during virus replication. Parental DNA, progeny DNA and DNA in nucleocapsids were analysed. Parental DNA was examined after infection of Vero cells with 32P- or 3H-thymidine-labelled HSV-1. Progeny DNA was examined after HSV-1-infected Vero cells were pulse-labelled with 3H-thymidine during HSV-1 DNA synthesis. In both cases, nuclei were isolated and digested with micrococcal nuclease. Digestion products were analysed by agarose or polyacrylamide gel electrophoresis (PAGE). Most parental DNA remained as intact molecules. However, a small amount was degraded into fragments which were heterogeneous in size or the size of nucleosomal cell DNA. These two classes of fragments were also produced upon digestion of progeny DNA. The heterogeneous fragments and nucleosomal fragments comprised major and minor fractions, respectively, of digested progeny DNA. When digested DNA from HSV-1-infected cells was transferred from composite polyacrylamide-agarose gels to diazobenzyloxymethyl paper, nucleosomal fragments hybridized to 32P-labelled HSV-1 DNA as well as to 32P-labelled Vero cell DNA. Therefore, nucleosomal fragments contained HSV-1 DNA sequences. HSV-1 DNA in nucleocapsids was analysed by micrococcal nuclease digestion after nucleocapsids were disrupted with pH 9.3 buffer, pyridine, Sarkosyl or NaCl/urea. Only fragments of heterogeneous size were produced. Thus, HSV-1 DNA is found predominantly in structures other than nucleosomes during virus replication.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large, complex virus. The virus particle contains about 24 proteins (Spear & Roizman, 1972) and double-stranded (ds)DNA with a mol. wt. of about 100 × 10⁶ (Keiff et al. 1972). As many as 50 proteins are induced during infection (Hones & Roizman, 1973). The site of HSV-1 DNA synthesis and assembly is the nucleus of the cell. Very little is known about the proteins and other molecules which are associated with HSV-1 DNA during virus replication. In the virus particle, VP21, a minor protein, may be associated with HSV-1 DNA since it is found only in full capsids (those which contain DNA) but not in empty capsids (Gibson & Roizman, 1972). Spermine may also be associated with HSV-1 DNA in the virus particle (Gibson & Roizman, 1971).

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Micrococcal nuclease digestion has provided useful information about the structure of DNA in eukaryotic cells. This DNA is folded in repeating units, nucleosomes. A nucleosome consists of 140 to 200 base pairs (bp) of DNA wrapped around a complex of eight histone molecules (Kornberg, 1977). When nuclei are digested with micrococcal nuclease for short times, discrete DNA fragments are produced which are integral multiples of the basic nucleosomal repeat unit. After long digestions, fragments the size of DNA in mononucleosomes are observed. Finally, a core fragment of 140 bp and other specific sub-nucleosomal fragments are found. These fragments represent regions of DNA which are protected from digestion by association with histones.

The structure of the DNA of different viruses has also been probed by micrococcal nuclease digestion. When nucleoprotein complexes of the papovaviruses simian virus 40 and polyoma virus are isolated from nuclei of infected cells or virus particles and are digested with micrococcal nuclease, a series of nucleosomal DNA fragments is produced (Cremisi et al. 1976; Ponder et al. 1978). When adenovirus type 2 (Ad2) is disrupted and digested with micrococcal nuclease, a heterogeneous population of DNA fragments results (Tate & Philipson, 1979). However, nucleosomal DNA fragments are found when intranuclear Ad2 parental DNA is digested (Sergeant et al. 1979; Tate & Philipson, 1979). Thus, Ad2 DNA is organized in nucleosomes during only a part of the virus replication cycle while papovavirus DNA is organized in this manner throughout the replication cycle.

In this study, as an initial approach to the characterization of the structures in which HSV-1 DNA is found during virus replication, three different forms of HSV-1 DNA were analysed by micrococcal nuclease digestion: parental DNA and progeny DNA in nuclei and DNA in nucleocapsids. The results of this study suggest that during virus replication most HSV-1 DNA is organized in structures which differ from nucleosomes.

