JOURNAL OF VIROLOGY, Oct. 1972, p. 565-572
Vol. 10, No. 4

Printed in U.S.A.

Separation of the Herpesvirus Deoxyribonucleic Acid Duplex into Unique Fragments and Intact Strand on Sedimentation in Alkaline Gradients

NIZA FRENKEL AND BERNARD ROIZMAN
Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

Received for publication 6 June 1972

Deoxyribonucleic acid (DNA) extracted from herpes simplex virions forms multiple partially overlapping bands upon denaturation and centrifugation in alkaline sucrose density gradients. The most rapidly sedimenting DNA corresponds to an intact strand 48 $\times$ 10^6 daltons in molecular weight. In this study, we analyzed the DNA fragments generated in alkaline sucrose gradients with respect to size and uniqueness of base sequences. The distribution of sedimentation constants of the various fragments obtained in numerous gradients showed that the fragments smaller than the whole strand fall into six distinct classes ranging in molecular weight from 10 $\times$ 10^6 to 39 $\times$ 10^6 daltons. Four types of DNA strands can be reconstructed from the whole strand and six fragments on the basis of their molecular weights. DNA from each of the bands self-hybridizes to a lower extent than unfractionated viral DNA, indicating that each of the bands preferentially contains sequences from one unique strand. The data permit reconstruction of four possible types of DNA duplexes differing in the positions of the strand interruptions. Analysis of viral DNA extracted from nuclei of cells labeled with ^{3}H-thymidine for intervals from 3 to 120 min showed that nascent DNA is invariably attached to small fragments and that the fragments become elongated only upon prolonged incubation of cells. The experiments suggest that viral DNA replication begins at numerous initiation sites along each strand and that the elongation beyond the size of the replication unit involves repair or ligation, or both. Since newly made DNA yields more fragments than viral DNA extracted from mature virions, it is suggested that the fragmentation of mature DNA on denaturation with alkali arises from incomplete processing of specific initiation sites. Comparison of viral DNA extracted from nuclei with that extracted from mature cytoplasmic virions in cells labeled for 120 min indicates that packaged DNA is not randomly selected from among the nuclear DNA population but rather represents DNA molecules which in alkaline gradients yield a minimal number of fragments.

The genetic information contained in herpes simplex viruses (HSV) is encoded in one double-stranded molecule (99 $\pm$ 5) $\times$ 10^6 daltons in molecular weight (9, 12). Studies in our laboratory and in others have indicated that deoxyribonucleic acids (DNA) extracted from HSV (1, 12) and from herpesviruses associated with Marek's disease (14) and with human lymphoproliferative diseases (15) form multiple partially overlapping bands containing intact strands and fragments, respectively, on sedimentation through alkaline sucrose density gradients. Extensive analyses in our laboratory (1, 12) indicated that the fragmentation of HSV DNA in alkaline solutions is independent of the host (human or simian cells) in which the virus is produced and is not a consequence of manipulative procedures or of a putative nuclease present in the intact virion. Thus, the data excluded the possibility that the DNA is cleaved by nucleases contained in the virion because (i) intact and sonically disrupted herpesvirus virions and nucleocapsids do not convert the supercoiled simian virus 40 form I DNA to the circular form II nor release nucleotides from labeled *Escherichia coli* DNA under various conditions of *p*H and divalent cation concentrations, and (ii) high-molecular-weight nonencapsidated DNA extracted from nuclei of cells late in infection generated the same frag-

ments as the DNA from fully enveloped nucleocapsids (1, 12).

Interest in the structure and function of herpesvirus DNA arises from two considerations. First, herpesvirus DNA is made in the same cellular compartment as host DNA; the study of the structure and function of viral DNA may shed light on cellular DNA synthesis and function. Second, the question arises whether the structure of the viral DNA is in any way related to the oncogenic potential displayed by these viruses. This paper deals with the nature and origin of the fragments generated upon sedimentation of HSV DNA in alkaline sucrose density gradients.

MATERIALS AND METHODS

Solutions and chemicals. Neutral DNA buffer consisted of 1 N NaCl, 0.01 M disodium (ethylenedinitrilo) tetraacetate (EDTA), and 0.05 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, *p*H 7.5. Alkaline DNA buffer consisted of 0.7 N NaCl, 0.3 N NaOH, and 0.01 M EDTA. Phosphate buffer was made of equimolar concentrations of Na_2HPO_4 and NaH_2PO_4 (final *p*H 6.8). ^{3}H-methyl thymidine (specific activity, 82.6 mCi/mg) was obtained from New England Nuclear Corp., Boston, Mass. The nonionic detergent Nonidet P-40 was a gift of Shell Chemical Co., New York, N.Y. Sarkosyl (NL 97) was obtained from Geigy Chemical Co., Ardsley, N.Y. Hydroxyapatite powder (Bio-Gel, HTP, DNA grade) was obtained from BioRad Laboratories, Richmond, Calif.

