The Synthesis and Substructure of Herpesvirus DNA: the Distribution of Alkali-labile Single Strand Interruptions in HSV-1 DNA

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SUMMARY

Denatured DNA of herpes simplex virus was released from the particles using an alkaline detergent, Decon-75. The largest single strands sedimented on alkaline sucrose gradients with a mol. wt. of \(47.2 \pm 0.33 \times 10^6\), slightly less than half the value calculated for the intact duplex \((104 \times 10^6)\). About 50% of the DNA was found in fragments which sedimented slower than this in a heterogeneous manner. On agarose gel electrophoresis the largest strands migrated with a mol. wt. of \(40 \times 10^6\). The reason for this difference is not known but since the individual strands of T4 DNA were shown to migrate with slightly different \(R_e\) values, factors other than mol. wt. may affect the migration of single-stranded DNA. Two fragments of mol. wt. \(35 \times 10^6\) and \(30 \times 10^6\) were observed but the rest of the fragments remained unresolved by this technique. Virus DNA associated with the nucleus of infected cells had a much lower mol. wt. than particle DNA \((3.2 \times 10^6)\). Although this value increased if the DNA was isolated from intact cells, the average sedimentation coefficient of nuclear virus DNA was never as high as the largest strands of particle DNA.

When the largest single strands from particles were prepared by sucrose gradient fractionation they exhibited a unimodal mol. wt. distribution after both sedimentation and electrophoretic analysis. These ‘intact’ single strands were annealed and analysed by banding in CsCl gradients and by analyses employing the Neurospora crassa endonuclease. The results show that the ‘intact’ strands reassociated with the same kinetics and to the same extent as total virus DNA, suggesting that both strands of the duplex were present in equal amounts.

INTRODUCTION

The DNA of herpes simplex virus type I (HSV-I) has been shown to be a linear duplex, mol. wt. about \(100 \times 10^6\) (Becker, Dym & Sarov, 1968; Kieff, Bachenheimer & Roizman, 1971). The work of Kieff et al. (1971) and our own studies on the replication and substructure of the virus DNA, which had started early in 1971 showed that native HSV-I DNA could be isolated as intact molecules, although alkali-denatured DNA contained a considerable number of fragments smaller than intact single-strands. This situation has precedents in the bacteriophage field where it has been shown that the genome of phage T5 is a linear duplex with one intact strand and with 3 to 4 uniquely positioned interruptions in the other strand (Bujard, 1969; Jaquemin-Sablon & Richardson, 1970; Hayward & Smith, 1972a; Labedan et al. 1973). On the other hand, Reznikoff & Thomas (1969) showed that about
half of both strands of the duplex of phage SP 50 contained interruptions apparently located at random. Our initial studies using sucrose gradient fractionation and annealing indicated that the situation with HSV-I resembled that of SP 50, but Frenkel & Roizman (1972) proposed that only one strand of the HSV-I duplex was ever intact, although both strands could have interruptions giving rise to seven unique bands on sucrose gradients sedimentation (Frenkel & Roizman, 1972). Since the arrangement and nature of single strand interruptions could obviously have great relevance to the replication mechanism and/or transcription of the herpes virus genome, our investigations were extended to high resolution gel electrophoresis combined with molecular hybridization studies employing the *Neurospora crassa* endonuclease. The results of these various studies are reported.

**METHODS**

**Cells and viruses**

*Baby hamster kidney cells (BHK cells).* BHK 21 (C 13) cells were grown in monolayer cultures in Eagle's medium supplemented with 10% tryptose phosphate and 10% calf serum (ETC 10%) in slowly rotating 80 oz bottles as previously described (Macpherson & Stoker, 1962), or in 50 mm diam. plastic (Nuncion) Petri dishes in the same culture medium.

**Infection with herpes virus.** Cells were infected at an input multiplicity of 5 p.f.u./cell with herpes simplex virus type 1, strain α (HSV-I) by adding to the confluent cell sheet in each bottle (4 x 10^5 cells) 20 ml of virus suspension in Eagle's medium supplemented with tryptose phosphate and 5% calf serum (ETC 5%). The bottle was rotated for one h at 37 °C, the cell sheet washed with 20 ml medium and the incubation continued in a further 70 ml of medium. The same protocol was adopted for Petri dishes (4 x 10^6 cells) but infection was in a vol. of 0.2 ml and the final incubation in a vol. of 5 ml. Zero time was considered to be at the end of the adsorption period. In order to label DNA, [5-3H]-methylthymidine ([3H]-TdR; sp. act. 15 Ci/m-mol, Amersham) was added to the medium at various times after infection to a final concentration of 2 or 5 µCi/ml. When the period of labelling was more than 3 h, unlabelled TdR was added to a final concentration of 1 µM.

