Organization of Herpes Simplex Virus Type 1 Deoxyribonucleic Acid during Replication Probed in Living Cells with 4,5′,8-Trimethylpsoralen†

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ABSTRACT: The structure of herpes simplex virus type 1 (HSV-1) DNA in the nuclei of living infected cells was studied with the DNA photoaffinity probe 4,5′,8-trimethylpsoralen. The rate of photobinding to HSV-1 DNA was compared to that of a suitable internal control at different times during infection. The rates of photobinding to DNA packaged in virions, capsids, and prereplicative and postreplicative DNA were characteristically different. By 4 h after infection, after the initiation of DNA replication, the rate of photobinding to HSV-1 DNA increased 4 times relative to the rate of binding to the host DNA. The enhanced rate of photobinding to HSV-1 DNA was maintained at all later times during infection and was not affected when frequent single-strand breaks were introduced in HSV-1 DNA by γ irradiation of infected cells. The results suggest that the bulk of the replicating herpes DNA is free of torsional tension and that the differing rates of photobinding are attributable to changes in accessibility of the HSV-1 DNA. The results are compatible with previous proposals, based on in vitro studies, that intranuclear HSV-1 DNA is primarily free of nucleosomal organization and suggest that there are few, if any, unrestrained DNA supercoils averaged over the entire HSV-1 genome.

Recent studies of herpes simplex virus type 1 (HSV-1) DNA from infected tissue culture cells have indicated that only a small fraction of the intranuclear DNA is organized in nucleosome structure (Mouttet et al., 1979; Shaw et al., 1979; Leinbach & Summers, 1980). This is unlike other eukaryotic DNA viruses where the intranuclear DNA is packaged via nucleosomes (Cremisi et al., 1976; Ponder et al., 1978; Tate & Philipson, 1979). Thus it seems possible that the higher order structure of HSV-1 DNA may be more analogous to that seen in prokaryotic systems where only a small part of the DNA in bacterial chromosomes and probably in replicating phage DNA is coiled in nucleosomal-like structures (Pettijohn & Pfenninger, 1980; Sinden et al., 1980). In these prokaryotic chromosomes superhelical turns remain unrestrained, and the packaged DNA is therefore maintained in a state of torsional tension. Quantitative analysis of the number of unrestrained DNA supercoils has been made possible by the development of new procedures using psoralen derivatives as photoaffinity probes (Sinden et al., 1980). The rate of photobinding to DNA by these probes is dependent on both the accessibility of the DNA (the fraction of the DNA free of nucleosomes or nucleosomal-like structures) and the amount of torsional tension in the helical winding of the DNA. It is possible to distinguish between these by relaxing any torsional strain through the introduction of single-strand breaks in the DNA (Sinden et al., 1980).

Evidence supporting the nonnucleosomal structure of herpes DNA is based on the observation that the replicating DNA in isolated nuclei is preferentially sensitive to micrococcal nuclease digestion and that the DNA is degraded randomly into heterogeneous nonnucleosomal-sized fragments (Mouttet et al., 1979; Shaw et al., 1979; Leinbach & Summers, 1980). In purified nuclei, the viral DNA is also preferentially degraded by endogenous nucleases and fails to fractionate with the cellular chromatin (Francke, 1977b). All these studies have been performed in vitro with isolated nuclei and required extensive disruption of the integrity of the nuclear DNA. Here we describe in vivo studies utilizing the interaction of the trimethylpsoralen photoaffinity probe with HSV-1 DNA in nuclei of intact infected cells. It should be emphasized that the study focuses on properties of the HSV-1 DNA in its natural state and the results should be independent of any effects of nicks, gaps, or double-strand breaks introduced in the DNA during isolation. Our goals were to determine if the HSV-1 DNA is indeed preferentially accessible to the photobinding probe, as predicted from the in vitro nuclease digestion studies, and to determine if the DNA is packaged with unrestrained torsional tension.

Materials and Methods

Viruses, Cells, and Infection Conditions. HSV-1 (Glasgow strain 17) ts^+ syn^+ was grown and titered on BHK 21 (C13) as described previously (Francke, 1977a,b). Vero cells used in this study were grown on plates at 37 °C in ME (minimum Eagle's) medium (Grand Island Biological Co.) supplemented with 5% fetal calf serum (FCS) and 50 units/mL penicillin and 50 μg/mL streptomycin. HeLa cells were grown at 37 °C on plates or in spinner flasks in RPMI Medium 1640 (Grand Island Biological Co.) supplemented with 7.5% FCS and 50 units/mL penicillin and 50 μg/mL streptomycin. For infection, confluent monolayers of Vero cells were washed once with PBS (0.137 M NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 8.1 mM Na_2HPO_4, and 1.5 mM NaH_2PO_4, pH 7.5), and virus, diluted in PBS containing 10% FCS, was absorbed to the cells at a multiplicity of infection of 20 PFU/cell at 37 °C. Plates were rocked every 15 min. After 60 min, the inoculum was removed, the cells were washed once with PBS, and fresh ME medium containing 2% FCS and 1 mM arginine was added. In some experiments 0.02 μCi/mL [^{14}C]thymidine (51 mCi/mmol, New England Nuclear) was added 2 h after infection. HeLa cells grown to (5–10) × 10^6/mL in spinner flasks were sedimented, resuspended in PBS containing 1% GG-free horse serum, 1% glucose, 0.1 mM CaCl, and 0.5 mM $MgSO_4$, and infected at a multiplicity of 20 or 1000 PFU/cell (as noted in text) for 1 h at 37 °C with constant shaking. Cells were washed twice with PBS and resuspended in fresh medium containing 1 mM arginine.