Cells. Human epidermoid carcinoma no. 2 (HEp-2) cells were originally obtained from Flow Laboratories, Rockville, Md. The cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum heated to 50 C for 30 min.

Virus and infection of cells. A strain of HSV subtype 1 (HSV-1) was used in all of the studies reported here. The methods of propagation and assay have been reported elsewhere (19).

In the experiments described here, cells grown in monolayer cultures were infected at 37 C with 5 to 30 plaque-forming units of HSV-1. The viral inoculum was replaced with mixture 199 supplemented with 1% calf serum, and incubation was continued at 37 C.

Labeling. DNA was labeled by incubation of infected cells in EMEM supplemented with 1% dialyzed calf serum and 5 to 10 μCi of ^{3}H-methyl thymidine per ml of medium.

Preparation of native viral DNA. The method for purification of HSV DNA was essentially the same as described earlier by Kieff et al. (12). The purification method made use of the fact that mature virions accumulate in the cytoplasm whereas the bulk of host DNA is restricted to the nucleus. Cells infected with HSV-1 at an effective multiplicity of 5 plaque-forming units per cell were harvested by scraping and were centrifuged at 800 $\times$ *g* in a PR-2 refrigerated centrifuge for 10 min at 4 C. The cell pellet was washed with phosphate-buffered saline and resuspended in 0.01 M Tris-hydrochloride, *p*H 7.4, and 0.0025 M EDTA containing 0.5% NP-40. Detergent lysis was allowed for 10 min at 4 C. The nuclei were removed by centrifugation of the cell lysate at 800 $\times$ *g* for 10 min at 4 C in a PR-2 centrifuge. The cytoplasm was layered directly on top of a 36-ml linear gradient made of 5 to 20% (w/w) sucrose in neutral DNA buffer containing 0.15% Sarkosyl. Centrifugation was at 25,000 rev/min and 20 C for 6 hr in an SW27 rotor. DNA gradients were collected and fractionated in an Isco fractionator equipped with an ultraviolet light absorbance analyzer. Fractions containing intact viral DNA were pooled, phenol-extracted, made 0.3 N with respect to NaOH, and allowed to stand at room temperature for 6 hr (to hydrolyze contaminating ribonucleic acid). The DNA was sheared by sonic treatment for 45 sec at 0 C in alkaline solution in an MSE model 100 sonic oscillator. The sheared DNA was dialyzed extensively against the buffer used for hybridization. Viral DNA prepared by this method is free from host DNA by the following criteria: (i) it does not hybridize with excess host DNA (13), (ii) it forms a single band upon isopycnic centrifugation in a model E ultracentrifuge (1, 12), and (iii) it has a kinetic complexity of (95 $\pm$ 1) $\times$ 10^6 daltons (9).

Preparation of DNA derived from velocity centrifugation in alkaline sucrose density gradients. The procedure followed the steps outlined above with the following modifications: the cytoplasmic fraction was lysed by exposure to final concentrations of 2% Sarkosyl, 1% sodium dodecyl sulfate (SDS), and 0.3 N NaOH on top of a 5 to 20% (w/w) sucrose gradient in alkaline DNA buffer containing 0.15% Sarkosyl; the treatment with 0.3 N NaOH for 6 hr was omitted.

RNA renaturation in a Gilford spectrophotometer. Sheared DNA in 0.3 M phosphate buffer was denatured by heating to 110 C for 10 min. Renaturation was followed at 25 C below the T_m in a Gilford model 2000 spectrophotometer equipped with an automatic absorbance and temperature recorder. After the reassociation was complete, the temperature in the cuvette chamber was raised so as to determine the maximal absorbance of denatured DNA.

DNA hybridization followed by fractionation on hydroxyapatite columns. DNA was hybridized in 0.4 M phosphate buffer at 75 C. Fractionation on hydroxyapatite (3, 4) was done in water-jacketed columns heated to 50 C. Approximately 0.5 g of hydroxyapatite powder was suspended in the initial elution buffer, boiled, and poured into the column. All elution buffers were boiled prior to use. Gradient elution was done with a linear gradient of 0.02 to 0.25 M potassium phosphate buffer (120 ml) followed by a 0.4 M phosphate buffer wash (20 ml). The fractions (2 ml) were collected and precipitated with trichloroacetic acid.