In order to label virus DNA with [32p] the cells were infected in ETC 5%-PO4 containing 10^{-6} M-phosphate, and exposed to 5 µCi/ml [32P]-orthophosphate from 2 to 17 h post-infection.

*Escherichia coli.* *Escherichia coli B,* for infection with phage T 4, were grown with aeration at 37 °C in synthetic TCG medium (Kozinski & Szybalski, 1959) with the phosphate omitted. For infection with phage T 5, *E. coli F* were grown in the defined MGM medium of Lanni (1961) containing only 15 mM-glucose and with added 10^{-2} M-CaCl2.

**Production of [32P]-labelled phage T 4.** Phage T 4 particles were grown in *Escherichia coli B* and purified by differential centrifuging, as described by Ritchie (1970) and Ritchie & Malcolm (1970). Carrier-free [32P]-orthophosphate (Amersham) was added to the cell culture 1 h before infection.

**Production of [3H]-labelled phage T 5.** *Escherichia coli F* were grown to a concentration of 2 x 10^8 cells per ml and [3H]-DL-glucose (168 mCi/m-mol, Amersham) was added to a final concentration of 1 µCi/ml. Growth was continued until the cells reached 4 x 10^8 per ml when they were infected at a calculated input multiplicity of 5 p.f.u./cell with phage T 5. After cell lysis the [3H]-labelled phage particles were purified by precipitation with polyethylene glycol and dextran sulphate as described by Albertsson (1967). The partially purified particles were finally centrifuged in CsCl and dialysed into a suitable buffer.
Fractionation of cells. Incubation of BHK monolayers was terminated at various times after infection and the cell sheets washed twice with ice-cold phosphate buffered saline (PBS; Dulbecco & Vogt, 1954). All subsequent operations were carried out at 0 °C. The cell sheet was loosened by brief treatment with 2.5 mg/ml trypsin. The cells were then washed carefully with PBS, and suspended in a small vol. of PBS. The cells were collected by centrifuging and lysed by resuspension in 0.01 M-KCl, 0.0015 M-MgCl₂, 0.01 M-tris-HCl, pH 7.5, (RSB) containing 0.5 % Nonidet P-40 (NP 40, BDH), at a concentration of 1.2 × 10⁷ cells/ml for cells from Petri dishes, and 2 × 10⁸ cells/ml for cells from bottles. After 5 min nuclei were separated from the cytoplasm by centrifuging, washed once in RSB plus 0.5 % NP 40 and finally resuspended in the same buffer at a concentration of 1.2 × 10⁷ or 2 × 10⁸ nuclei/ml, respectively. Separated nuclei and cytoplasm were stored in small vol. at −70 °C. The whole procedure took 20 min.

Virus was prepared from the infected cell supernatant fluid (cell released virus) by sedimenting at 15000 rev/min for 2 h at 4 °C in the 8 x 50 rotor of the MSE 18 centrifuge. The pellet was resuspended in a small vol. of RSB and stored at −70 °C.

Release of DNA from virus particles and cell fractions

Neutral release of DNA. Cell fractions were made 0.01 M with respect to EDTA and 2 % with respect to SDS. An equal vol. of phenol saturated with 0.01 M-EDTA, 0.01 M-tris-HCl, pH 7.5, was added and the solution gently agitated for 15 min at room temperature. The aqueous phase was separated by centrifuging at 12000g for 10 min at room temperature and carefully re-extracted with phenol. Phenol could be removed by dialysis against 0.001 M-EDTA, 0.01 M-tris-HCl, pH 7.5.

Alkaline release of DNA. The cell fraction was made 0.01 M with respect to EDTA. The alkaline detergent Decon-75 (BDH) was then added to a final concentration of 5 % followed by NaOH to 0.1 M. The final pH was 13. In some experiments the lysate was further extracted with water saturated phenol.

Fractionation of DNA

Sucrose gradients. Fifty to 200 µl samples of alkaline DNA extracts were centrifuged through linear gradients of 5 to 20 % sucrose in 0.9 M-NaCl, 0.001 M-EDTA, adjusted to pH 12.5 with NaOH, at 40000 rev/min for 95 min at 18 °C in the Beckman SW 65 Ti rotor. Fractions were sampled for the determination of acid-precipitable radioactivity by the paper disc method of Bollum (1959). Neutral DNA extracts were analysed in the same way on gradients of 5 to 20 % sucrose in 1.0 M-NaCl, 0.001 M-EDTA, 0.01 M-tris-HCl, pH 7.5.