**METHODS**

*Preparation of labelled virus.* ³H-thymidine-labelled virus was prepared by infecting confluent monolayers of Vero cells in four roller bottles (650 cm², approx. 5 × 10⁷ cells/bottle) with HSV-1 strain KOS (10 to 20 p.f.u./cell). Virus was added to each roller bottle in 10 ml of virus medium (Eagle's minimum essential medium containing 2% foetal calf serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin). After 2 h at 37 °C in a 5% CO₂ atmosphere, an additional 10 ml of virus medium was added to each roller bottle. ³H-thymidine (5 µCi/ml, New England Nuclear, Boston, Mass., U.S.A.) was added at 5 h p.i. and incubation was continued. Cells were harvested at 24 h p.i. and suspended in 1.5 ml of 10 mM-tris-HCl, pH 7.4, 5 mM-MgCl₂. After incubation at 4 °C for 10 min, a cell extract was prepared with a Dounce homogenizer and centrifuged at 800 g for 10 min. The resulting supernatant fraction was made 100 mM in NaCl and was digested with 50 µg/ml each of DNase I and pancreatic RNase for 30 min at 37 °C. This fraction was layered on a linear 35 ml 12 to 52% (w/v) sucrose gradient in 10 mM-tris-HCl, pH 7.4, 150 mM-NaCl and was centrifuged in a Beckman SW27 rotor at 24000 rev/min for 45 min at 4 °C. After centrifugation, fractions were collected from the bottom of the tube and assayed for ³H-thymidine-labelled material and infectious virus. A peak containing the maximum amount of ³H-thymidine-labelled material and infectious virus was found approximately two-thirds down the gradient. These fractions were pooled. Phosphate-buffered saline (PBS) was layered over them and they were centrifuged at 24000 rev/min for 1 h at 4 °C. The resulting visible pellet was suspended by sonic treatment (3 min in an ultrasonic cleaner) in Medium 199 containing 10% foetal calf serum. Virus was stored at −70 °C.

³²P-labelled HSV-1 was prepared in a similar way except that Vero cells were pre-labelled in 30 ml of ³²P-labelling medium (phosphate-free Dulbecco’s modified minimum
essential medium containing 2% dialysed foetal calf serum and 80 μCi 32P-phosphoric acid, New England Nuclear) for 12 h. Cells were infected in 10 ml of 32P-labelling medium. After 2 h, the remainder of the 32P-labelling medium was returned to the cells and incubation continued.

**Preparation and disruption of nucleocapsids.** Nucleocapsids were prepared from cytoplasmic virus. Cells were infected and labelled as above. At 24 h p.i., cells were harvested, suspended in 10 mM-tris-HCl, pH 7.4, 5 mM-MgCl2, incubated in the presence of 1% Nonidet P-40 for 10 min at 4 °C and disrupted with a Dounce homogenizer. After centrifugation at 800 g for 10 min, the supernatant fraction was treated with deoxycholate, DNase I, pancreatic RNase (50 μg/ml), Brij-58 and urea and clarified as previously described (Gibson & Roizman, 1972). This fraction was centrifuged on 12 to 52% sucrose gradients as described above. Nucleocapsids sedimented as a broad peak one-half to two-thirds down the gradient as determined by electron microscopy and the location of the 3H-thymidine-labelled DNA. These were pelleted and suspended in 2 ml of 10 mM-tris-HCl, pH 7.4.

Freshly prepared nucleocapsids were used in disruption experiments. Nucleocapsid suspensions were treated with (i) 150 mM-tris-HCl, pH 9.3 (4 °C), 20 mM-dithiothreitol; (ii) 10% pyridine; and (iii) 1% Sarkosyl as previously described for studies with polyoma virus (Ponder et al. 1978) and Ad2 (Prage et al. 1970; Brown et al. 1975). In addition, nucleocapsid suspensions were dialysed against 0.01 M-tris-HCl, pH 7.4, 0.5 M-NaCl, 5 M-urea for 12 h at 4 °C. After treatment, nucleocapsids were dialysed against 0.01 M-tris-HCl, pH 8, 0.25 M-sucrose or were layered on sucrose gradients.

**Infection of cells for examination of parental and progeny HSV-1 DNA.** A confluent monolayer of Vero cells in one roller bottle was infected with 32P- or 3H-thymidine-labelled HSV-1 (1 to 40 p.f.u./cell) in 10 ml of virus medium for analysis of parental DNA. After 2 h, 10 ml of virus medium was added and incubation continued. At various times after infection, cells were washed twice with cold PBS, harvested by scraping and pelleted.

For analysis of progeny HSV-1 DNA, Vero cells were infected with HSV-1 at 20 to 100 p.f.u./cell as described above. Between 12 and 16 h p.i., virus medium was removed and 10 ml of 3H-thymidine labelling medium (virus medium containing 10 μCi/ml of 3H-thymidine) was added. After 1 h the cells were harvested by scraping.