Purification of Host DNA Free HSV-1. For obtainment of a HSV-1 preparation free of host DNA, infected cells 48 h after infection were sedimented, washed once with PBS,

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resuspended in PBS containing 1% GG-free horse serum, 1% glucose, 0.1 mM $CaCl_2$, and 0.5 mM $MgSO_4$, and dounce homogenized 8–10 strokes in a tight-fitting homogenizer, and the nuclei were sedimented by centrifugation at 3000*g* for 10 min at 4 °C. The supernatant contained HSV-1 free of detectable host cell DNA.

^{60}Co Irradiation of Cells. For irradiation on plates, Vero cells were washed once with cold PBS, and 2 mL of cold PBS was added to each plate. For irradiation of a cell suspension, HeLa or Vero cells were sedimented and resuspended in cold PBS and γ irradiated in an open glass vial in an ice bath. Cells were irradiated with a Gamma Cell 220 or a Gamma Cell 60 ^{60}Co source (Atomic Energy Canada) with dose rates of 4.8 or 1.7 kilorads/min, respectively.

Determination of Yield of Breaks Introduced by ^{60}Co Irradiation in HSV-1 and Host Chromosomal DNA. Vero cells were infected at a MOI[1] of 20 as described above, and [^{3}H]thymidine (10 μCi/mL) was added at 2 h postinfection. At 12 h postinfection cells were harvested, resuspended in PBS, and exposed to various doses of γ irradiation. Nuclei were isolated and DNAs purified as described by Francke & Garrett (1982) for the purification of the HSV replicative DNA complex. The HSV and host cell DNA peaks from CsCl gradients were pooled and dialyzed. DNA molecular weights were determined by sedimentation analysis on neutral or alkaline sucrose gradients as described by Lydersen & Pettijohn (1977) and Rupp & Howard-Flanders (1968). ^{14}C-Labeled bacteriophage T4 and T7 DNAs were used as molecular weight markers.

4,5′,8-[^{3}H]Trimethylpsoralen Photobinding Conditions. [^{3}H]Me$_3$psoralen and the photobinding conditions have been described in detail elsewhere (Sinden et al., 1980). The specific activity of the Me$_3$psoralen was 3.77×10^5 cpm/μg (counting efficiency about 13%). Briefly, Me$_3$psoralen was added to cells in suspension or on plates at a concentration of 1.2 μg/mL in PBS and equilibrated for 2 min at 4 °C before irradiation. Samples were irradiated with a Burton Model 9110 lamp (Cavitron Corp., Van Nuys, CA) or with two General Electric F15T8/BLB bulbs at incident light intensities of 1 and 2.5 kJ/(m^2·min), respectively, as measured with a Blak-ray ultraviolet meter Model J221 (Ultra-violet Products, Inc., San Gabriel, CA). The dose range used in these experiments was from 5 to 15 kJ/m^2.

Measurement of [^{3}H]Trimethylpsoralen Bound to HSV-1 and Host DNA in Infected Cells. Following photobinding of [^{3}H]Me$_3$psoralen, cells were washed twice with cold PBS and lysed in 1 mL/10^7 cells in NTE buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA) by the addition of 1% NaDodSO$_4$. Proteinase K was added (1 mg/mL), and the lysate was incubated 1 h at 37 °C. The lysate was vortex mixed at high speed for 30 s to reduce the viscosity. The solution was extracted twice with phenol and 3 times with chloroform–isoamyl alcohol (24:1), the solution was adjusted to 0.3 M sodium acetate, and the nucleic acids were precipitated by the addition of two volumes of ethanol. RNA was separated from DNA as described previously (Sinden et al., 1980). The HSV-1 and host DNAs were separated by centrifugation to equilibrium in CsCl (1.700 g/mL) in a VTi 65 rotor (Beckman) for 12 h at 40000 rpm at 20 °C. Gradients were pumped from the tube bottoms, and the A_{260} of each fraction was determined with a Gilford 2400 spectrophotometer. A sample of each fraction was counted for radioactivity.

Capsid Purification. Infected cells collected after various times of infection were washed once with PBS, resuspended at a concentration of (2–5) $\times 10^7$ cells in 10 mM $NaHPO_4$, pH 7.5, containing 1% deoxycholate (DOC) and 1% Nonidet P40 (BDH Chemicals, Ltd.), and dounce homogenized 8–10 strokes. Nuclei were sedimented by centrifugation at 3000*g* for 10 min at 4 °C. The supernatant was layered over 30% sucrose in 12 mM Tris, 12 mM NaCl, and 2 mM EDTA, pH 7.5, and centrifuged 4 h at 4 °C at 20000 rpm in a SW 27 rotor (Beckman). The capsid pellet was redissolved in PBS. DNA from the capsid pellet contained no contaminating host chromosomal DNA when centrifuged to equilibrium on CsCl gradients.