Stepwise elution was done in a similar manner except that the DNA was loaded on the column in 0.03 M sodium phosphate buffer and eluted with 0.19 and 0.3 M sodium phosphate buffers. The choice of buffers (0.19 and 0.3 M, respectively) for the elution of single-stranded DNA and double-stranded DNA was based on preliminary experiments with heat-denatured DNA and native sheared DNA singly and in artificial mixtures.

Extraction and sedimentation of nuclear DNA. Infected cells were pulse-labeled for 3, 7, 30, 80, and 120 min between 4.5 and 6.5 hr postinfection with

HSV-1 at multiplicity of 30 plaque-forming units per cell. At the end of the labeling period, the nuclei were harvested with 0.5% NP-40 as above, sedimented, and lysed by storage for 4 hr at 4 C and 4 hr at room temperature in 0.01 M Tris, 0.025 M EDTA, 0.2 N NaOH, 1% SDS, and 2% Sarkosyl. The nuclear lysates were then layered on top of 36 ml of 5 to 20% (w/w) sucrose gradient prepared in alkaline DNA buffer containing 0.15% Sarkosyl and centrifuged as above.

RESULTS

Size of the fragments generated in alkaline sucrose density gradients. The purpose of these experiments was to determine whether the DNA fragments generated in alkaline sucrose density gradients are random in length. In these experiments, several batches of HSV DNA labeled with ^{3}H-thymidine were centrifuged in neutral and alkaline sucrose density gradients alone or in artificial mixture with T4 DNA labeled with ^{14}C-thymidine. Pertinent to the analysis of results are the following data.

(i) In neutral sucrose density gradients, HSV-1 DNA forms a single band slightly above the T4 DNA. A typical profile of HSV-1 DNA banded in neutral sucrose density gradients is shown in the top panel of Fig. 1. On the basis of numerous centrifugations, our laboratory previously reported that HSV-1 has a sedimentation constant of 55*S* and a molecular weight of $(99 \pm 5) \times 10^6$ daltons (12). This figure is in excellent agreement with the molecular weight of the DNA calculated from measurements of its length by use of an electron microscope (2) and with the kinetic complexity of the DNA ($[95 \pm 1] \times 10^6$ daltons) measured from its reassociation kinetics (9).

(ii) As previously reported (12), both HSV-1 DNA and HSV-2 DNA form multiple bands on centrifugation in alkaline sucrose density gradients. An example of the profile of the HSV-1 DNA banded along with T4 DNA in alkaline sucrose density gradients is shown in the bottom panel of Fig. 1. T4 DNA invariably formed one band; HSV-1 DNA invariably formed one major band (designated as band 1) sedimenting slightly slower than T4 DNA and several minor bands (designated as bands 2, 3, etc., in order of decreasing sedimentation rate). Previous studies from our laboratory (12) based on analyses of numerous gradients have shown that the sedimentation constant of the DNA in band 1 was 69*S* and that the average molecular weight of the HSV-1 band 1 DNA, calculated according to the equation and coefficient of Studier (20), relative to that of T4 DNA was $(48 \pm 3) \times 10^6$ daltons. The molecular weight of the DNA therefore corresponds to one-half the molecular weight of

