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Infectious DNA from Herpes Simplex Virus: Infectivity of Double-stranded and Single-stranded Molecules

(*N. crassa* endonuclease/sucrose gradient centrifugation/hydrodynamic shear)

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ABSTRACT **The infectious units in native and alkali-denatured preparations of DNA of herpes simplex virus were characterized with respect to their sensitivity to *Neurospora crassa* endonuclease, their sedimentation properties in high-salt, neutral sucrose gradients, and their sensitivity to hydrodynamic shearing forces. Infectious molecules in native preparations were resistant to *N. crassa* endonuclease, sedimented at 56 S, and were highly sensitive to shearing forces. After alkaline denaturation, infectious molecules became sensitive to the *N. crassa* enzyme, sedimented at 200 S, and were relatively resistant to shear. We conclude that both intact duplex molecules ($\simeq 100 \times 10^6$ daltons) and intact single strands ($\simeq 50 \times 10^6$ daltons) are capable of initiating productive infection.**

The chromosome of herpes simplex virus (HSV) is a linear, double-stranded DNA molecule of $\simeq 100 \times 10^6$ daltons (1–4). A preliminary report (5) presented evidence that HSV DNA, isolated by mild extraction procedures, could infect primary rabbit-kidney cells or rabbit-skin fibroblasts in the presence of diethylaminoethyl-dextran. The infectious unit was shown to be DNase-sensitive, resistant to anti-HSV serum, and to have a density in CsCl of $\simeq 1.7$ g/cm^3. Additional studies (Sheldrick and Laithier, in preparation) have shown that infectivity is protease-resistant, and that progeny virus issuing from DNA-infected cells carry genetic markers present in the infecting DNA.

In this communication, we describe experiments that further characterize the infectious unit in native preparations as a molecule of HSV DNA. Moreover, we present evidence that intact single strands ($\simeq 50 \times 10^6$ daltons) of HSV DNA are also infectious.

MATERIALS AND METHODS

Culture Medium. Eagle's minimal essential medium (Eurobio, Paris) was supplemented with 3.2 g of glucose, 2.7 g of tryptose phosphate (Difco), and 2.0 g of $NaHCO_3$ per liter. All incubations using this medium were performed in an atmosphere of 5% CO_2.

Cells. The strain of rabbit-skin fibroblasts, RS537, used in this study was isolated and kindly provided by Dr. G. Orth (6). The cells were propagated at 37° in medium supplemented with 10% calf serum in the absence of antibiotics. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to the medium for the growth of virus and for plaque assays. Cultures were trypsinized twice weekly and reseeded at $^1/_4$–$^1/_5$ the original cell concentration. The experiments described here were performed with cells between their 40th and 70th passages.

Virus. The strain of HSV (subtype 1) used here is designated A_{44} and has been described (7). It produces a syncytial cytopathic effect in RS537 cultures and, in this sense, is similar to the MP variant described by Hoggan and Roizman (8).

Virus Growth and Purification. Confluent cultures of RS537 cells in 1-liter Roux bottles (about 2 to 3 $\times 10^7$ cells per bottle) were infected with 1 to 2 $\times 10^7$ plaque-forming units (PFU) of virus in 50 ml (per bottle) of medium supplemented with 5% calf serum. After 24 hr of incubation at 37°, the cultures were frozen (−70°) and thawed three times. The lysate (2 to 5 $\times 10^7$ PFU/ml) was clarified by centrifugation at 1200 $\times g$ for 10 min, and the virus was sedimented by centrifugation at 15,000 $\times g$ for 1 hr at 4°. The viral pellet (70–90% recovery of PFU) was resuspended in a small volume (usually $^1/_{50}$ the lysate volume) of 1 M NaCl–1 mM EDTA–0.01 M sodium phosphate (pH 7.5) clarified by low-speed centrifugation, and layered onto 2.5-ml CsCl step gradients [successive 0.5-ml volumes of 33, 26, 20, 13, and 7% (w/v) CsCl in 1 mM EDTA–0.01 M sodium phosphate (pH 7.5)] in nitrocellulose tubes of a SW41 rotor (Beckman). After centrifugation at 25,000 rpm for 1 hr (15°), a broad light-scattering band in the upper half of the CsCl gradient (about 90% of input PFU) was collected by piercing the side of the tube with a needle and syringe. The virus band was dialyzed at 4° against two changes of 0.1 M NaCl–1 mM EDTA–0.01 M sodium phosphate (pH 7.5) and stored at 4°.