**Isolation of nuclei.** Nuclei were prepared by a modification of the procedure of Anderson et al. (1977). Freshly harvested cells from one roller bottle were suspended in 2 ml of nuclei buffer (10 mM-tris-HCl, pH 8, 5 mM-MgCl2, 0.5 mM-dithiothreitol, 1 mM-phenylmethylsulphonyl fluoride, 0.5 mM-spermidine, 0.3 mM-spermine) supplemented with 5 mM-KCl. After incubation at 4 °C for 10 min, they were disrupted with a Dounce homogenizer and diluted 10-fold with Triton buffer (nuclei buffer with 0.02% Triton X-100 and 0.25 M-sucrose). This extract was layered over 6 ml of 20% (w/v) Ficoll in nuclei buffer. After centrifugation in a Sorvall HS-4 rotor, at 6000 rev/min for 10 min at 4 °C, the nuclear pellet was washed in 30 ml of suspension buffer (10 mM-tris-HCl, pH 8, 50 mM-KCl, 5 mM-MgCl2, 0.5 mM-dithiothreitol, 0.5 mM-spermidine, 0.3 mM-spermine, 250 mM-sucrose). The final nuclear pellet was suspended in 0.25 ml of nuclease digestion buffer (10 mM-tris-HCl, pH 8, 1 mM-CaCl2, 0.5 mM-spermidine, 0.3 mM-spermine, 250 mM-sucrose) and incubated for 5 min at 37 °C before digestion. Nuclei were prepared from frozen uninfected cells when they were used as internal controls for micrococcal nuclease digestion of disrupted nucleocapsids.

**Micrococcal nuclease digestion.** For analysis of parental and progeny HSV-1 DNA, nuclei were digested for various times with 300 units/ml of micrococcal nuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Digestion was terminated by addition of 0.25 ml of 10% trichloroacetic acid (TCA) or 0.1 vol. of 100 mM-EDTA, 10% Sarkosyl.
For analysis of DNA in disrupted nucleocapsids, 5 \mu l each of 100 mM-CaCl₂, 50 mM-spermidine and 30 mM-spermine, 25 \mu l of a suspension of nuclei as described above and 10 \mu l of micrococcal nuclease (15 units/\mu l) were added to 0.5 ml samples of nucleocapsids. The reaction was terminated by addition of 50 \mu l of 100 mM-EDTA, 10% Sarkosyl at the appropriate times.

**Fractionation of DNA by electrophoresis.** DNA was extracted from nucleocapsid digests by deproteinization with Pronase (1 mg/ml for 2 h at 37°C) and water-saturated phenol. Samples were then digested with pancreatic RNase (0.1 mg/ml for 1 h at 37°C) and again extracted with water-saturated phenol. The DNA solution was made to 0.3 M in sodium acetate and precipitated with 2.5 vol. of 95% ethanol overnight at -70°C. Air-dried precipitates were dissolved in 0.01 x electrophoresis buffer (electrophoresis buffer is 40 mM-tris-acetate, 20 mM-sodium acetate, 2 mM-EDTA, pH 7.8). One-tenth vol. of 0.1% bromophenol blue, 0.05% xylene cyanol FF, 75% glycerol was added before electrophoresis.

DNA (0.5 to 10 \mu g) was fractionated on vertical 2% agarose or 8% polyacrylamide (0.27% methylene bisacrylamide) slab gels in electrophoresis buffer containing 0.5 \mu g/ml of ethidium bromide. Electrophoresis was for about 3 h at 100 V (agarose), 14 h at 50 V (polyacrylamide) or until the bromophenol blue tracking dye (fast dye) migrated out of the gel. When 3H-thymidine-labelled DNA was analysed, polyacrylamide gels were subjected to fluorography (Bonner & Laskey, 1974), dried on Whatman 540 paper and exposed to pre-flashed Kodak XR-5 film at -70°C (Laskey & Mills, 1975). When 32P-labelled DNA was analysed, gels were dried and exposed to the film at -70°C in the presence of a Cronex Lightning-Plus intensifying screen (Dupont, Wilmington, Del., U.S.A.).

G4 RF I DNA was used to prepare DNA standards. The DNA was labelled by nick-translation for 1 h at 12°C using 3H-dATP or \( \alpha^{32P} \)-dATP (Grindley & Godson, 1978) and was digested with restriction endonucleases AluI, HhaI or HinfI.

**Transfer and hybridization.** DNA was transferred from composite polyacrylamide-agarose gels (7.4% acrylamide, 0.6% N,N'-diallyltartardiamide, 0.6% agarose) to diazo-benzoyloxymethyl (DBM) paper as described (Reiser et al. 1978). After transfer, the DBM paper was treated with 0.45 M-NaCl, 0.045 M-sodium citrate, 0.2% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, 0.1% sodium dodecyl sulphate, 1% glycine and 50 \mu g/ml of denatured calf thymus DNA for 24 h at 65°C. Hybridizations were performed in the same buffer minus glycine for 48 h at 65°C. The DBM paper was then washed as described (Jeffreys & Flavell, 1977).

The HSV-1 DNA which was used as a probe for the hybridizations was extracted from virus purified from the cytoplasm of infected Vero cells and was further purified by CsCl density-gradient centrifugation. Cell DNA was extracted from nuclei of uninfected Vero cells. DNA was labelled by nick-translation using \( \alpha^{32P} \)-dCTP (600 Ci/mmol, Amersham Corp., Arlington Heights, Ill., U.S.A.). For hybridizations, \( 3 \times 10^6 \) to \( 8 \times 10^6 \) ct/min of \( ^{32P} \)-labelled DNA was used.