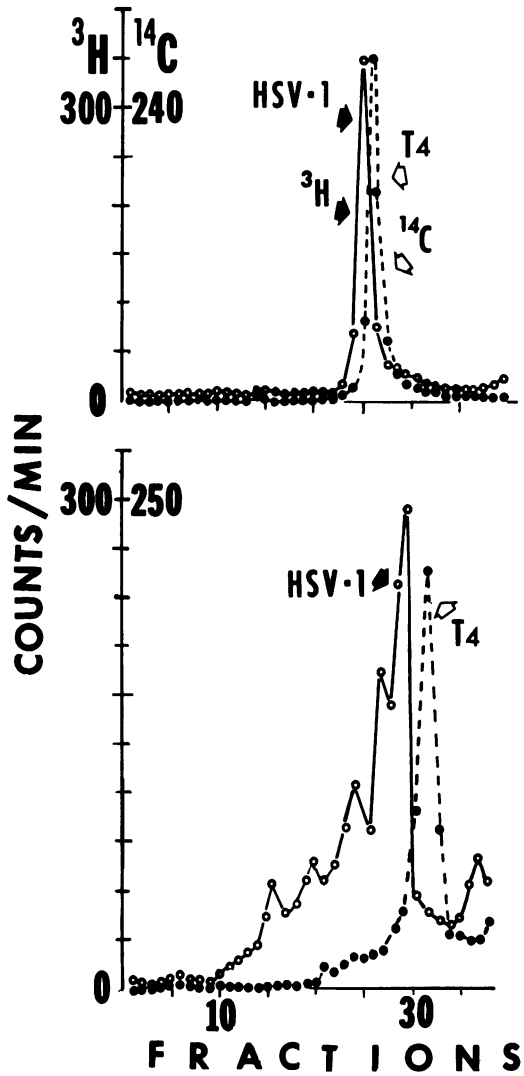

FIG. 1. *Zone sedimentation of HSV DNA in neutral and alkaline sucrose density gradients. ^{3}H-labeled HSV-1 DNA was mixed with ^{14}C-T4 DNA and co-sedimented in 5 to 20% (w/w) neutral (top panel) and alkaline (bottom panel) sucrose density gradients. The ^{14}C-T4 DNA was a gift of Robert Haselkorn. HSV-1 DNA was prepared from HSV-1 nucleocapsids grown in human epidermoid carcinoma (HEp-2) cells and purified as described in Materials and Methods. Direction of sedimentation is to the right. Solid line, ^{3}H-labeled DNA; dashed line, ^{14}C-labeled DNA. The calculated sedimentation constants for the intact HSV-1 DNA duplex based on co-sedimentation with T4 DNA was 55S, corresponding to a molecule 99 million daltons in molecular weight (12). The most rapidly sedimenting HSV-1 DNA band in the alkaline sucrose gradient had a sedimentation constant of 68S and corresponded in size to a molecule one-half that of the intact DNA duplex (12).*

the DNA duplex, indicating that band 1 contains intact single strands.

In the studies described in this paper, we calculated the sedimentation constants of the HSV-1 DNA fragments relative to the sedimentation constant of band 1 DNA calculated in previous studies (12). The frequency distribution of the sedimentation constants of DNA bands collected in numerous alkaline density gradients is shown in Fig. 2. The data show that the sedimentation constants of HSV-1 DNA smaller than the intact strand (band 1) form six clusters corresponding to six DNA bands with average relative sedimentation values ranging from 38 to 63S. The molecular weights of the DNA in bands 2 to 7 were calculated from the sedimentation constants by use of the equation of Studier (20). The data (Table 1) are of interest from two points of view. First, the frequency distribution indicates that the fragments are nonrandom in size, i.e., the length of the fragments is ordered. The second finding of particular interest is that the fragments appear as if they originated from single breaks of intact strands. Thus, fragment 7 complements fragment 2 to make one strand, as do fragments 6 plus 3 and 4 plus 5.

Uniqueness of HSV DNA fragments. These experiments were concerned with the question of whether the nonrandom-sized fragments originated from one unique strand or randomly from either strand. To differentiate between these possibilities, the peak fractions containing DNA from the various bands were collected, and allowed to self-associate as described in Materials and Methods. Three different experiments were done. In the first experiment, the reassociation of band 1 DNA was monitored continuously in a Gilford recording spectrophotometer. As a control, we used an identical amount of DNA banded in a neutral sucrose density gradient and handled subsequently in exactly the same way as the band 1 DNA. The results of this experiment, shown in Fig. 3, indicate that band 1 DNA reassociated at a slower rate than the DNA banded in the neutral sucrose density gradient. Whereas approximately 86% of the neutral

TABLE 1. *Size and sedimentation of the intact strand (band 1) and of the fragments (bands 2–7) banded in alkaline sucrose density gradients*

Band no.	Relative distance sedimented	Sedimentation constant	Molecular wt (daltons)
1	1.000	68[a] ± 1.5	48[b] ± 2.6 × 10^6
2	0.92 ± 0.02	62.3 ± 2.7	39.0 ± 4.2 × 10^6
3	0.86 ± 0.02	58.3 ± 2.6	32.9 ± 3.6 × 10^6
4	0.79 ± 0.02	53.8 ± 2.5	26.6 ± 3.1 × 10^6
5	0.72 ± 0.02	48.8 ± 2.4	21.1 ± 2.6 × 10^6
6	0.63 ± 0.02	42.6 ± 2.3	15.1 ± 2.0 × 10^6
7	0.53 ± 0.01	36.0 ± 1.5	9.8 ± 1.01 × 10^6

[a] Based on co-sedimentation with T4 DNA in alkaline sucrose density gradients in these and other (1, 12) studies.

[b] Calculated value based on the coefficient of Studier (20).