Isolation of Rapidly Sedimenting Replicating HSV-1 DNA Complex. The isolation procedure that yields a replicative DNA complex that is selectively labeled with [^{3}H]thymidine was previously described (Francke & Garrett, 1982). Briefly, infected cells, continuously labeled with [^{14}C]thymidine throughout infection, were harvested at 12 h postinfection. The isolated nuclei were incubated with proteinase K in the presence of nonionic detergents, polyamines, and 1 M NaCl. After treatment with sodium dodecyl sulfate the DNA was centrifuged to equilibrium in a CsCl density gradient. Fractions from this gradient containing the viral DNA were pooled and dialyzed, and the DNA was sedimented through a 30-mL 5–20% sucrose gradient containing 1 M NaCl, 20 mM Tris buffer, pH 7.5, and 5 mM EDTA (SW 27 rotor, 20000 rpm for 3 h at 20 °C). Under these conditions, replicating DNA sedimented rapidly onto a saturated CsCl cushion present at the bottom of the sucrose gradient.

Results

In the experiments described here the organization of HSV-1 DNA was probed inside living cells by measuring the rate of 4,5′,8-trimethylpsoralen photobinding to the HSV-1 DNA. Me$_3$psoralen enters cells readily, intercalates reversibly in the cellular DNA, and on absorption of 360-nm light binds covalently at a rate directly proportional to the negative torsional tension in the DNA (Sinden et al., 1980). DNA coiled about nucleosomes cannot photobind the psoralen probe (Hanson et al., 1976; Cech et al., 1977). Therefore, the tension in internucleosomal DNA is being probed; however, this tension should be in equilibrium with the tension between other nucleosomes in the same domain. For accurate detection of differences in the rate of Me$_3$psoralen photobinding to DNA in living cells, it is desirable to develop a parameter that can provide an internal control to normalize for any changes in the rate of Me$_3$psoralen photobinding that may be due to changes in cell permeability, ionic strength, or competitive binding of the Me$_3$psoralen probe to other cellular components. For this reason measurements of the rate of Me$_3$psoralen photobinding to viral DNA were normalized to that of the host DNA and are expressed as a ratio *R*. It is assumed that any influence of internal variables on Me$_3$psoralen photobinding to intranuclear HSV-1 DNA will equally affect the binding to host DNA. Control experiments described below showed that the rate of photobinding to host DNA before infection was not altered up to 12 h after virus infection (Figure 2B). Thus it appears that the permeability of cells and the nucleosomal organization of the host chromosome were not altered by infection as also suggested by previous studies (Leinbach & Summers, 1980).

Me$_3$psoralen Photobinding to HSV-1 DNA and Host DNA in Vitro and in Vivo. The rate of Me$_3$psoralen photobinding to HSV-1 DNA relative to host DNA was determined. Vero cells were infected, labeled with [^{14}C]thymidine, and processed

[1] Abbreviations: MOI, multiplicity of infection; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