DNA Extraction. Purified virus preparations made 0.01 M in EDTA and 1% in Na dodecyl sulfate were heated to 37° for 3 min before addition of an equal volume of redistilled phenol previously equilibrated with 0.1 M sodium phosphate (pH 7.5). The phases were partially mixed by gentle rocking for 15 min at ambient temperature and separated by centrifugation at 13,000 $\times g$ for 15 min. The aqueous phase was carefully removed from the phenol phase and (abundant) interphase by means of a broken (bore diameter $\simeq 3$ mm) Pasteur pipette, reextracted with an equal volume of phenol, and dialyzed at 4° against two changes of 0.2 M NaCl–2 mM

Abbreviations: HSV, herpes simplex virus; PFU, plaque-forming units; PBS, phosphate-buffered saline (pH 7.0).

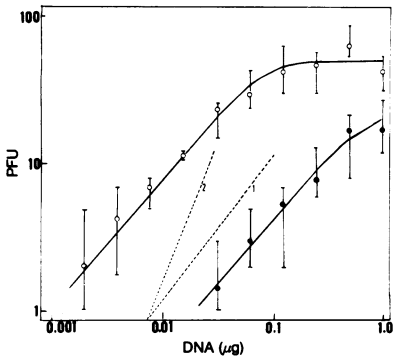

FIG. 1. Dose-response curves of native and denatured infectious HSV DNA. Triplicate cell monolayers were treated with 1:2 dilutions of native (○——○) or alkaline-denatured (●——●) HSV DNA. Results are expressed as average PFU of the triplicate series with scatter represented by *vertical bars*. The *dashed lines* illustrate theoretical first-order (*1*) and second-order (*2*) dose responses. The specific infectivity of the native DNA preparation was ≃1000 PFU/μg.

EDTA–0.02 M Na phosphate (pH 7.5) and two changes of 0.1 mM EDTA–0.01 M Tris·HCl (pH 7.4).

Subsequent manipulations of all DNA preparations were performed either by pouring, or with 1-ml sterile plastic pipettes (bore diameter ≃ 1 mm). The flow rate during pipetting was not allowed to exceed ≃ 0.05 ml/sec.

DNA concentrations were estimated from the absorbance at 260 nm, taking one A_{260} unit as corresponding to 50 μg of native, or 40 μg of denatured DNA. Native DNA preparations had A_{260}/A_{280} ratios of 1.96 ± 0.05 and banded at $\rho \simeq$ 1.727 g/cm³ in analytical (Beckman, model E) CsCl gradients. Specific infectivities of fresh preparations of native HSV DNA varied from 500–3800 PFU/μg. This variability was due to low-molecular-weight DNA (see Fig. 3, *Results*) associated with the virus preparations.

DNA Denaturation. One-tenth volume of 1 M NaOH was carefully mixed with the DMA preparation and held at ambient temperature for 5 min. After the sample was cooled to 0° in iced water, $^1/_{10}$ volume of 1 M KH_2PO_4 was added to bring the final pH to about 8. Repeated analyses of DNA preparations denatured in this way, either in analytical CsCl gradients or in sucrose gradients with radioactive material failed to show detectable quantities (<1%) of native DNA. For experiments with *Neurospora crassa* endonuclease, which is inhibited by phosphate ions (9), the alkaline DNA solution was neutralized (pH 7–8) with $^1/_5$ volume of 3:2 (v/v) 0.5 M Tris (pH 7.4)–1 M HCl. Experiments comparing the behavior of native and denatured DNA were done with both preparations adjusted to the same ionic environment by addition of the appropriate volume of premixed base and neutralizing agent to the native preparation.