**RESULTS**

**Analysis of parental HSV-1 DNA**

Since transcription and replication of HSV-1 DNA occur in nuclei, these were analysed by micrococcal nuclease digestion to examine the structure of functional parental HSV-1 DNA. In initial experiments the kinetics of uptake of HSV-1 material into nuclei were measured. Vero cells were infected with \( ^{32P} \)-labelled HSV-1 at 2 p.f.u./cell. At various times after infection, cells were harvested and nuclei were prepared to determine the amount of \( ^{32P} \)-labelled virus material present. Uptake increased through 10 h p.i. when 10% of the total input label was found in nuclei (Fig. 1). Uptake was a function of m.o.i.
Micrococcal nuclease digestion of HSV-1 DNA

Fig. 1. Kinetics of uptake of $^{32}$P-labelled HSV-1 into nuclei. Vero cells (approx. $1 \times 10^7$ cells/culture) were infected with $^{32}$P-labelled HSV-1 at 2 p.f.u./cell in 10 ml of virus medium. This inoculum was not removed during the experiment. At the indicated times, cells were washed and harvested and nuclei were prepared as described in Methods. The radioactivity in the final suspension of nuclei was measured to determine the amount of uptake into nuclei. Percentage uptake was calculated based on the fact that a total of $6 \times 10^6$ ct/min of $^{32}$P-labelled HSV-1 was used to infect each culture. At least 80% of this input radioactivity was recovered from each culture.

Fig. 2. Kinetics of micrococcal nuclease digestion of $^{32}$P-labelled HSV-1 DNA ($\bigcirc$--$\bigcirc$) and $^3$H-thymidine-labelled cell DNA ($\bullet$--$\bullet$) in nuclei. Vero cells were pre-labelled with 1 $\mu$Ci/ml of $^3$H-thymidine for 12 h. The medium was then removed and $^{32}$P-labelled HSV-1 was added at 1 p.f.u./cell. At 10 h p.i., nuclei were prepared and digested with micrococcal nuclease (300 units/ml). At the indicated times, samples were removed to 10% TCA and centrifuged for 1 min in an Eppendorf microcentrifuge. The amount of radioactivity in the supernatant (fraction A) and in the pellet (fraction B) was determined. About 60% of the $^{32}$P in fraction B was $^{32}$P-labelled HSV-1 DNA based on the comparison, at time 0, of the amount of $^{32}$P found in fraction B before and after treatment with Pronase, phenol, RNase and ethanol as described in Methods. Therefore, for HSV-1 DNA, percent acid soluble = $\frac{[^{32}\text{P ct/min fraction A at time } t] - [^{32}\text{P ct/min fraction A at time } 0]}{[^{32}\text{P ct/min fraction A at time } t]} \times 100$. For cell DNA, percent acid soluble = $\frac{[^3\text{H ct/min fraction A at time } t] - [^3\text{H ct/min fraction A at time } 0]}{[^3\text{H ct/min fraction A at time } t]} \times 100$. A total of $7.5 \times 10^6$ ct/min of $^{32}$P and $2 \times 10^6$ ct/min of $^3$H were analysed for each time point. At time 0, $2000$ ct/min of $^{32}$P and $150$ ct/min of $^3$H were found in fraction A. CsCl density-gradient centrifugation of the DNA extracted from time 0 samples indicated that undetectable amounts of HSV-1 DNA were labelled with $^3$H-thymidine.

When cells were infected with HSV-1 at 2 and 10 p.f.u./cell, 5% and 24% of the total input label, respectively, was found in nuclei at 4 h p.i. This nuclear material was not virus which bound to nuclei during isolation. When $^{32}$P-labelled HSV-1 was mixed with nuclei and nuclei were re-isolated, less than 1% of the virus co-purified with nuclei.

Thus, based on these experiments, Vero cells infected with $^{32}$P-labelled HSV-1 (1 p.f.u./cell) were harvested at 10 h p.i. and nuclei were prepared and digested with micrococcal nuclease. Before infection the Vero cells were pre-labelled with $^3$H-thymidine so that the kinetics of solubilization of cell and HSV-1 DNA could be compared. These kinetics were different (Fig. 2). HSV-1 DNA was digested less extensively than cell DNA. After 20 min only about 10% of the HSV-1 DNA became acid soluble while about 60% of the cell DNA became acid soluble.

This resistance of parental DNA to solubilization by micrococcal nuclease was observed throughout infection. Vero cells infected with $^{32}$P-labelled HSV-1 at 2 p.f.u./cell were harvested at 0.5, 1, 2, 4 and 10 h p.i. Nuclei were prepared and digested with micrococcal
nuclease for 5 min. About 5% of the total $^{32}$P-labelled parental DNA became acid soluble. This result was also obtained when nuclei from Vero cells infected with 1 p.f.u./cell of HSV-1 were analysed at 10 h p.i. (Fig. 2).