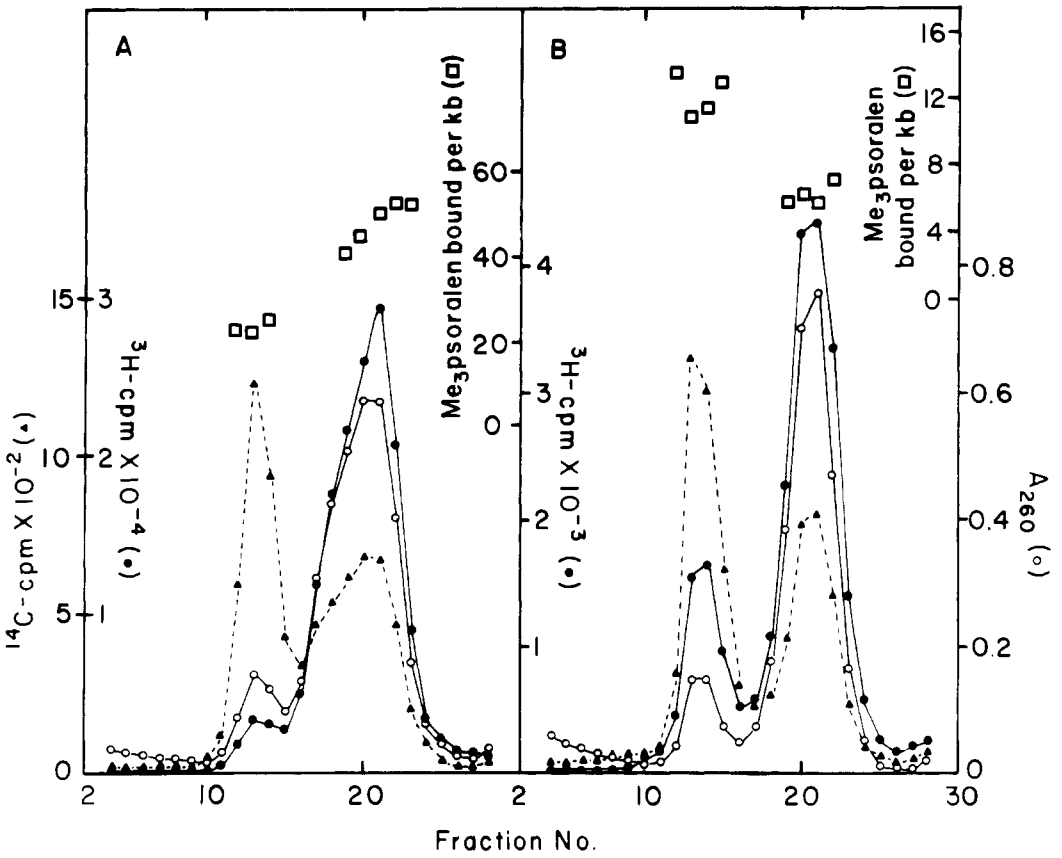

FIGURE 1: Analysis on equilibrium CsCl density gradients of Me$_3$psoralen photobinding to HSV-1 and host DNA. (A) DNA purified from Vero cells 12 h after infection with HSV-1 was subsequently treated with [^{3}H]Me$_3$psoralen and light in vitro with a light flux of 10 kJ/m^2. (B) DNA purified from the same cells as used in panel A but treated with Me$_3$psoralen and light in vivo prior to DNA purification. Cells had been labeled with [^{14}C]thymidine from 2 to 12 h after infection. Purified DNAs were centrifuged to equilibrium in CsCl gradients in a VTi 65 rotor 12 h at 40000 rpm at 20 °C. Shown above are (○) A_{260}, (▲) ^{14}C cpm, (●) [^{3}H]Me$_3$psoralen cpm, and (□) molecules of Me$_3$psoralen bound per 10^3 bp.

at 12 h after infection. Figure 1 shows the final purification and resolution of HSV-1 and host DNA by equilibrium centrifugation in CsCl gradients. In one case (panel A) the DNA had been purified from the infected cells prior to the photobinding while in the other (panel B) the photobinding was to DNA in intact cells. Since HSV infection reduces host DNA synthesis after infection, the ^{14}C cpm in Figure 1 are not representative of the amounts of viral DNA (dense peak) and cellular DNA (light peak), respectively. For the quantitation of Me$_3$psoralen binding, therefore, the absorbancy (A_{260}) profiles were used. The amount of Me$_3$psoralen bound per microgram of HSV-1 DNA was 0.5 that to the host DNA (Figure 1A). Since the amounts of covalently bound Me$_3$psoralen increase nearly linearly with time of irradiation at the low doses of 360-nm light used here (Sinden et al., 1980), the results indicate that the rate of photobinding for purified HSV-1 DNA was 0.5 that of purified host DNA. The Me$_3$psoralen bound per unit of DNA increased slightly across the DNA band toward less dense DNA fractions. This may reflect a preferential photobinding of Me$_3$psoralen to AT-rich molecules, although little difference in the intercalative binding to DNAs of different base compositions has been observed (Dall'Acqua et al., 1978).

When Me$_3$psoralen was photobound to DNA in intact infected cells 12 h after infection, the rate of photobinding to HSV-1 DNA was twice that of the host DNA (Figure 1B). The amounts of Me$_3$psoralen bound, averaged across the peaks, are shown in Table I (lines 1, 4). These results show that the rate of Me$_3$psoralen photobinding to HSV-1 DNA relative to the host DNA is about 4 times greater when the photobinding is done in intact infected cells at 12 h after infection rather than in purified DNAs.

Me$_3$psoralen Photobinding to Host and HSV-1 DNA during the Replication Cycle. The amounts of Me$_3$psoralen photobound at constant light flux to intracellular RNA, host DNA, and HSV-1 DNA at various times after infection are shown in Figure 2. The rate of photobinding to host DNA did not change after infection. The rate of binding to RNA decreased somewhat after 6 h of infection, which may reflect changes in the intracellular RNA population. For measurement of Me$_3$psoralen binding to HSV-1 DNA, the DNA was isolated from purified nuclei to ensure only intranuclear HSV-1 molecules were analyzed. Experiments measuring incorporation of [^{3}H]thymidine into HSV DNA showed that DNA replication under these conditions began between 2 and 4 h after infection, independent of the multiplicity of infection, similar to the time viral replication begins in cells infected at even lower multiplicities (Hirsch et al., 1976). At early times after infection the rate of photobinding to intranuclear HSV-1 DNA was 0.6–0.8 times the rate of binding to the host DNA. At 4 h, after replication had begun, the R value increased to 1.7. Between 6 and 12 h HSV-1 DNA bound Me$_3$psoralen at a rate about twice that to the host DNA. These results and data in Tables I and II show that the rate of Me$_3$psoralen photobinding to HSV-1 DNA changes during the course of infection, while the rate of photobinding to other DNA in the same cells does not. Presumably this represents changes in the intranuclear organization of the HSV-1 DNA or in its state of torsional tension.

Rate of Me$_3$psoralen Photobinding to HSV-1 DNA in

Table I: [^{3}H]Trimethylpsoralen Photobinding to HSV-1 DNA in Cells, Capsids, and Virions[a]

	time after infection (h)	Me_3psoralen molecules bound per 10^3 bp: HSV-1 DNA total	HSV-1 DNA capsids	HSV-1 DNA virions	host DNA	R (Me_3psoralen bound to HSV-1 DNA/Me_3psoralen bound to host DNA)
in vivo						
1	12	11.91			6.07	1.96
2	20	3.32			1.96	1.69
3	20		1.08		1.96	0.55
in vitro						
4	12	23.1			47.90	0.48
5	20		4.66		19.75	0.24
6	20			3.83	20.74	0.18

[a] In different experiments Me_3psoralen was photobound to DNA in living infected cells (lines 1–3) or purified host cell DNA mixed with either purified HSV-1 DNA (line 4), purified HSV-1 capsids (line 5), or purified virions (line 6). The time after infection is noted for each experiment using infected cells; for the in vitro experiments the time after infection refers to the cells from which DNA capsids or virions were isolated. After photobinding the DNAs were purified or repurified and separated on equilibrium CsCl density gradients as in Figure 1. Amounts of Me_3psoralen bound to each DNA were then determined. In the different experiments the absolute amounts of Me_3psoralen photobound per kilobase of DNA differ because the flux of photoactivating light differs somewhat and also because of differences in free probe concentration in vitro and in vivo; however, it is assumed that the internal control host DNA compensates for these variations.