Infectivity Assays. DNA. The assay is based on that described by Pagano and Vaheri (10), using the carboxymethylcellulose overlay technique of Russell (11). Plastic petri dishes (5-cm; Nunclon) were seeded with 10^6 (per dish) freshly trypsinized RS537 cells in 5 ml of culture medium (10% calf serum) and incubated at 37° for 24 hr. The just-confluent monolayers were washed once with phosphate-buffered saline (pH 7.0) lacking Ca^{++} and Mg^{++} (PBS) and treated with 0.2 ml of a 1:1 mixture of the DNA solution (in PBS) and a solution of DEAE-dextran (molecular weight ≃ 2 × 10^6; Pharmacia) at 400 μg/ml in PBS. After 1 hr at ambient temperature, the monolayers were washed twice with PBS and covered with 5 ml of culture medium (10% calf serum) containing 1% sodium carboxymethylcellulose (Hercules). Plaques 0.5–1.0 mm in diameter developed after 3 days at 37° and were scored after the cell layers were stained with 3 ml (per dish) of 0.05% neutral red in PBS. All infectious DNA assays were performed in duplicate or triplicate.

Virus. Washed RS537 monolayers, prepared as described above, were inoculated with 0.2 ml of the virus suspension in PBS. After a 20-min adsorption period (ambient temperature) 5 ml of carboxymethylcellulose-containing medium was added directly to the monolayers. Plaques ≃ 3 mm in diameter developed after 3 days at 37°.

Sedimentation in sucrose gradients. DNA solutions were gently pipetted onto 11-ml linear 5–20% (w/w) sucrose gradients (polyallomer tubes) prepared in 1 M NaCl–1 mM EDTA–0.01 M sodium phosphate (pH 7.5) and sedimented for 1.5 hr at 30,000 rpm in an SW41 rotor at 18°. Fractions (≃ 300 μl) were collected in glass tubes containing 200 μl of H_2O by piercing the tube bottom with an 18-gauge hypodermic needle. Part (100 μl) of each fraction was diluted in PBS for infectivity assays; the rest (400 μl) was used for monitoring A_{260}.

RESULTS

During the course of our studies on infectious HSV DNA, we observed that alkaline denaturation seldom completely abolished the infectivity of native preparations. As a rule, 5–20% of the original infectivity remained associated with denatured DNA. Dose-response curves for infectious HSV DNA before and after alkaline denaturation are shown in Fig. 1. At low DNA concentrations, the dependence of infectivity on DNA concentration for both native and denatured preparations was most consistent with a first-order relationship, indicating that infection was initiated by a single DNA molecule. Measured in the first-order response region, the denatured preparation was approximately 10% as infectious as the native. The saturation plateau at high DNA

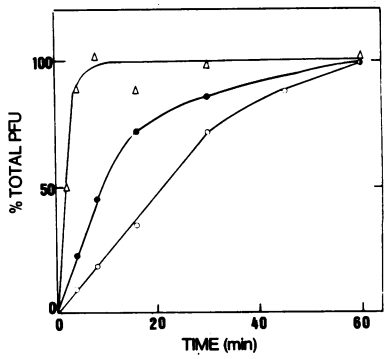

FIG. 2. Time-course of the irreversible attachment to RS537 cells of infectious units in preparations of virus, native DNA, and denatured DNA. Duplicate cell monolayers were treated for various times with DEAE-dextran solutions of virus (△——△; ≃60 PFU per petri dish), or native DNA [○——○; ≃50 PFU (0.05 μg) per dish], or alkaline-denatured DNA [●——●; ≃60 PFU (0.5 μg) per dish] and processed as described in *Methods*.

concentrations might be related to the finding (12, 13) that high-molecular-weight DNA tends to aggregate in the presence of DEAE–dextran. According to this interpretation, denatured HSV DNA, for which the linear response region extends to higher concentrations than in the case of native HSV DNA, should have less tendency to aggregate in DEAE-dextran. Alternatively, the saturation plateau could be due to a limited number of "susceptible" cells in the population. Whatever the origin of the saturation plateau, in practice we have rarely been able to obtain more than 100 plaques per 1.5×10^6 cells (5-cm petri dish).

We compared the rates at which the infectious unit in preparations of virus, native DNA, and denatured DNA became irreversibly fixed to cells. Cell monolayers were exposed to each of the three preparations in the presence of DEAE-dextran and, after various periods of time, were washed with PBS. The results in Fig. 2 show that, at similar concentrations, the infectious unit in viral preparations became irreversibly cell-associated five times more rapidly than that in denatured DNA, and 10 times more rapidly than that in native DNA. The 2-fold (initial) rate difference between native and denatured DNA preparations suggested to us that the infectious units in the two preparations might not be equivalent (i.e., native DNA molecules). Although direct evidence is lacking, it is tempting to suppose that the compact spatial conformation of the denatured, as compared to the native, infectious molecule (see below) might facilitate its uptake by the target cell.