![Agarose gel electrophoresis of cell DNA (upper panel) and $^{32}$P-labelled parental DNA (lower panel) after micrococcal nuclease digestion of nuclei from HSV-1-infected Vero cells. Upper panel, ethidium bromide-stained gel; lower panel, autoradiogram of the same gel. Samples of nuclei from the experiment described in Fig. 2 were analysed. Micrococcal nuclease digestion was terminated with 100 mM-EDTA, 10% Sarkosyl and DNA was extracted and subjected to electrophoresis as indicated in Methods. The times of digestion (min) are indicated. The material observed is DNA because, after extraction, a portion of the 0 min sample was digested extensively by DNase I (D). The DNA markers produced after AluI and HhaI digestion of a mixture of unlabelled and $^{32}$P-dAMP-labelled G4 RF I DNA are shown (G4). The sizes of some of these are indicated in base pairs (bp).]
Micrococcal nuclease digestion of HSV-1 DNA

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Fig. 4. Fluorogram of micrococcal nuclease-digested ³H-thymidine-labelled parental HSV-1 DNA and Vero cell DNA from nuclei. DNA was fractionated by PAGE. For analysis of parental HSV-1 DNA, Vero cells were infected with ³H-thymidine-labelled HSV-1 (40 p.f.u./cell). At 8 h.p.i., nuclei were prepared and digested. Uninfected Vero cells were labelled with ³H-thymidine (5 μCi/ml) for 1 h before analysis. The digestion times for the DNA from uninfected (U) and infected (I) samples are indicated. The fragments produced by HindI digestion of ³H-dAMP-labelled G4 RF I DNA are shown (G4).

The DNA fragments produced during micrococcal nuclease digestion were analysed. DNA was extracted from samples of nuclei from the experiment described in Fig. 2 and was analysed by electrophoresis on a 2% agarose gel (Fig. 3). Cell DNA was identified by ethidium bromide staining of the gel. Parental HSV-1 DNA was identified by autoradiography of the same gel. In the absence of nuclease, at zero time, cell DNA was of high mol. wt. and was found at the top of the gel. In the presence of micrococcal nuclease, after 0.5 to 2 min of digestion, the cell DNA was digested into a series of fragments which were multiples of about 190 bp. This is the pattern of DNA fragments expected after micrococcal nuclease digestion of DNA organized in nucleosomes (Kornberg, 1977). The digestion products of parental HSV-1 DNA were quite different. Throughout the entire digestion period most of the parental DNA remained as high mol. wt. DNA. This was
Fig. 5. Kinetics of micrococcal nuclease digestion of \(^3\)H-thymidine-labelled progeny HSV-1 DNA in infected nuclei (●—●), Vero cell DNA in uninfected nuclei (○—○) and naked Vero cell DNA (▲—▲). For analysis of progeny HSV-1 DNA, Vero cells were infected with 20 p.f.u./cell of HSV-1. At 16 h p.i., the culture was labelled with \(^3\)H-thymidine for 1 h. Uninfected cultures were labelled in parallel. Nuclei were prepared and digested with micrococcal nuclease (300 units/ml). For the naked DNA sample, DNA was extracted from nuclei before digestion. Digestions were stopped at the indicated times with 10\% TCA and percent solubilization of \(^3\)H-thymidine-labelled DNA was calculated as described in Fig. 2.

Fig. 6. Fluorogram of \(^3\)H-thymidine-labelled progeny HSV-1 DNA and Vero cell DNA after digestion with micrococcal nuclease in nuclei from infected (I) and uninfected (U) cells, respectively. Vero cells were infected with HSV-1 at 20 p.f.u./cell. At 12 h p.i., the culture was labelled with \(^3\)H-thymidine. Uninfected Vero cells were labelled in parallel. At 13 h p.i., nuclei were prepared and digested. DNA was then extracted and subjected to PAGE. Times of digestion are indicated. G4 \(HinfI\) DNA markers are shown.

intact DNA as determined by analysis on 0.7\% agarose gels with intact HSV-1 DNA as a marker (data not shown).

A small amount of parental DNA was digested into small fragments which migrated to the bottom of the 2\% agarose gel (Fig. 3). In order to investigate the nature of these small DNA fragments, parental DNA was analysed on 8\% polyacrylamide gels. Moreover, Vero cells were infected with \(^3\)H-thymidine-labelled HSV-1 at 40 p.f.u./cell to increase the amount of material available for analysis. At 8 h p.i., nuclei were isolated and digested with micrococcal nuclease. The resulting DNA fragments were analysed by polyacrylamide gel electrophoresis (PAGE) (Fig. 4). For purposes of comparison uninfected Vero cells were labelled with \(^3\)H-thymidine and also analysed. Cell DNA from uninfected nuclei was digested into a series of fragments typical of nucleosomal DNA. The major one observed was the 140 bp nucleosomal core fragment. The small amount of parental DNA which was digested into fragments was found in this region of the gel. A fragment the size of nucleosomal core DNA was observed as well as a smear of smaller fragments.
Micrococcal nuclease digestion of HSV-1 DNA