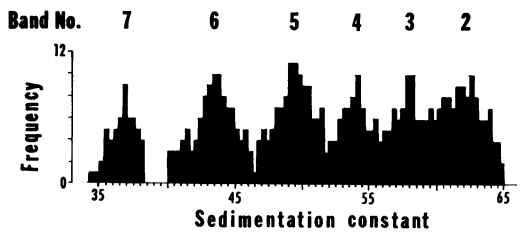

FIG. 2. *Sedimentation of HSV-1 DNA in alkaline sucrose density gradients. The sedimentation constants of the fragments smaller than the intact strands were calculated from the distance of sedimentation relative to that of the band containing the intact strand (band 1) according to the equation of Burgi and Hershey (8). The frequency distribution of the sedimentation constants in numerous gradients obtained in several independent experiments is shown in the figure. The calculations of the sedimentation constant of band 1 are as described in the legend to Fig. 1.*

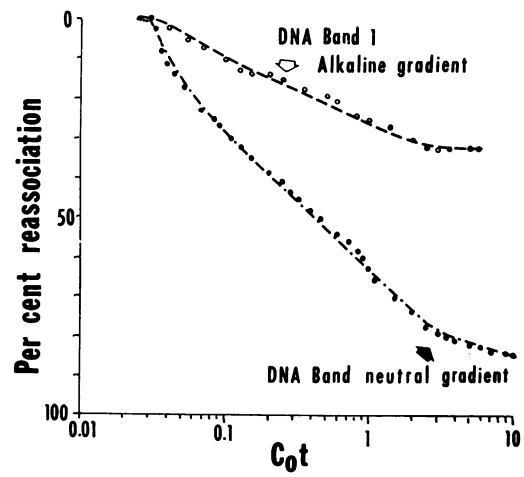

FIG. 3. *Comparison of the self-reassociation of the DNAs in alkaline density gradient band 1 (intact strand) and the DNA banded in neutral sucrose density gradient. The DNAs were sonically disrupted in alkaline solutions to fragments 5S. The hybridization tests were done at 75 C in 0.3 M phosphate and were monitored in a Gilford recording spectrophotometer. The data were adjusted for 0.12 M phosphate according to the relationship given by Britten and Smith (6) to enable comparison with the renaturation studies published previously (9).*

DNA was in hybrid form, only 32% of the total DNA in band 1 ended up in duplex form. The C_0t_{50} of the hybridizable fraction of band 1 DNA is similar to or slightly lower than that of the neutral DNA, and both are in agreement with the value obtained from the studies of reassociation kinetics of HSV DNA (9). The results of this experiment indicate that the bulk of the DNA in band 1 consisted of one, unique strand. The observed reassociation could be accounted for by contamination of band 1 DNA with DNA fragments from adjacent bands. Thus, the observed amount of band 1 DNA in duplex form could be accounted for by 16% of the DNA in this band being in fact contaminants from slower bands that arise from the opposite strand.

In the second and third experiments (Table 2), we monitored the extent of reassociation of the DNAs in bands 1, 3, 4, 5, and 6 by chromatography on hydroxyapatite columns. In the second experiment, the single-stranded DNA and the DNA-DNA hybrids were eluted from the hydroxyapatite columns sequentially with a continuous linear potassium phosphate gradient. In the third experiment, the DNAs were eluted stepwise with sodium phosphate buffers of different molarities as described in Materials and Methods. The results may be summarized as follows. Band 1 DNA appears to be two to three times more extensively reassociated as measured by hydroxyapatite chromatography (experiments 2 and 3) than by optical density measurements (experiment 1). As demonstrated by Britten and Kohne (5), the reason for the observed discrepancy between the rate of reassociation measured on hydroxyapatite and that measured optically is that a partial hybrid (with free single-stranded ends) appears as a partial hybrid in optical measurements but behaves as a complete hybrid on hydroxyapatite columns. Nevertheless, in each experiment the reassociation of the DNAs in bands 1 to 6 is considerably less than that of the DNA banded in a neutral sucrose density gradient. Moreover, the apparent degree of reassociation of the DNA in bands 3, 4, 5, and 6 is similar to that of band 1 DNA. This means that if the extent of reassociation were measured optically the DNA in duplex form would not exceed 30 to 40% of the total DNA in each band. The data indicate that at least a portion of the DNAs in bands 3, 4, 5, and 6 is unique. As with band 1 DNA, it is very likely that the self-association of the DNAs in these bands is due to overlapping of adjacent bands containing DNAs from opposite strands. The data exclude the possibility that the larger DNA fragments arose by nonrandom cleavage of both strands. Thus, the degree of reassociation is less than would be predicted were band 3 (35×10^6 daltons) to contain fragments originating from both strands.