Gel electrophoresis. Agarose gel electrophoresis was carried out as described by Hayward & Smith (1972a): 0.6 % agarose (Behringwerke, Germany) dissolved in TPS buffer (0.03 M-tris, 0.03 M-NaH₂PO₄, 1 mM-EDTA, 0.05 % SDS, pH 8.0) was refluxed for 10 min, then cooled to 45 °C, poured into 12 mm (internal diam.) perspex tubes (17 cm lengths) and allowed to set.

Fifty to 150 µl samples containing up to 50 µg of DNA were applied as 5 % sucrose solutions to the tops of the gels and electrophoresis was for 14 h at 3 V/cm and about 7.5 mA/gel in TPS buffer at 4 °C.

Gels containing radioactive DNA were fractionated into 1 mm slices using a Mickle gel slicer. Slices containing [³²P]-labelled DNA were dried on 25 mm discs of Whatman No. 1 paper and counted in toluene-based scintillation fluid. Slices containing [³H]- or [¹⁴C]-labelled DNA were oxidized in the Intertechnique Ltd Oxymat and the separate isotopes counted (efficiency 35 % [³H]; 65 % [¹⁴C]).
Unlabelled DNA could be detected by immersing the gels in 5% Stainsall (Eastman) diluted with 50% formamide. Visible bands of DNA appeared after destaining in 50% formamide.

CsCl gradients. DNA determinations were carried out on a Beckman Model E analytical ultracentrifuge using u.v. optics. Buoyant density determinations in CsCl were made as described by Schildkraut, Marmur & Doty (1962), using 0.5 to 2 μg of DNA plus 1 μg Clostridium perfringens DNA (ρ = 1.6915) as a density marker. Separation of radioactive DNA on CsCl gradients was carried out by centrifuging Oakridge tubes containing 9 ml gradients at 40000 rev/min for 3 days at 18 °C in the Ti 50 angle rotor of the Spinco model L.

Annealing studies. DNA–DNA annealing was carried out at DNA concentrations of between 0.5 and 1 μg/ml. The concentration of DNA in small vol. containing small amounts of DNA was determined by CsCl banding analysis in the analytical ultracentrifuge against a known standard of Clostridium perfringens DNA. DNA to be annealed was dialysed into 1 x SSC, 40% formamide, heated at 95 °C for 2 min and then held at 50 °C for various periods of time. In some experiments annealing was carried out at 75 °C in either 1 x SSC or 0.9 M NaCl, 1 mM-EDTA, 0.01 M-tris-HCl, pH 7.5, containing 10 to 15% sucrose.

Hybrid DNAs were analysed by banding in CsCl gradients or by testing with Neurospora crassa endonuclease as described below.

Neurospora crassa endonuclease tests. An endonuclease specific for polynucleotides lacking an ordered structure was isolated from the conidia of Neurospora crassa (Miles Chemicals) by the method of Linn & Lehman (1965). Purification was taken as far as the dialysed acetone fraction and the enzyme was stored at 4 °C. The preparations were found to have activities of about 0.6 units/ml when tested against denatured DNA by assay A of Linn & Lehman (1965). The preparations also contained an activity against native DNA (also described by Linn & Lehman, 1965), which was, however, less than 5% of the activity against denatured DNA.

DNA–DNA hybrids could then be tested for their degree of double-strandedness. Unlabelled native DNA from Krebs ascites cells (the gift of M. Wilkie) was added to each reaction in excess in order to reduce the activity against double-stranded DNA. The single-stranded DNA substrate was supplied by denatured ascites DNA and the radioactive DNA under test was present in only small amounts. The standard assay contained in a vol. of 100 μl; 200 μg native ascites DNA; 10 μg denatured ascites DNA, less than 0.1 μg of radioactive test DNA; 1 μ-mol of MgCl₂; 10 μ-mol tris-HCl, pH 8.0 and 0.003 units of enzyme activity. Incubation was carried out at 40 °C for up to 90 min and duplicate samples were taken for the measurement of acid precipitable radioactivity by the paper disc method of Bollum, 1959.