Analysis of progeny HSV-1 DNA

Vero cells were infected with HSV-1 at 20 p.f.u./cell and were labelled with ³H-thymidine for 1 h at various times after infection. The DNA which was labelled during this pulse was analysed by CsCl density-gradient centrifugation to determine the amount of the ³H-thymidine-labelled DNA which was HSV-1 DNA. When HSV-1-infected Vero cells were pulse-labelled from 16 to 17 h p.i., 90% of the ³H-thymidine-labelled DNA was HSV-1 DNA. Therefore, HSV-1-infected Vero cells were labelled with ³H-thymidine during this interval and nuclei were isolated and digested with micrococcal nuclease. Uninfected Vero cells were analysed in parallel. Progeny DNA was solubilized more rapidly and extensively than uninfected cell DNA (Fig. 5). After 40 min of digestion, about 90% of the progeny DNA had become acid soluble compared to only 50% of the uninfected cell DNA. The progeny DNA was degraded to almost the same extent as naked DNA.

In this experiment, since 90% of the ³H-thymidine-labelled DNA was HSV-1 DNA, the kinetics of solubilization of the ³H-thymidine-labelled DNA was essentially that of progeny DNA. However, because labelling was performed late in infection, a number of cells detached when virus medium was removed and ³H-thymidine labelling medium was added. Therefore, HSV-1-infected cells were labelled with ³H-thymidine from 12 to 13 h p.i. for analysis of the DNA fragments produced after micrococcal nuclease digestion. During this labelling period 70% of the ³H-thymidine-labelled DNA was HSV-1 DNA.

The DNA fragments produced after micrococcal nuclease digestion of nuclei from these labelled HSV-1-infected cells were quite different from those produced after digestion of nuclei from labelled uninfected cells (Fig. 6). Only specific nucleosomal fragments were observed for DNA from uninfected cells. In contrast, a smear of fragments as well as specific nucleosomal fragments were observed for DNA from infected cells. After 40 min of digestion, it was apparent that the former and latter comprised the major and minor fractions, respectively, of digested progeny DNA.

Examination of micrococcal nuclease-digested DNA by hybridization

The ³H-thymidine-labelled nucleosomal fragments which were identified in the analysis of progeny HSV-1 DNA (Fig. 6) might have originated from a small amount of cell DNA which was synthesized during the labelling period. Alternatively, a small amount of progeny DNA might be organized in nucleosomes. Likewise, a small amount of parental DNA might be organized in nucleosomes since ³H-thymidine-labelled nucleosomal DNA fragments were identified after micrococcal nuclease digestion of nuclei from cells infected with ³H-thymidine-labelled HSV-1 (Fig. 4). Thus, to determine if intranuclear HSV-1 DNA exists in nucleosomes, micrococcal nuclease-digested DNA from nuclei of HSV-1-infected cells was fractionated on composite polyacrylamide–agarose gels, transferred to DBM paper and hybridized to either ³²P-labelled HSV-1 DNA or ³²P-labelled cell DNA. Bands of about 280 and 140 bp were detected with the HSV-1 DNA probe for the 15 and 60 min digests (Fig. 7a; channels 3 and 4). These bands were also observed after hybridization with the cell DNA probe (Fig. 7b; channels 3 and 4). Therefore, both HSV-1 DNA and cell DNA sequences were present in nucleosomal DNA fragments.

The DNA probes used for hybridization were specific by two criteria: (i) the ³²P-labelled HSV-1 DNA probe hybridized to DNA fragments produced after Smal digestion of HSV-1 DNA while the ³²P-labelled cell DNA probe did not (Fig. 7a, b; channel 6) and (ii) the cell DNA probe hybridized to micrococcal nuclease-digested DNA from nuclei of uninfected cells while the HSV-1 DNA probe did not (Fig. 7a, b; channel 5).
Fig. 7. Autoradiogram of DNA fragments which hybridized to $^{32}$P-labelled HSV-1 DNA (a) or $^{32}$P-labelled Vero cell DNA (b) after micrococcal nuclease digestion of nuclei. Nuclei were prepared from HSV-1-infected Vero cells (m.o.i. of 100) at 12 h p.i. or from uninfected Vero cells and were digested with micrococcal nuclease (300 units/ml). After extraction of DNA, equal amounts of DNA samples were fractionated on a composite polyacrylamide-agarose gel for transfer to DBM paper and hybridization to (a) HSV-1 DNA or (b) cell DNA. (a, b) Channel 1 shows $^{32}$P-labelled HinfI DNA fragments; channels 2 to 4 indicate 2, 15 and 60 min digests of DNA from infected nuclei; channel 5 shows a 4 min digest of DNA from uninfected nuclei; channel 6 indicates HSV-1 SmaI DNA fragments.