Table II: Trimethylpsoralen Photobinding to HSV-1 DNA at Different Times after Infection in HeLa Cells[a]

time after infection (h)	kilorads of ^{60}Co	Me_3psoralen molecules bound per 10^3 bp: HSV-1 DNA in nuclei	HSV-1 DNA total	host DNA	R (Me_3psoralen bound to HSV-1 DNA/Me_3psoralen bound to host DNA)
1	0		2.45	2.98	0.82
2	0	0.86		0.99	0.87
2	30	0.65		0.93	0.70
3	0	1.12		1.82	0.62
3	30	1.21		1.88	0.64
4	0	1.57		0.95	1.65
4	30	1.53		0.88	1.74
8	0		1.20	0.60	2.00
8	10		1.14	0.63	1.81
8	20		1.17	0.58	2.02
8	0		1.29	0.68	1.90
8	30		1.17	0.49	2.39
8	40		1.21	0.51	2.37
8	0		1.09	0.56	1.95
8	50		0.97	0.51	1.90
10	0		0.54	0.24	2.25
10	5		0.50	0.24	2.08
10	25		0.45	0.25	1.80
10	50		0.47	0.25	1.88
10	0		0.60	0.25	2.40
10	100		0.53	0.27	1.96
10	0		0.54	0.23	2.35
10	150		0.57	0.29	1.97
12	0		0.58	0.35	1.66
12	10		0.84	0.48	1.75
12	30		0.74	0.48	1.54

[a] HeLa cells were grown in suspension cultures, sedimented, and infected with HSV-1. Infection in the 1-, 2-, 3-, and 4-h samples was at a multiplicity of 1000. The 8-, 10-, and 12-h experiments were infected at a multiciplicity of 20. Trimethylpsoralen photobinding was performed with intact cells, with or without prior γ irradiation for the times indicated. For the high-multiplicity infections (up to 4 h) the DNA was extracted from purified nuclei to remove excess parental virus. The DNAs were purified and fractionated on CsCl gradients, and the amount of Me_3psoralen bound was determined for each DNA.

Capsids and Virions. For investigation of the rate of Me_3psoralen photobinding to HSV-1 DNA in capsids, the capsids were purified from cells 20 h after infection following treatment in vivo with [^{3}H]Me_3psoralen and light. HSV-1 DNA extracted from purified capsids was centrifuged to equilibrium in a CsCl gradient, as were the nuclear DNAs from the same cells, and the amount of [^{3}H]Me_3psoralen bound to each DNA was determined. The rate of Me_3psoralen photobinding to HSV-1 DNA in capsids was 0.55 times the rate of binding to the host DNA (Table I). In this experiment the rate of photobinding to the bulk of the HSV-1 DNA was 1.7 times the rate of binding to the host DNA. In other experiments, between 8 and 12 h, the rate of binding to HSV-1 DNA was twice of the host DNA. These results showed that HSV-1 DNA packaged into capsids photobinds the Me_3psoralen probe at a rate 3–4 times less than unencapsidated HSV-1 DNA in nuclei of infected cells.

When purified capsids were mixed with purified host DNA before treatment in vitro with Me_3psoralen, the relative rate of photobinding to HSV-1 DNA was 0.24 that of the host DNA. This is 0.5 times the rate of binding to purified HSV-1 DNA and suggests that HSV-1 DNA in purified capsids is somewhat more accessible for Me_3psoralen photobinding than encapsidated DNA in infected cells. The rate of binding to DNA in virions was 0.18 times that to purified host DNA. Other data (not shown here) demonstrated that at 12 h after infection about 15% of the total HSV-1 DNA was encapsidated in infected cells, which is consistent with the finding that

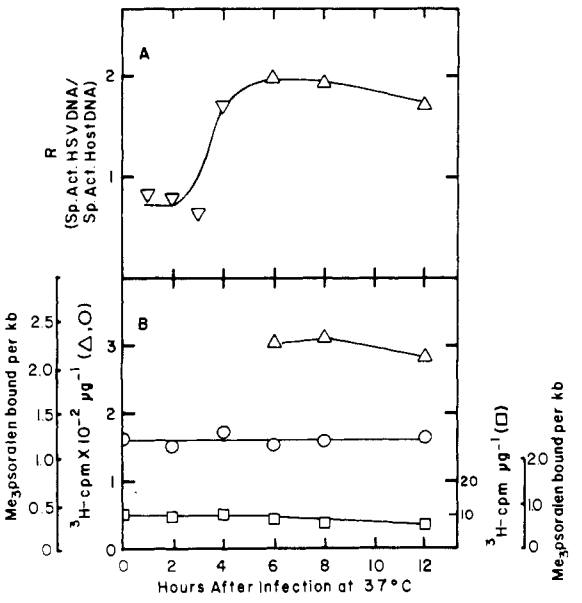

FIGURE 2: Analysis of Me_3psoralen photobinding in vivo to host and HSV-1 DNA at various times after infection. (A) *R*, ratio of Me_3psoralen photobound to HSV-1 DNA to Me_3psoralen photobound to host DNA. (B) Specific activities of Me_3psoralen labeling to nucleic acids: (△) HSV-1 DNA, (○) host DNA, and (□) RNA. Data in panel B are from a control experiment in which the photobinding to host DNA, HSV-1 DNA, and RNA were measured at a constant 360-nm light flux in the same population of HeLa cells before and after infection at a multiplicity of infection of 20. DNA and total RNA were purified, the DNAs were separated on CsCl gradients as in Figure 1, and the amount of Me_3psoralen bound was determined as described under Materials and Methods. In panel A the 6-, 8-, and 12-h data points (△) are from the experiment shown in panel B. Data for the 1-, 2-, 3-, and 4-h time points (▽) were from HeLa cells infected at a multiplicity of 1000, necessary to provide enough input HSV-1 DNA for accurate measurement of the Me_3psoralen photobinding.

the rate of photobinding to total intranuclear HSV-1 DNA was much greater than to encapsidated DNA.