A trivial reason for the residual infectivity of denatured HSV DNA would be the presence of undenatured or renatured molecules in the preparation. This possibility was tested by incubating native and denatured preparations with *N. crassa* endonuclease, which specifically degrades polynucleotides lacking secondary structure (9). The results in Table 1 show that the infectivity of native HSV DNA was relatively resistant to the enzyme, while the infectivity of denatured DNA was rapidly inactivated. Thus, in denatured, but not in native, DNA preparations the infectious molecules were, partially or entirely, in a single-stranded state.

TABLE 1. *Effect of N. crassa endonuclease on the infectivity of native and denatured HSV DNA*

Enzyme (units)*	Min of incubation	Native DNA PFU/ml	Native DNA % Survival	Denatured DNA PFU/ml	Denatured DNA % Survival
None	0	11,000	100	3500	100
	20	—†	—	3100	89
	60	10,300	94	3000	85
0.120	5	—	—	<5	<0.1
	10	9,500	86	<5	<0.1
	20	—	—	<5	<0.1
	60	5,000	46	<5	<0.1
0.006	5	—	—	50	1.4
	10	—	—	5	0.1
	20	—	—	<5	<0.1
	60	—	—	<5	<0.1

The reaction mixtures contained in 1.30 ml: 25 mM NaCl, 100 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 12 μg of native or alkaline-denatured HSV DNA, and the indicated amounts of *N. crassa* endonuclease (≃1000 units/mg; kindly provided by Dr. B. Acharia). After incubation at 35° for the indicated times, 0.2-ml aliquots were diluted in PBS at 0° and assayed for infectivity.

* A unit of enzyme is that amount which catalyzes the formation of 1 μmol of acid-soluble nucleotide in 30 min (37°) in the assay conditions of Linn and Lehman (9).

† *Dashes*, not done.

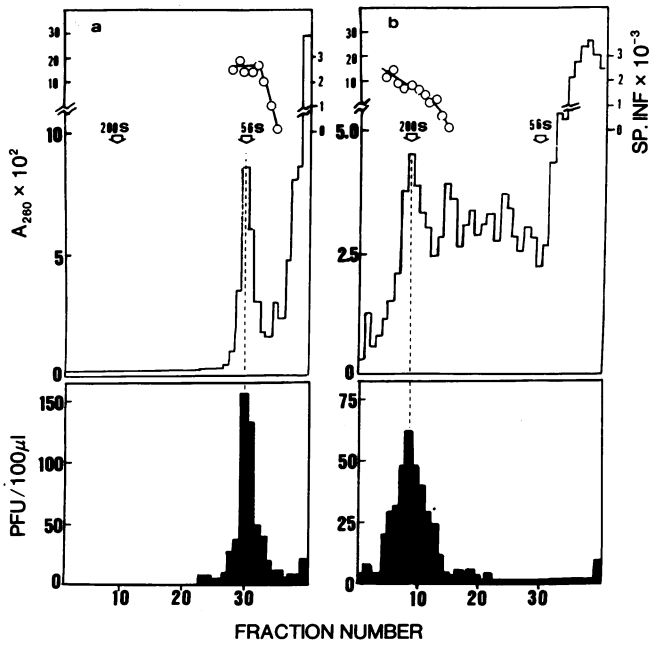

FIG. 3. Sucrose density gradient centrifugation of native and denatured infectious HSV DNA. (*a*) Native DNA, 14 μg (≃12,000 PFU) in a volume of 300 μl, and (*b*) alkaline-denatured DNA, 40 μg (≃8000 PFU) in a volume of 300 μl, were centrifuged. The *open histograms* refer to A_{260} (numbers have been multiplied by 10^2), the *solid histograms* to infectivity, and the *open circles* to specific infectivity (PFU/μg) (numbers have been multiplied by 10^{-3}).

To more completely characterize the physical structure of infectious molecules, we analyzed native and denatured HSV DNA preparations in high-salt, neutral sucrose density gradients (Fig. 3). Native HSV DNA (Fig. 3*a*) sedimented as a single sharp band whose sedimentation coefficient was determined, relative to T4 and T5 st(0) DNAs (experiments not shown), to be 56 S. This value corresponds to a double-stranded DNA molecule of ≃ 100×10^6 daltons (14) and is in agreement with previous estimates of the molecular weight of HSV DNA. The material sedimenting at the top of the gradients in Fig. 3 consisted of native DNA fragments of both viral and cellular origin (Kermici and Sheldrick, unpublished), and appeared in variable amounts in our DNA preparations. Small DNA fragments have also been observed by others in some DNA preparations derived from HSV (15) and Marek's disease virus (16).