**Analysis of HSV-1 DNA in nucleocapsids**

$^{32}$P-labelled nucleocapsids were treated with either pH 9-3 buffer, pyridine, Sarkosyl or NaCl/urea. After dialysis to remove the disrupting agent, the samples were digested with micrococcal nuclease. When DNA was extracted from the digests and fractionated by PAGE, a smear of DNA fragments was found (data not shown). When nucleocapsids were dialysed against 0.5 M-1 M- or 2 M-NaCl, the HSV-1 DNA present was not degraded by the nuclease (data not shown).

The pyridine-treated nucleocapsids were analysed in greater detail. Pyridine-treated $^{3}$H-thymidine-labelled nucleocapsids were fractionated on 5 to 20% sucrose gradients with a 77% sucrose cushion. Conditions of centrifugation were such that free HSV-1 DNA sedimented into the top third of the gradient. All the $^{3}$H-thymidine-labelled DNA in the pyridine-treated nucleocapsid sample sedimented into the sucrose cushion as did the $^{3}$H-thymidine-labelled DNA in the untreated nucleocapsid sample (data not shown). This result suggested that HSV-1 DNA was still associated with nucleocapsids after pyridine treatment. Electron microscopy of samples from the cushion region of these gradients
Micrococcal nuclease digestion of HSV-1 DNA

Fig. 8. Electron micrographs of (a) untreated and (b) pyridine-treated HSV-1 nucleocapsids. 

indicated that pyridine-treated nucleocapsids were similar in size to the untreated nucleo-
capsids (Fig. 8). However, the former were completely permeable to stain while the latter
were not.

The \(^{3}\text{H}\)-thymidine-labelled nucleocapsid samples in the cushion fractions were digested
with micrococcal nuclease and analysed by PAGE. The DNA in the pyridine-treated
nucleocapsid sample was digested into a heterogeneous population of fragments whose
size decreased with digestion time (Fig. 9). Most of the DNA in the untreated nucleocapsid
sample was not degraded, although a small amount was, probably due to disruption of a
limited number of nucleocapsids during their preparation. Naked HSV-1 DNA was
digested more rapidly than DNA in the pyridine-treated nucleocapsids (Fig. 9).

DISCUSSION

Parental and progeny HSV-1 DNA and DNA in nucleocapsids were probed by micro-
coccal nuclease digestion to obtain information about the structures in which HSV-1 DNA
resides during virus replication. Since transcription and synthesis of HSV-1 DNA occur
in nuclei, parental and progeny DNA were analysed in nuclei so as to decrease the chance
of disrupting these structures. Digestion of nuclei also allowed for analysis of cell DNA
during HSV-1 infection. Ethidium bromide-stained gels of digested DNA from nuclei of
infected cells revealed fragment patterns typical of nucleosomal DNA (e.g. Fig. 3). Thus,
HSV-1 infection does not disrupt the nucleosomal structure of cell DNA.

Parental DNA was examined between 0.5 and 10 h p.i. by micrococcal nuclease digestion
of nuclei prepared from Vero cells infected with \(^{32}\text{P}\)-labelled HSV-1 at 1 to 2 p.f.u./cell.
After 5 min digestion about 95% of the parental DNA remained acid insoluble. Most of
this appeared to be intact HSV-1 DNA. Thus, these results suggest that, throughout
virus replication, only a few parental DNA molecules are uncoated and available as tem-
plates for transcription and DNA replication. This inefficient uncoating could explain the
observation that only a small amount of parental DNA participates in HSV-1 DNA
synthesis (Jacob & Roizman, 1977).
Fig. 9. Fluorogram of DNA from $^3$H-thymidine-labelled HSV-1 nucleocapsids after digestion with micrococcal nuclease and PAGE. Fractions from the cushion region of the 5 to 20% sucrose gradient described in the text were digested. Samples of DNA from a G4 HindIII digest (G4), naked DNA extracted from nucleocapsids before digestion (DNA), untreated nucleocapsids (UN) and pyridine-treated nucleocapsids (PYR) are indicated together with digestion times.

The small amount of parental DNA which was uncoated was digested into fragments which were heterogeneous in size or the size of nucleosomal DNA. These two classes of DNA fragments were also observed when progeny DNA was analysed. The major fraction of digested progeny DNA consisted of the heterogeneous fragments while the minor fraction contained the nucleosomal fragments.