We conclude from these data that the bulk of the DNA in each band represents fragments of DNA of ordered size and unique sequence, each arising from a unique strand. Moreover, on the

TABLE 2. *Self-association of DNA banded in neutral and alkaline sucrose density gradients*[a]

Expt	DNA tested	Observed percent remaining single-stranded	Percent ssDNA in alkaline band/percent ssDNA in neutral
1. Change in optical density during hybridization	Neut[b]	14.0	1.0
	Band 1[c]	68.0	4.8
2. Separation of ssDNA and dsDNA on hydroxyapatite by gradient elution	Neut	12.6	1.0
	Band 1	31.0	2.5
	Band 3	24.6	2.0
	Band 4	32.5	2.6
	Band 5	26.2	2.1
3. Separation of ssDNA and dsDNA on hydroxyapatite by stepwise elution	Neut	16.2	1.0
	Band 1	42.3	2.6
	Band 3	42.0	2.6
	Band 5	31.3	1.9
	Band 6	27.4	1.7

[a] The DNAs from the alkaline sucrose density gradient bands and the neutral DNA were allowed to reassociate to the same C_0t values in each of the experiments. The measurements based on changes in optical density (experiment 1) are the same as those shown in Fig. 3 at the end of hybridization. The fractionation on hydroxyapatite columns was done as outlined in Materials and Methods. ssDNA = single-stranded DNA; dsDNA = double-stranded DNA.

[b] DNA banded in neutral sucrose density gradient.

[c] In alkaline sucrose density gradient.

basis of these data, together with the observation that the intact strand represents less than 50% of the total DNA and the finding that bands 4, 5, and 6, although far removed from band 1, do reassociate to some extent, it seems clear that the fragments are derived uniquely from both strands.

How do the fragments arise? In this section, we present evidence that newly made viral DNA yields more fragments than mature viral DNA and that, as the viral DNA matures, the number of fragments generated in alkaline gradients actually decreases. In these experiments, we took advantage of the fact that, in HEp-2 cells infected with HSV, host DNA synthesis ceases almost completely within the first 3 hr after infection and that after 4 hr postinfection only viral DNA is made (16, 18). Infected cells were pulse-labeled for 3, 7, 30, 80, and 120 min in the interval between 4.5 and 6.5 hr postinfection. The nuclei were then extracted from infected cells with NP-40, collected by centrifugation, lysed, and centrifuged on alkaline sucrose density gradients as described in Materials and Methods. To facilitate interpretation of the data, we co-centrifuged with each nuclear extract a sample of ^{14}C-DNA extracted from purified virions collected 20 hr postinfection. For purposes of comparison, we also centrifuged the DNA extracted from virions contained in the cytoplasm of the cells labeled for 120 min. The acid-precipitable counts in the sucrose density gradient fractions are shown in Fig. 4. The salient feature of the data is that the DNA labeled for 3 min was roughly 0.5×10^6 to 10×10^6 daltons in molecular weight. As the duration of the labeling period increased, the DNA became larger. Similar results were obtained by Horowitz (11) on