Effect of γ Irradiation on Rate of Me_3psoralen Photobinding to HSV-1 DNA in Vivo. The introduction of nicks into DNA by γ irradiation has been shown to reduce the rate of Me_3psoralen photobinding to DNA wound with negative torsional tension since the rate of binding is proportional to the negative superhelical tension in DNA (Sinden et al., 1980). Superhelical tension in HSV-1 DNA was probed for by exposing infected Vero cells to different doses of γ irradiation 12 h after infection before photobinding the Me_3psoralen probe. The DNAs were purified and separated on CsCl gradients as in Figure 1 and analyzed to determine the amount of Me_3psoralen bound. Since the rate of photobinding to host DNA in vivo does not change when nicks are introduced by γ irradiation (Sinden et al., 1980), the rate of binding to HSV-1 DNA relative to the binding to the host cell DNA was determined. There was no difference in the rate of Me_3psoralen photobinding to HSV-1 DNA even after extensive γ irradiation.

Similar experiments were performed on Hela cells grown in suspension culture, which provided a more uniform sample than individual plates (Table II). In this case there was also little or no difference in the rate of Me_3psoralen photobinding to HSV-1 DNA following γ irradiation at different times after infection. With this assay negative unrestrained torsional tension in HSV-1 could not be detected at any time after infection.

In the data of Tables I and II the amounts of Me_3psoralen bound vary from one experiment to another. This is attributable to differences in the photobinding conditions (360-nm light dose, Me_3psoralen concentrations, or cell type). When data are normalized, expressed as the ratio of photobinding to HSV-1 relative to the host DNA, the results are independent of the actual amounts of bound Me_3psoralen over the range examined.

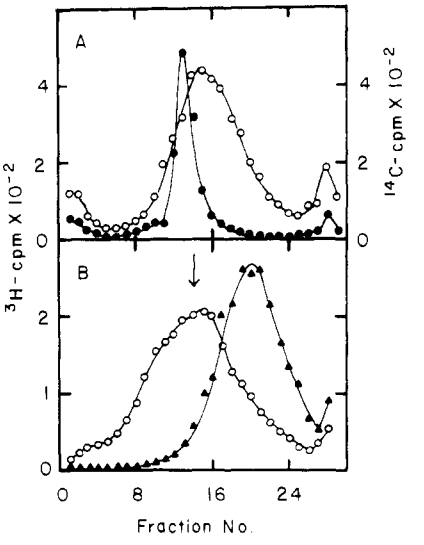

FIGURE 3: Neutral and alkaline sedimentation analysis of HSV-1 DNA isolated from cells 12 h postinfection. Vero cells were infected at a MOI of 20, the DNA was labeled with [^{3}H]thymidine, and cells were harvested 12 h postinfection. HSV-1 DNA isolated from gently lysed cells was sedimented through neutral sucrose, in a SW 50.1 rotor (Beckman) for 2 h at 30000 rpm at 20 °C as shown in panel A: (○) ^{3}H-labeled HSV-1 DNA; (●) ^{14}C-labeled bacteriophage T4 DNA. Panel B shows HSV-1 DNA from the same sample but sedimented in alkaline sucrose (○). Also shown in panel B is the sedimentation profile of HSV-1 DNA isolated from a sample of the same infected cells that was exposed to 80 kilorads of ^{60}Co irradiation before purification of HSV-1 DNA (▲). The arrow shows the sedimentation position of T7 DNA in these gradients. Alkaline gradients were centrifuged 3 h at 40000 rpm at 20 °C in a SW 50.1 rotor. Sedimentation is from right to left.

Quantitation of Alkali-Labile Sites in HSV-1 DNA following γ Irradiation. For determination of the number of nicks introduced into HSV-1 DNA by γ irradiation, Vero cells 12 h after infection were exposed to various doses of ^{60}Co irradiation, and the HSV-1 and host DNAs were isolated from CsCl gradients by the procedure described by Francke & Garret (1982) for the isolation of rapidly sedimenting HSV-1 DNA. The sedimentation of nonirradiated HSV-1 DNA isolated by this procedure in neutral sucrose is shown in Figure 3A; HSV-1 sediments as a broad peak slightly slower than bacteriophage T4 DNA [M_r 106 × 10^6 (Friefelder, 1970)]. Sedimentation of HSV-1 DNA from the same sample in alkaline sucrose is shown in Figure 3B. Single-strand HSV-1 DNA isolated 12 h after infection cosediments with bacteriophage T7 DNA [M_r 12.6 × 10^6 (Fiefelder, 1970)]. From the analysis described by Rupp & Howard-Flanders (1968) this represents a single-strand average M_r of 7.8 × 10^6 or 12.8 single-strand breaks per HSV-1 DNA molecule. This is consistent with findings of other laboratories that HSV DNA fragments in alkali (Kieff et al., 1971; Frenkel & Roizman, 1972; Wilkie, 1973; Biswal et al., 1974; Hirsh et al., 1976). Following γ irradiation the molecular weight of HSV DNA was reduced (Figure 3B). From analysis of the sedimentation in alkaline sucrose gradients following various doses of ^{60}Co irradiation, the yield of breaks in HSV-1 DNA was one break per 2.7 × 10^8 daltons per kilorad of ^{60}Co irradiation. The yield of breaks in host Vero DNA in infected cells was one break per 5.9 × 10^8 daltons per kilorad of ^{60}Co irradiation. Data