Since small amounts of nucleosomal DNA fragments were found when parental and progeny HSV-1 DNA were analysed, transfer and hybridization experiments were performed to determine if HSV-1 DNA sequences were contained in these fragments. HSV-1 DNA sequences were detected. However, quantification of the amount of intranuclear HSV-1 DNA present in nucleosomes was not possible from these experiments. DNA
Micrococcal nuclease digestion of HSV-1 DNA

fragments were transferred from gels to DBM paper instead of to nitrocellulose paper to ensure that small DNA fragments would be retained on the paper for hybridization (Reiser et al. 1978). However, small DNA fragments still presented a problem. When samples of ³H-thymidine-pulse-labelled, nuclease-digested DNA from nuclei of HSV-1-infected cells were fractionated on gels and then analysed by either fluorography or transfer and hybridization, different results were obtained. Fluorography revealed DNA fragments smaller than 130 bp, as in Fig. 6. These were not observed after transfer and hybridization as in Fig. 7. Thus, these small DNA fragments either were lost during treatment of the gel in preparation for hybridization or did not hybridize efficiently. Both problems have been noted previously (Reiser et al. 1978).

Although an estimate of the amount of intranuclear HSV-1 DNA which exists in nucleosomes cannot be made from the transfer and hybridization experiment of Fig. 7, an estimate of the amount of progeny HSV-1 DNA in nucleosomes can be made from the fluorogram in Fig. 6. After 40 min of digestion, only one major nucleosomal DNA fragment is observed. Most of the heterogeneous fragments are smaller in size than this major nucleosomal fragment. Thus, a densitometer tracing of this sample was used to calculate the amount of nucleosomal DNA relative to the total amount of DNA in the gel. The major nucleosomal DNA fragment comprised about 10% of the total DNA. However, the amount of nucleosomal progeny HSV-1 DNA is less than this because (i) 30% of the ³H-thymidine-labelled DNA was cell DNA and (ii) very small DNA fragments would have migrated out of the gel. Thus, a minor fraction, less than 10%, of progeny HSV-1 DNA may be organized in nucleosomes between 12 and 13 h p.i. in cells infected with HSV-1 at 20 p.f.u./cell.

While these experiments were in progress, two other groups reported results on micrococcal nuclease digestion of herpesvirus DNA. Mouttet et al. (1979) probed ³H-thymidine-pulse-labelled progeny HSV-1 DNA in nuclei and found random cleavage, as was found in this study. However, no evidence was obtained for progeny DNA in nucleosomes. Perhaps this class of progeny DNA was not detected because it is such a minor species. Furthermore, it may not have been detected because, although a similar m.o.i. was used in the two experiments, Mouttet et al. (1979) labelled HSV-1-infected cells from 6 to 7 h p.i. while we labelled cells from 12 to 13 h p.i. Thus, it may be that small amounts of progeny HSV-1 DNA are organized in nucleosomes at late but not early times during virus DNA synthesis.

Shaw et al. (1979) analysed the DNA of another herpesvirus, Epstein-Barr virus (EBV). Two kinds of EBV-transformed cell lines exist; one kind produces virus while the second does not. The structure of EBV DNA in these two kinds of lines was compared. In the non-virus-producing Raji cell line, EBV DNA was organized in nucleosomes. In the virus-producing P3HR-1 cell line, most of the EBV DNA was not found in nucleosomes. When nuclei from P3HR-1 cells were digested with micrococcal nuclease, a smear of DNA fragments was found together with a small amount of nucleosomal fragments.

Thus, it appears that the major portion of nuclear progeny herpesvirus DNA produced during virus replication is not found in nucleosomes and is not associated with histones. Therefore, some other mechanism must exist for folding this DNA in the nucleus of the infected cell. The small amount of intranuclear HSV-1 DNA which is organized in nucleosomes may be associated with cell DNA as is the case for EBV DNA in transformed cell lines (Shaw et al. 1979). There is evidence that small amounts of both progeny and parental HSV-1 DNA may be associated with cell DNA during infection (Biegeleisen et al. 1977; Yanagi et al. 1979).

The organization of HSV-1 DNA in nucleocapsids was also analysed by micrococcal nuclease digestion. When nucleocapsids were treated with pH 9·3 buffer, pyridine, Sarkosyl
or NaCl/urea and then digested with micrococcal nuclease, a smear of DNA fragments resulted. Thus, HSV-1 DNA in nucleocapsids is not organized in nucleosomes since treatment of polyoma virus with pH 9.3 buffer and micrococcal nuclease results in the production of discrete nucleosomal fragments rather than a smear of fragments (Ponder et al. 1978).

Treatment of Ad2 with pyridine results in the release of a core particle containing DNA in association with virus polypeptides VII and V (Brown et al. 1975). When HSV-1 nucleocapsids were treated with pyridine, no DNA was released. Thus, no direct information was obtained about the structure of HSV-1 DNA in nucleocapsids. Nevertheless, the fact that HSV-1 DNA in pyridine-treated nucleocapsids was digested more slowly than naked DNA indicates that capsid proteins and perhaps a core protein limit the accessibility of the DNA to micrococcal nuclease. Additional studies on the architecture of HSV-1 nucleocapsids are in progress.

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