RESULTS

Kinetics of virus DNA synthesis

The time sequence of the appearance of virus DNA in BHK cell fractions after infection with HSV-I is shown in Fig. 1. Petri dish cultures exposed to 2 μCi/ml [3H]-thymidine and 1 μM unlabelled thymidine at 2 h post-infection were harvested and fractionated as described. Nuclei were lysed by addition of EDTA to 0.01 M and SDS to 0.5%, digested with 500 μg/ml pre-incubated pronase at 37 °C for 3 h and samples from all cell fractions were TCA-precipitated and counted. After the addition of [3H]-thymidine to the cell culture at 2 h post-absorption, a rapid increase of nuclear DNA synthesis levelled off and stopped at 8 h. Analysis on neutral CsCl gradients indicated that 90% of this labelled DNA banded in the position of HSV-I DNA and the remaining 10% banded as BHK cell DNA. Almost imme-
Fig. 1. Kinetics of the appearance of HSV-I DNA in cell fractions. The results are expressed as the average of duplicates but the nuclear DNA scale is 10 times that of the others. ×—×, nuclear DNA; •—•, cytoplasmic DNA; ○—○, cell-released DNA; △—△, infective virus. * Scale: \( \times 10^{-9} \) for cytoplasmic and cell-released DNA; \( \times 10^{-4} \) for nuclear DNA.

Immediately after the appearance of labelled nuclear DNA, \(^{3}H\)-DNA began to accumulate in the cytoplasm, levelling off at 9 to 10 h. After a delay of about 1 h virus DNA also began to appear in the culture medium, reaching its maximum level, which was somewhat higher than that of cytoplasmic DNA, at 14 h. The ratio of cell-released to cytoplasmic DNA at the end of the infectious cycle varied somewhat from experiment to experiment but was usually about 1 : 1. All of the cytoplasmic and cell-released DNA banded with the density of virus DNA in CsCl.

In the experiment shown in Fig. 1 only about 10% of the total DNA labelled between 2 and 24 h appeared in the combined extra nuclear fractions. (Note that the nuclear DNA scale is 10 times that of the others.) In other experiments this could be as much as 25% but in all cases the major portion of the virus DNA remained associated with the nucleus.

**Decon release of virus DNA**

In order to examine the physical properties of the virus genome it is most important to preserve the integrity of the DNA strands. Release of single-stranded DNA from cell fractions with detergent at pH 12.5 to 13.0 should confer the advantage of protection from nuclease attack during lysis. Initial studies showed that DNA could not be released satisfactorily from herpes virus particles with alkali alone nor with alkali plus SDS. However, the alkaline detergent Decon-75 released single-stranded virus DNA in good yield and with a sharp banding profile in CsCl gradients, (Fig. 2). Figs. 3 and 4 also show that Decon-75 also released DNA from phage T 4 and T 5 which could be subjected directly to sucrose gradient centrifuging or gel electrophoresis.
Fig. 2. Alkaline release of HSV-I DNA from virus particles. Twenty μl samples were made (a) 0.01 M with respect to EDTA then 2% with respect to SDS; (b) 0.01 M with respect to EDTA, 0.1 M with respect to NaOH and then 2% with respect to SDS; (c) 0.01 M with respect to EDTA and then 5% with respect to Decon-75, pH 12.5. Each solution was diluted with 0.6 ml CsCl, adjusted to pH 12.5 with NaOH and the densities were adjusted to 1.725. The samples were analysed in the analytical ultracentrifuge as described.

Sucrose gradient fractionation

Native DNA from mixed [32P]-T 4 virus particles and [3H]-cytoplasm from HSV-I infected cells was co-extracted and analysed by zone sedimentation through linear gradients of 5 to 20% sucrose (Fig. 3a). The HSV DNA sedimented in a single homogeneous band slightly slower than the T 4 marker DNA. The formula of Burgi & Hershey (1963) for neutral sucrose gradients, \[ \frac{D_1}{D_2} = \left( \frac{M_1}{M_2} \right)^{0.35} \], was used to relate the mol. wts. \((M_1\) and \(M_2\)) of the two DNA species to the distance travelled \((D_1\) and \(D_2\)). Assuming that the mol. wt. of T 4 DNA is \(120 \times 10^6\) (Luria & Darnell, 1968), the results of determinations on six different preparations gave a value of \(103.5 \pm 3.76 \times 10^6\) for HSV-I DNA.