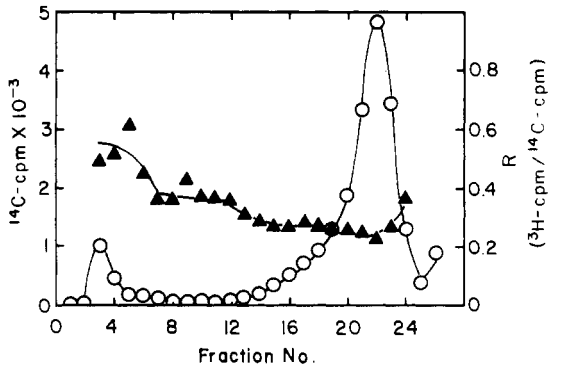

FIGURE 4: Analysis of Me_3psoralen photobinding to rapidly sedimenting HSV-1 DNA molecules. Vero cells were infected at a multiplicity of 20, the DNA was labeled with [^{14}C]thymidine, and at 12 h after infection cells were treated on plates with [^{3}H]-Me_3psoralen and light. HSV-1 DNA from gently lysed cells was centrifuged to equilibrium in CsCl gradients, collected, and dialyzed as described under Materials and Methods. HSV-1 DNA was sedimented 3 h at 20000 rpm through a 5–20% (w/v) sucrose gradient containing a saturated CsCl cushion (up to fraction 3). Sedimentation is from right to left. The rapidly sedimenting complex is in fractions 3–5. (○) ^{14}C cpm; (▲) [^{3}H]Me_3psoralen cpm/^{14}C cpm.

were nearly identical in two repeats of this experiment. Thus, at the highest doses of ^{60}Co irradiation used, one break was introduced into HSV-1 DNA on average every 1.8×10^6 daltons or one single-strand break every 2700 bp.

Rate of Me_3psoralen Photobinding to Rapidly Sedimenting HSV-1 DNA. Following HSV-1 DNA replication a small fraction of the total HSV-1 DNA can be isolated as a rapidly sedimenting complex that presumably represents newly replicated DNA (Ben-Porat et al., 1976; Jacob & Roizman, 1977; Ben-Porat & Rixon, 1979; Jacob et al., 1979). HSV-1 DNA from gently lysed cells isolated from CsCl gradients, dialyzed, and sedimented on sucrose gradients as described by Francke & Garrett (1982) contains about 5% of the HSV-1 DNA as a rapidly sedimenting DNA complex (Figure 4). In the experiments shown in Figure 4, [^{14}C]thymidine labeling conditions were such to ensure equal specific radioactivity for the rapid and slow sedimenting viral DNA components. Since rapidly sedimenting DNA apparently represents replicating HSV-1 DNA molecules, we wished to analyse its Me_3psoralen photobinding and to probe for unrestrained torsional tension in vivo. As shown by the ^{3}H/^{14}C ratio in Figure 4, 12 h after infection, the rapidly sedimenting HSV-1 DNA bound Me_3psoralen in vivo at a rate twice that of the bulk of the HSV-1 DNA. In all repeats of this experiment the rate of in vivo photobinding was 2–3 times greater to the rapidly sedimenting HSV-1 DNA relative to the slower sedimenting HSV-1 DNA. However, γ irradiation of infected cells even at the highest doses described above did not effect the rate of photobinding to the rapidly sedimenting HSV-1 DNA, suggesting that the DNA contained little or no superhelical tension (data not shown).

Discussion

The results reported here show that different states of HSV-1 DNA packaging in infected cells can be identified from studies of Me_3psoralen photobinding to the DNA. The Me_3psoralen probe can photobind to HSV-1 DNA in virions, in capsids, and in its pre- and postreplicative states in nuclei. The rates of photobinding are characteristically different in each of these states. Although the differences are not large, reliable analyses can be obtained when an internal standard is employed as in the described procedures. Thus, it becomes possible to characterize these states of DNA packaging in intact cells and to examine changes occurring during the course of infection.