Infectivity sedimented with the 56S band (Fig. 3*a*), indicating that the infectious unit in native preparations was an intact molecule of HSV DNA. Also plotted in the figure are the specific infectivities calculated for each fraction across the 56S band. These values remain constant at 2600 PFU/μg and abruptly decrease at the band's trailing edge due to the presence of low-molecular-weight, noninfectious DNA in this region. A specific infectivity of 2600 PFU/μg corresponds to

TABLE 2. *Effect of hydrodynamic shear on the infectivity of native and denatured HSV DNA*

	Native DNA			Denatured DNA		
	Specific infectivity*			Specific infectivity*		
Solvent	Unsheared	Sheared	% Survival	Unsheared	Sheared	% Survival
PBS	3800	<0.5	<0.02	720	70	10
DEAE-dextran	2300	<0.5	<0.02	230	25	11

Stock preparations (10 μg/ml) of native or denatured HSV DNA were serially diluted (1:3) in PBS. One dilution series was then made to 200 μg/ml of DEAE-dextran. Shearing was performed with 0.5-ml aliquots in 1 × 10-cm Pyrex tubes rotated at maximum speed for 3 min (ambient temperature) with a Vortex mixer. Infectivity was determined as described in *Methods*.

* PFU/μg.

one infectious molecule per 2 to 3 × 10^6 molecules of 100 × 10^6 daltons.

Alkaline-denatured HSV DNA (Fig. 3*b*) displayed a complex sedimentation pattern, suggesting the presence of nonrandom single-strand interruptions in the duplex molecule. This feature of denatured HSV DNA has been observed independently and studied extensively in alkaline sucrose gradients by Roizman and his collaborators (2, 17). The gradient illustrated in Fig. 3*b* shows six bands ranging from 200 S to 70 S, or, in terms of molecular weight as calculated by the relation of Studier (14), from ≃ 60 × 10^6 to ≃ 10 × 10^6 daltons. The precise relationship of the six bands shown here to the seven fragments described in alkaline gradients by Frenkel and Roizman (17) is uncertain, since sedimentation rates of single DNA strands in neutral sucrose gradients can depend on conformational differences in collapsed molecules‡ (18).

As shown in Fig. 3*b*, the infectivity of denatured HSV DNA was associated solely with the 200S band. Slower sedimenting fragments exhibited little, if any, biological activity; the low levels of infectivity following the 200S band were probably due to some trailing during collection of the gradient. No infectivity was detected in the (56S) region of the gradient where intact duplex molecules would be found, thus directly excluding undenatured molecules as the source of the infectivity of denatured HSV DNA. In contrast to native DNA, the specific infectivities of fractions across the 200S band constantly decreased from a maximum of ≃ 2000 PFU/μg (1 infectious molecule per 6 × 10^6 molecules of 50 × 10^6 daltons) at the leading edge to ≃ 1000 PFU/μg at the trailing edge. We take this as an indication that noninfectious single-stranded fragments closely follow the intact, infectious molecules in the gradient. Whether these correspond to the 40 × 10^6-dalton fragment described by Frenkel and Roizman (17) remains to be determined.

The behavior of infectious molecules in denatured HSV DNA preparations, both toward *N. Crassa* endonuclease and in neutral sucrose gradients, demonstrated that they could at least pass through a single-stranded state and remain infectious, but did not rigorously exclude the possibility that renaturation to form infectious duplex molecules occurred at some later stage in the manipulations before uptake by a cell. It has been reported, for example, that DEAE-dextran enhances the self-association (dimerization) of poly(uridylic acid) (12), and lowers the melting temperature of native DNA (14). Since these observations suggested that DEAE-dextran might promote renaturation of single-stranded HSV DNA in our experiments, we sought a means to determine whether infectious HSV DNA remained single-stranded in solutions of DEAE-dextran.