When [32P]-T 4 particles were mixed with HSV-I infected cell cytoplasm and the single-stranded DNA released by Decon analysed on alkaline 5 to 20% sucrose gradients, the T 4 DNA still sedimented as a homogeneous band (Fig. 3b). However, the HSV DNA had a sharp leading band followed by smaller mol. wt. fragments which sedimeted in a heterogeneous manner. Using the relationship of Abelson & Thomas (1966) for alkaline sucrose gradients, \[ \frac{D_1}{D_2} = \left( \frac{M_1}{M_2} \right)^{0.38} \], the average of 16 determinations on separate preparations gave a value of \(47.2 \pm 0.33 \times 10^6\) for the leading band of HSV-I DNA. This is less than half the value calculated for double-stranded DNA, but it is not known whether the difference is real or whether it is due to some basic discrepancy in the method of measuring the mol. wts. of double- and single-stranded DNA of widely different base composition. Double-
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Fig. 3. Sucrose gradient analysis of HSV-I DNA. (a) Native cytoplasmic DNA on a neutral gradient. (b) Decon-released cytoplasmic DNA on an alkaline gradient. (c) Fraction 16 from Fig. 3 (b) dialysed against 0.1 M-NaOH and re-run on an alkaline gradient. (d) Alkaline gradient of Decon-released DNA from cytoplasm stored for 1 week at -70 °C. (e) Decon-released nuclear DNA on an alkaline gradient. (f) The infected cell monolayer was treated directly with 5% Decon-75, 0.01 M-EDTA, 0.1 M-NaOH and analysed on an alkaline gradient with Decon-released [32P]-T4 DNA. •--•, [3H]-HSV-I DNA; ○--○, [32P]-T4 DNA. Direction of sedimentation from right to left.

stranded and Decon-released DNA from cell-released virus gave the same patterns as cytoplasmic DNA.

When the DNA from the leading edge of the largest HSV-DNA band was re-run on an alkaline gradient (Fig. 3 (c)) it sedimented in a homogeneous manner. After nine determinations on different preparations the mol. wt. of this band was calculated to be 46.0 ± 0.52 x 10^6. There was no indication of contamination with smaller mol. wt. fragments. Until further evidence is forthcoming, we assume that this corresponds to intact single strands of the virus genome.

The amount of cytoplasmic or cell-released HSV-DNA which sedimented as fragments varied from experiment to experiment. In the best cases almost 50% of the DNA could be accounted for as intact single strands and in others a much smaller proportion appeared in this fraction. One factor which influenced this was found to be the length of storage of the cell fractions at -70 °C. Fig. 3 (d) shows that the pattern obtained when another sample of the cytoplasm used for Fig. 3 (b) was stored for 1 week and the experiment repeated. This time a much larger proportion of the virus DNA appeared in the fragments. This was not merely due to freezing and thawing because over a period of 2 weeks a steady deterioration in the condition of DNA from samples frozen only once has been noted. The reason for this breakdown is not known, but freshly isolated cell fractions were used in all of the work reported here.

Fig. 3 (e) shows that, in contrast to extranuclear virus DNA, the bulk of nuclear DNA released by Decon sedimented very slowly with very little material in the position expected for intact strands (intact [32P]-T4 particles added before lysis). The average of five deter-
Fig. 4. Agarose gel electrophoresis. (a) Decon-released [14C]-T 5 DNA (●●) and [32P]-T 4 DNA (○○○) run on parallel gels. (b) Decon-released [3H]-cytoplasmic HSV-1 DNA. (c) Fraction 16 from Fig. 3(b) dialysed against 0.1 M-NaOH before application to the gel.

Minations gave a value of $3.2 \pm 0.04 \times 10^6$ daltons for the main peak. However, if the DNA from infected cell monolayers was decon released prior to cell fractionation there was an appreciable increase in the average S value and more materials sedimented as fast as or even faster than intact DNA (intact [32P] T 4 particles added after lysis). Since about 80% of the total cell DNA was associated with the nuclei, this experiment suggests that despite the speed and care with which the cells were fractionated, some nuclease activity had occurred during the isolation of the nuclei.
Fig. 5. Neutral CsCl gradient analysis of annealed HSV-I DNA. In each case [32P]-labelled native HSV-I DNA and T 4 DNA were added before analysis to act as density markers. (a) [3H]-labelled alkali denatured cytoplasmic HSV-I DNA. (b) [3H]-labelled intact single stranded HSV-I DNA from neutralized sucrose gradient fractions as in Fig. 3(b). (c) As in Fig. 4(b) but the DNA annealed overnight at 75 °C (approximately 0.61 M-NaCl, 0.0067 M-EDTA and 10 % sucrose). (d) Endonuclease-treated hybrid. EDTA was added to a final concentration of 0.01 M before analysis.