Early in infection prior to the onset of viral DNA replication, the intranuclear HSV-1 DNA photobinds Me_3psoralen at a modest rate slightly reduced from that averaged over all host DNA sequences. Later after the onset of HSV-1 DNA replication there is a 3–4-fold increase in the rate of photobinding to the viral DNA. The magnitude of this difference is comparable to that between relaxed and tensive prokaryotic chromosomes (Sinden & Pettijohn, 1981). However, the enhanced binding rate of replicated HSV-1 DNA, unlike that of tensive bacterial DNA, was not affected by the introduction of frequent single-strand DNA breaks, implying that the enhanced binding rate is probably attributable to an increased accessibility of the HSV-1 DNA to the probe after the onset of DNA replication. This interpretation is compatible with prior in vitro studies of HSV-1 DNA in isolated nuclei (Lienbach & Summers, 1980) where it was shown that the viral DNA prior to DNA replication is resistant to digestion by micrococal nuclease but the replicated DNA is quite sensitive compared to host DNA. The enhanced sensitivity to micrococal nuclease in the in vitro analysis is apparently attributable to the nonnucleosomal structure of the replicated herpes DNA. Viral DNA free of nucleosomes is also expected to be more accessible to the Me_3psoralen probe. Thus, our studies of HSV-1 DNA in vivo substantiate the earlier in vitro studies. Probably the reduced photobinding rate of the HSV-1 DNA immediately after infection (prior to DNA replication) is attributable to the encapsidated state of most of the intranuclear DNA. Newly replicated HSV-1 DNA is primarily free of capsids, as well as nucleosomes, and therefore has an enhanced photobinding rate. Earlier results (Jacob & Roizman, 1977; Friedmann et al., 1977; Leinbach & Summers, 1980) indicated that only 5–10% of the infecting HSV-1 DNA molecules are uncoated and initiate replication, so the enhanced binding rate may only be attained after substantial DNA replication.

Models for the initiation of DNA replication in prokaryotic and eukaryotic systems have suggested a requirement for unrestrained negative torsional tension in DNA (Mattern & Painter, 1979; Liu et al., 1980). Recent studies of chromosome organization in living bacterial cells have shown that DNA is wound with negative torsional tension (Sinden et al., 1980) and that only a fraction of the superhelical turns are restrained in nucleosome-like structures (Pettijohn & Pfenninger, 1980). In contrast, torsional tension in the DNA of living eukaryotic cells could not be detected (Sinden et al., 1980), implying that all or nearly all of the superhelical turns of eukaryotic DNA are restrained in nucleosomes. In this sense, herpes simplex virus appears to be an exception among eukaryotic systems since as noted above, the replicated DNA is not organized in nucleosomes. Thus, the HSV-1 DNA appears more prokaryotic-like, raising the possibility that if torsional tension is required during HSV-1 DNA replication, the bulk of the HSV-1 DNA may be maintained in a tensive state. Isolated replicating herpes DNA has been shown to contain gaps and nicks (Kieff et al., 1971; Frenkel & Roizman, 1972; Wilkie, 1973; Hirsch et al., 1976; Biswal et al., 1974; Wadsworth et al., 1976), and under our conditions an alkali-labile site every 7.8×10^6 daltons was observed. However, appropriate organization of the genome into separate topological domains could permit the segregation and maintenance of superhelical tension in the DNA in a major part of the herpes virus chromosome. Such a stable configuration has been demonstrated in nicked prokaryotic chromosomes (Sinden & Pet-

tijohn, 1981). Also, it is not certain that the gaps and nicks found in the isolated HSV-1 DNA actually exist in vivo. Since the photobinding studies described here were done on the HSV-1 DNA in vivo, results should reflect the true tensive state of the DNA independent of when the gaps and nicks occur in isolated DNA. In addition, recent results from this laboratory have shown that torsional tension can be detected in the linear bacteriophage T4 genome in infected cells by using the Me_3psoralen photobinding probe method (Sinden & Pettijohn, 1982). In support of the idea that tension in DNA may be involved in replication, it was recently reported that herpes DNA synthesis is sensitive to inhibitors of DNA gyrase (Francke & Margolin, 1981).

To investigate the possibility that tension exists in herpes chromosomes, we analyzed the rate of Me_3psoralen photobinding to HSV-1 DNA at all stages of the replication cycle. HSV-1 DNA from nuclei analyzed at 2 and 3 h postinfection represented parental molecules prior to the initiation of DNA replication. At 4 h the nuclear HSV-1 DNA population represented both parental and newly replicated molecules. At later times primarily replicated, unencapsidated molecules were analyzed. In all experiments averaged over the entire HSV-1 DNA population there was no evidence for unrestrained negative superhelical tension. Since probably less than 10% of parental nuclear HSV-1 DNA molecules initiate DNA repliation (Friedmann et al., 1977; Jacob & Roizman, 1977; Leinbach & Summers, 1980), tension in replicating molecules may not be detectable averaged over the entire HSV-1 DNA population. Similarly, late in infection only a small fraction of the total HSV-1 DNA may be undergoing replication, and tension, if present only in these molecules, may not be detectable.

To enrich for replicating HSV-1 DNA molecules at 12 h after infection, we analyzed replicating structures isolated as rapidly sedimenting DNA molecules. Despite a 2-fold higher Me_3psoralen binding level, compared to the bulk of the viral DNA, there was no evidence for tension in the DNA in these structures. In addition, tension could not be detected in wild-type HSV-1 DNA after inhibiting DNA replication with phosphonoacetic acid or in several mutants of HSV-1, temperature-sensitive for DNA synthesis following a shift from permissive to nonpermissive temperature at 15 h after infection (data not shown).

These results suggest that negative unrestrained torsional tension is not detectable averaged over the HSV-1 genome. Alternatively, the HSV-1 genome is segregated into domains of supercoiling much smaller than 2700 bp. We cannot rule out the possibility that torsional tension, if required for HSV-1 DNA replication, is maintained over a small region of the chromosome, presumably around the origin of DNA replication, as suggested for bacteriophage T4 (Liu et al., 1980), or that tension may exist in a very small fraction of the total HSV-1 DNA molecules. Further studies will be required to investigate these possibilities.

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