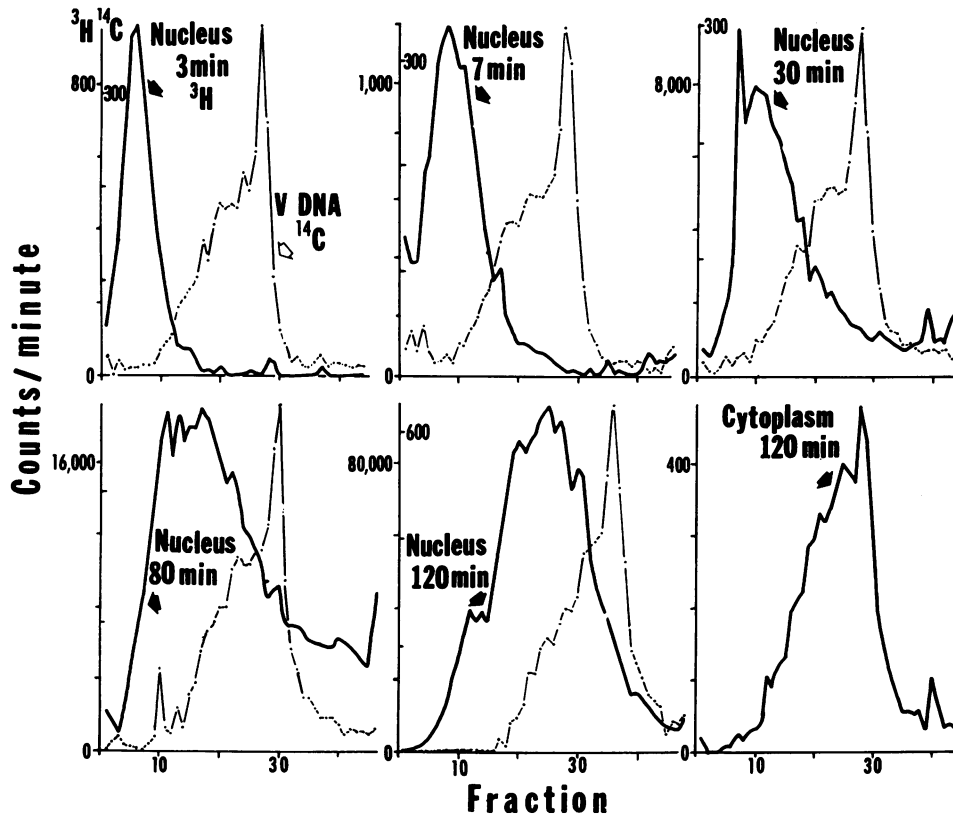

FIG. 4. *Profile of labeled DNA in nuclear and cytoplasmic lysates labeled with ^3H-thymidine (10 μCi/ml) for intervals ranging from 3 to 120 min between 4.5 and 6.5 hr postinfection with HSV-1. The nuclear and cytoplasmic lysates were prepared as outlined in Materials and Methods and were centrifuged in 5 to 20% (w/w) alkaline sucrose density gradients. The lower right panel shows the profile of the labeled DNA in the cytoplasm of cells labeled for 120 min. To facilitate comparison of the various profiles, ^{14}C-labeled HSV-1 DNA extracted from virions contained in cytoplasm of 24-hr infected cells was co-centrifuged with all nuclear lysates. Solid line, ^3H-labeled DNA; dashed line, ^{14}C-labeled DNA. Sedimentation from left to right.*

adenovirus DNA replication. Our data indicate that the growing end of DNA molecules is invariably attached to segments which are at most 10 million daltons long and that the elongation of the molecule beyond this size results from some process other than the addition of single nucleotides. It is noteworthy that appreciable amounts of intact strands were not present until 30 min postlabeling and that even in nuclei labeled for 120 min the bulk of the DNA was smaller than the intact strand. Nevertheless, comparison of the sucrose density gradient profiles of the labeled DNA in the nucleus and in the virion after 120 min of labeling indicates that the packaged DNA is not randomly selected from among the nuclear DNA population but rather it represents a population of DNA which in alkaline gradients yields a minimal number of fragments.

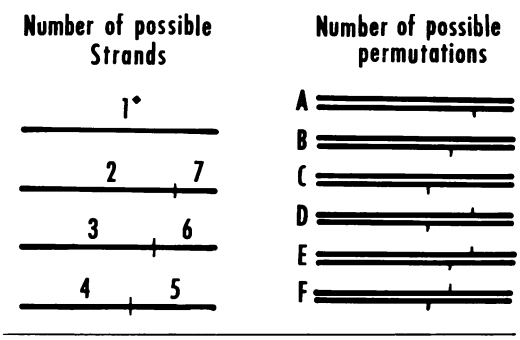

FIG. 5. *Diagrammatic reconstruction of single strands and duplexes of HSV DNA from the intact strand (band 1) and the various fragments contained in bands 2 to 7. The molecular weights of the fragments were taken from Table 1. The permutations shown describe types of duplexes without assigning polarity to the fragments. The number of permutations becomes 9 with the assigned polarity since molecules of the types D, E, and F could occur in two forms depending on whether the fragmented strands have the same base sequences as the intact strand or the complementary sequences. Further explanations are given in the text.*

DISCUSSION

Structure of HSV DNA. In this paper, we have presented evidence that on centrifugation in alkaline sucrose density gradients HSV-1 DNA forms seven bands containing, respectively, an intact strand and six fragments of nonrandom size and unique nucleotide sequence. Our studies were done on DNA molecules banded in alkaline sucrose density gradients.

As indicated diagrammatically in Fig. 5, the intact DNA strand and the six fragments fit four single strands. Based on the evidence presented earlier in the text, that the fragments arise uniquely from both DNA strands, the one intact and the four reconstructed strands could form no more than four duplexes out of the six permutations shown in Fig. 5. Thus, if fragments 2 and 7 originate from the same strand as band 1 DNA, the possible permutations are B, C, D, and E. Preliminary experiments based on hybridization in solution of the DNA with ribonucleic acid transcribed in the course of productive infection indicated that fragments 4 and 5 probably originate from the same strand as band 1 DNA and, therefore, that HSV DNA molecules exist in four forms, i.e., A, B, D, and F. The mechanisms which limit the number and specify the form are not clear. It should be noted that recent studies (summarized in detail in reference 10) indicated that T5 DNA also contains single-strand interruptions at unique and preferred sites.