- - [3H]-HSV-I DNA; O - - O, [32P]-HSV-I and T 4 DNA.

Agarose gel electrophoresis

Electrophoresis on agarose gels offers a considerable advantage over sucrose gradients in the resolution of single-stranded DNA (Hayward & Smith, 1972a, b). The distance migrated was found to be directly proportional to the log of the mol. wt. up to a tested mol. wt. of about $40 \times 10^6$. Single-stranded T 5 DNA, used as a marker in the present work, contains five major molecular species which have mol. wts. of 37, 45, 13.9, 5.1 and $3.8 \times 10^6$ and which give four discrete bands on agarose gel electrophoresis (Hayward & Smith, 1972a, and Fig. 4a). The technique is so reproducible that the marker can be used to estimate the mol. wt. of other DNA species co-electrophoresed on the same gel or run on a separate gel in the same experiment.

Decon-released T 4 DNA routinely gave two bands separated by about 2 to 3 mm
which ran with calculated mol. wts. of about $56 \times 10^6$ and $51 \times 10^6$, and which are assumed to be the separated intact strands of the parental T4 duplex (Hayward, 1972). This mol. wt. range is somewhat less than would be expected for single-stranded T4 DNA and it is possible that the strict linear relationship between distance migrated and log mol. wt. begins to break down about $40 \times 10^6$ daltons.

Decon-released cytoplasmic HSV-I DNA gave the electrophoretogram shown in Fig. 4(b). The sharp leading peak migrated with an $R_e$ equivalent to $40 \times 10^6$ daltons, some 6 to $7 \times 10^6$ daltons less than the value calculated from alkaline sucrose gradients. Two other distinct peaks at $35 \times 10^6$ and $30 \times 10^6$ daltons were repeatedly observed above a background of unresolved fragments which ranged from more than $30 \times 10^6$ to $5 \times 10^6$ daltons.

The ‘intact’ strands of HSV-I DNA purified from sucrose gradients (Fig. 3c) were also analysed and repeatedly found to migrate as a homogeneous band at an $R_e$ equivalent to $39 \times 10^6$ daltons (Fig. 4b). The reason for this small difference between total HSV-I DNA and the purified ‘intact’ strands is not known but the sharpness of the band reinforces our impression that the latter represents DNA with a unimodal mol. wt. distribution.

Re-association experiments

Denatured HSV-I DNA banded at a higher density in CsCl gradients than the native form (Fig. 5a). This is a general phenomenon and has been reported previously for a variety of DNA species by Szybalski (1968). Purified ‘intact’ single-stranded HSV-I DNA banded at the same density (Fig. 5b), but when it was annealed at 75 °C for 24 h in 0.6 M- NaCl, 1 mM-EDTA and 10 to 15% sucrose, reassociation occurred and the hybrid banded at a density still slightly higher than that of native DNA (Fig. 5c). The hybrid formed when denatured total HSV-I DNA was re-annealed behaved in exactly the same way; this result was also obtained when alkali denatured HSV-I DNA fragments, from which ‘intact’ strands had been removed, was annealed and banded in CsCl (not shown). When approximately 0.2 μg of the hybrid formed from re-associated ‘intact’ HSV-I DNA was also dialysed into 0.1 M-tris-HCl, pH 8.0, 0.15 M-NaCl, 0.002 M-MgCl2, 5 μg native Clostridium perfringens DNA added and the mixture digested for 15 min at 37 °C with 0.0025 units of Neurospora crassa endonuclease, most of the virus DNA was converted to a form with the same density as native DNA (Fig. 5d). This suggested the presence of tails or loops of single-stranded DNA before the enzyme treatment.

This experiment suggested that both strands of the duplex were present in purified ‘intact’ single-stranded HSV-I DNA in roughly stoichiometric amounts. The result was confirmed by further analysis of the hybrids with Neurospora crassa endonuclease. The characteristics of the reaction are shown in Fig. 6. Under the conditions selected, single-stranded virus DNA was rapidly hydrolysed to an acid-soluble form while 90 to 95% of the native DNA remained acid-precipitable for up to 90 min. Hybrids of either total or ‘intact’ HSV-I DNA gave an intermediate pattern. Using this reaction, therefore, the degree of double-strandedness of a DNA can be estimated.