For this purpose, we used hydrodynamic shear sensitivity as a measure of the molecular domain of the infectious molecule. At neutral pH in solvents of moderate ionic strength (e.g., 0.1 M Na^+), the three-dimensional conformation of single-stranded DNA is considerably more compact than that of double-stranded DNA (14, 18). Thus, the infectious unit should be more resistant to shearing forces in the single-stranded state than in the double-stranded state. The data in Table 2 show that the infectivity of denatured HSV DNA was more shear-resistant than that of native HSV DNA by at least a factor of 500. Control experiments (Sheldrick, unpublished) showed that, at similar DNA concentrations, these conditions of shearing reduced native HSV DNA to fragments of ≃ 25 × 10^6 daltons, while native T7 DNA (26 × 10^6 daltons, ref. 14) was not affected. Moreover, the shear sensitivities of the infectious molecules remained unchanged in the presence of DEAE-dextran. Thus, the conformation of infectious molecules in solutions of DEAE-dextran was not modified in a significant way, showing that renaturation of single strands to form duplex molecules did not occur.

DISCUSSION

Taken together, the results of the experiments presented here strongly support the view that HSV DNA can be infectious in both its double-stranded and single-stranded states. Resistance to *N. crassa* endonuclease, sensitivity to hydrodynamic shear, and a sedimentation coefficient of 56 S all serve to characterize the infectious unit in native DNA preparations as a duplex molecule of ≃ 100 × 10^6 daltons. On the other hand, after alkaline denaturation the infectious unit becomes highly sensitive to *N. crassa* endonuclease and relatively shear resistant, and sediments at 200 S. As previously mentioned, we surmise that the infectious 200S species is an intact DNA strand (rather than an aggregate of an intact strand "plus" a fragment), even though it sediments somewhat (1.3 ×) more rapidly in the neutral sucrose gradient than would be predicted (14) on the basis of its supposed molecular weight. Recently, we have obtained supporting evidence for this supposition by showing that in high-salt, alkaline sucrose gradients, where neither aggregation nor extensive folding of single-stranded DNA is likely to occur (18), infectious HSV DNA molecules sediment at 67 S (Buchini, Boccara, and Sheldrick, unpublished), consonant with a molecular weight of ≃ 50 × 10^6 (14, 2).

‡ We have, in experiments not shown here, obtained sedimentation patterns of our DNA preparations in alkaline sucrose gradients that were indistinguishable from those published by Frenkel and Roizman (17). In concordance with their results, the most rapidly sedimenting species corresponded to a single DNA strand of ≃50 × 10^6 daltons. Thus, it is assumed here that the 200S band in Fig. 3*b* also corresponds to an intact DNA strand. We tentatively attribute the anomalously high sedimentation coefficient of this species to enhanced "longe-range folding" (18) of the single strand, perhaps as a result of the high (68%) G + C content of HSV DNA.

Several of our observations argue against renaturation being responsible for the infectivity of single strands of HSV DNA. First, the high specific infectivity of the 200S species, approaching that of native duplex molecules (Fig. 3), would seem to require either that renaturation of single strands be highly efficient (which seems unlikely in view of the genetic complexity of HSV DNA; ref. 3), or that duplex molecules thus generated be inherently more infectious than native duplex molecules. Second, the shear resistance of infectious denatured DNA in solutions of DEAE-dextran (Table 2) indicates that the infectious molecule remains single-stranded at least until contact with the target cell. Third, if renaturation were occurring, say, at the surface (or interior) of the target cell, the dose-response relation for the infectivity of denatured DNA would be expected to show a higher-order dependence on DNA concentration, rather than the first-order dependence actually observed (Fig. 1). In addition, Frenkel and Roizman have given evidence that the intact strand of HSV DNA corresponds to only one of the complementary strands in the duplex molecule (17). Such a situation would of course further diminish the likelihood that the infectivity of the 200S species derives from renatured molecules.

The present findings imply that infection initiated with single HSV DNA strands could require a host-dependent capacity for DNA replication. Such a requirement exists for the bacterial viruses (ϕX174 and M13, where the infecting chromosome, a single DNA strand, is converted to a duplex molecule before gene expression (19, 20).

Finally, we note that while previous studies have defined an "intact" strand of HSV DNA in terms of physicochemical (sedimentation) measurements (2, 3), the present work permits a supplementary definition of "intactness"; namely, that 200S (or 67S in alkaline gradients) DNA strands are intact in that they carry a complement of genetic information both necessary and sufficient for productive viral replication.

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