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Purified ‘intact’ and denatured unfractionated HSV-I DNA were adjusted to the same concentration (about 1 μg/ml) and then annealed for various times at 50 °C in 1 x SSC, 40% formamide. The hybrid DNA was then digested with Neurospora crassa endonuclease and the content of duplex calculated as described in the legend to Fig. 7. The degree of reassociation was plotted against Cot (nucleotide concentration x time, mol-s/l; Britten & Kohne, 1968) and compared to the values obtained when the reassociation of 50 μg/ml unlabelled virus DNA was monitored optically at 275 nm in a Gilford 2400-S recording spectrophotometer (Fig. 7). Although the mol. wts. of the unlabelled DNA and the [3H]-
Fig. 6. Digestion of native, denatured and annealed HSV-I DNA with *Neurospora crassa* endonuclease. △—△, native HSV-I DNA; ×—×, denatured HSV-I DNA; ○—○, annealed total HSV-I DNA; •—•, annealed intact single strands of HSV-I DNA. (The position of duplicates and the mean are shown at each point.)

Fig. 7. Reassociation kinetics of HSV-I DNA. Hybrids of [³H]-DNA were tested with endonuclease as for Fig. 6. In each experiment the amount of digestion with double-stranded DNA was checked and single-stranded [¹⁴C]-T 5 DNA was included in each reaction mixture to monitor the digestion and the percentage of duplex DNA in the hybrids was calculated. The results are uncorrected for cation concentration and are presented in terms of Cot. ×—×, total HSV-I DNA monitored optically; △, total HSV DNA monitored with endonuclease; ○, intact single strands of HSV-I DNA monitored with endonuclease.
DNA were not identical, it can be seen that the endonuclease technique compared fairly well with the classical optical method. No difference could be detected in the reassociation rates of the [3H] total DNA and ‘intact’ single strands but the important point is that 80 to 85% of the intact strands become resistant to endonuclease digestion demonstrating that both strands of the duplex were present in equal proportions – unless each strand of the duplex exhibits extensive self-complementarity.

DISCUSSION

The mol. wt. of 103·5 ± 3·76 x 10⁶ found for native HSV-I DNA in this study is similar to previously published estimations by groups using sedimentation techniques and electron microscopy (100 x 10⁶; Becker et al. 1968) and sedimentation and reassociation studies (95 to 99 x 10⁶; Kieff et al. 1971; Frenkel & Roizman, 1972). In our best cases about 50% of alkali denatured DNA was found in ‘intact’ strands on sucrose gradient sedimentation and the remainder in fragments (Figs. 3c, 4b). Similar findings, showing, however, considerably more fragmentation, have been published for herpes simplex viruses types 1 and 2 and for Marek’s disease virus (Kieff et al. 1971; Lee et al. 1971). In the present study, ‘intact’ strands were found to sediment with a mol. wt. of only 47·2 ± 0·33 x 10⁶. The difference between this value and half the value for native HSV-I DNA (about 52 x 10⁶ daltons) might be due to the methods used. However, the possibility that alkali-labile bonds exist near the ends of both strands of every duplex cannot yet be excluded.

The use of alkaline detergent to lyse particles should ensure that DNA strands so obtained have the same length as when they pre-existed in virus. The finding of fragmented DNA therefore suggests that alkali-labile bonds also pre-existed in virus DNA. These could conceivably be single-strand breaks and/or RNA sequences. Any single-strand breaks cannot coincide in both strands of the duplex since native DNA was found to be intact (Fig. 3a).

Gel electrophoresis of alkali denatured DNA gave a mol. wt. of only 40 x 10⁶ for the ‘intact’ strands compared to the marker T 5 DNA (Figs. 4a, b). This is considerably smaller than the values found by the other methods already discussed and may be due to the high G +C content of HSV-I DNA compared to the other DNA species previously studied (Hayward & Smith, 1972a, b). On the other hand, the individual strands of the DNA from phage T 4 (this report), and phages T 2, T 1, T 7, λ and φ (Hayward, 1972) may be separated by agarose gel electrophoresis. Since each strand of the duplex has the same mol. wt. as its partner, this suggests that factors other than mol. wt. can influence the migration of single-stranded DNA. Individual strands of phage DNA are known to vary in G+C content, T+C content and in the distribution of pyrimidine-rich clusters (Thomas & McHattie, 1967), but at the moment we have no indication as to how these variations might affect the electrophoretic mobility. Only two major fragments could be distinguished at 35 x 10⁶ and 30 x 10⁶ daltons. This suggests that at least some of the interruptions may be distributed in a non-random (possibly unique) manner. The remainder of the fragments migrated over a wide range of mol. wt. and could not be resolved even by the high resolution available with gel electrophoresis. This is in direct contrast to the report by Frenkel & Roizman (1972), who claimed to distinguish seven unique fragments of HSV-I DNA by sucrose gradient sedimentation.

Nuclear virus DNA clearly sedimented with a lower average mol. wt. than particle DNA, even after direct extraction of the cell sheet with Decon-75 (Figs. 3e, f). The difference between the mol. wt. of nuclear DNA from isolated nuclei and directly extracted cells may indicate the presence of a powerful nuclease, but if the fragmented nature of nuclear DNA
is accepted at face value there is obviously a difficulty in understanding how mature virus DNA arises from it. A significant proportion of the nuclear DNA from Decon-lysed cell monolayers sedimented faster than intact virus DNA (Fig. 3f). Indeed with nuclear preparations, and especially with whole cell preparations, a variable proportion of the DNA sedimented to the bottom of the centrifuge tube. Although some of the T 4 marker DNA behaved in the same way in these experiments, the possibility that mature virus DNA originates from a high mol. wt. precursor cannot be ruled out. It is interesting that preliminary results indicate that about 20% of the low molecular nuclear DNA pulse-labelled during the period of active nuclear DNA synthesis can be chased into extranuclear virus particles which suggests the possible involvement of a ligase during replication and/or maturation of herpes simplex virus DNA. The significance of the large excess of nuclear DNA which never matures into extranuclear virus particles is not understood but the same phenomenon has been observed previously for HSV and other DNA viruses (Russell et al. 1964; Fenner, 1968).

There are two general models which could explain the alkaline denaturation profiles of HSV-I DNA. The first is that only one strand of the double helix can be found intact while the other always has alkali-labile interruptions. This situation has a precedent in the Escherichia coli phage T 5 (Bujard, 1969; Jacquemin-Sablon & Richardson, 1970; Hayward & Smith, 1972b), and a similar model has been proposed for HSV-I DNA by Frenkel & Roizman (1972). The second is that the virus population contains some virus particles in which either or both strands of the duplex are intact and others in which either or both strands have alkali-labile interruptions, so that both strands are present in the leading band of denatured DNA. This model has a precedent in the bacteriophage SP 50 (Reznikoff & Thomas, 1969). The situation can be resolved by molecular reassociation experiments. If model one is correct the leading DNA band cannot contain complementary sequences of DNA. If model two is correct the leading band of DNA should include both strands of the duplex and exhibit self-complementarily. Figs. 5 and 7 show that electrophoretically pure ‘intact’ single strands of HSV-I DNA reassociated to the same extent and at the same rate as denatured total HSV-I DNA. This suggests that model two is correct.

It is not clear why the present findings differ so radically from those of Frenkel & Roizman (1972), who suggested that only one strand of the duplex was intact although six fragments which arose ‘uniquely from both strands of the DNA’ were also detected. One difference was their reliance on hydroxy-apatite (as well as optical methods) to detect the reassociation of DNA strands. We feel that the use of endonuclease is a more powerful and specific tool in this respect. Another difference was the virus and cell lines used in the two reports, although it is not clear how this might affect the results. The arrangement and nature of the interruptions is obviously of great importance to considerations of the replication and transcription of herpes viruses in general. Studies are therefore currently under way in this laboratory to determine the presence (if any), nature and arrangement of single-strand interruptions in the DNA of viruses in the herpes group.

The data from Fig. 5 suggests that hybrids formed from ‘intact’ single-stranded HSV-I DNA contained some loops or tails of single strands. One trivial reason for this could be the random assortment and reassociation of fragments shorter than unit length. Indeed, preliminary studies on the reanalysis of hybrid DNA indicate that such fragments can be generated during the incubation. However, the possibility that the result is a consequence of an unusual structure of the virus DNA cannot be ignored. One possibility which has been discussed above is that single-strand interruptions exist in opposite strands of the duplex near the opposite ends of the molecule. After alkaline denaturation and sucrose
gradient sedimentation the small fragments would be lost and ‘intact’ single strands so isolated would have single-strand tails after annealing. An interesting alternative would be if HSV-I DNA were, like T 2 DNA, a circularly permuted, terminally redundant collection of linear duplexes (Thomas & McHattie, 1964; McHattie et al. 1967). Experiments are now in progress to distinguish these various possibilities.

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REFERENCES


**Synthesis and substructure of herpesvirus DNA**


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