Origin and nature of the single-strand interruptions in HSV DNA. Previous studies from our laboratory (1, 12) have shown that the fragmentation of HSV DNA in alkaline gradients is not due to manipulative procedures or to the action of a putative nuclease incorporated into the virion. In this study, we have shown that newly synthesized DNA generates more fragments than DNA extracted from virions and that the size of the fragments increases with time. Moreover, the DNA labeled during a relatively brief time (2 hr) and incorporated into virions cannot be differentiated from the DNA present in virions late in infection, but is readily differentiated with respect to the size and number of fragments generated in an alkaline sucrose gradient from the DNA labeled during the same interval but not yet incorporated into virions.

The explanation which best fits the observed data is as follows. (i) There are numerous initiation sites for DNA replication along each strand of viral DNA. (ii) DNA is elongated by addition of single deoxyribonucleotides in the segments between the initiation sites. (iii) Adjacent segments are linked by some process which could involve ligation alone or ligation preceded by excision of nucleotides which are linked by covalent bonds labile in alkaline solutions. Among such nucleotides could be ribonucleotides, possibly as a consequence of their putative function as primers in the initiation of DNA synthesis (7). (iv) Whatever the nature of the process, it is clearly selective. This conclusion emerges from the fact that only one strand is

completely processed and that the fragments are limited in number and unique with respect to size and nucleotide sequence. (v) Only maximally processed DNA ends up in enveloped nucleocapsids accumulating in the cytoplasm. This conclusion emerges from the fact that the DNA labeled for 120 min immediately before extraction from cytoplasmic virions cannot be differentiated from that extracted from virions late in infection.

Function of the interruptions in HSV DNA. Earlier in the text, we presented evidence suggesting that the fragmentation is a consequence of incomplete processing of the DNA strands after synthesis. We do not know why the DNA is incompletely processed or whether the interruption in processing fulfills a unique function required for the replication of this virus. The observations that the fragmentation of the DNA in alkaline solution is independent of the host in which the virus is grown (12) and that fragmentation appears to be a general property of herpesviruses varying widely in guanine plus cytosine content (1, 12, 14, 15) suggest that the interruption in processing fulfills a specific functional requirement. Co-sedimentation of the bands of HSV-1 and HSV-2 in alkaline sucrose gradients (12) showed that the DNAs generate fragments of similar sizes. In other studies (13, 17), it was shown that the two viruses share only 47% of their base sequences. Thus, it seems that there has been a selective pressure to maintain this property of the DNA. Also, on the basis of the evidence presented here, it seems that the DNAs of the two HSV subtypes are replicated in a similar manner and that at least some of the initiation sites for DNA synthesis may have been preserved. We do not know how the interruptions arose and what function they might have. Conceivably, the interruptions in processing could occur if the DNA site to be ligated or repaired, or both, is already occupied by another molecule. Such molecules could be ribonucleic acid polymerases or viral structural proteins involved in the condensation of DNA for packaging into virions, but more work will be needed to establish whether this is really the case.

ACKNOWLEDGMENTS

These studies were aided by grants from the American Cancer Society (NP-15G), the National Cancer Institute (CA-08494), and the National Science Foundation. We should like to acknowledge that some of the preliminary experiments on pulse-labeling of the viral DNA were done by Elliott Kieff and Steven Backenheimer. N. F. is a predoctoral fellow in the Committee on Virology sponsored by the Whitehall Foundation.

LITERATURE CITED

1. Bachenheimer, S. L., E. D. Kieff, L. Lee, and B. Roizman. 1972. Comparative studies of DNAs of Marek's disease and herpes simplex virus. Symposium on Oncogenesis and Herpes-Type Viruses, Cambridge, England, June, 1971, p. 24–81. International Agency Research Against Cancer, Lyon.
2. Becker, Y., H. Dym and I. Sarov. 1968. Herpes simplex virus DNA. Virology 36:184–192.
3. Bernardi, G. 1969. Chromatography of nucleic acids on hydroxyapatite. I. Chromatography of native DNA. Biochim. Biophys. Acta 174:423–434.
4. Bernardi, G. 1969. Chromatography of nucleic acids on hydroxyapatite. II. Chromatography of denatured DNA. Biochim. Biophys. Acta 174:435–448.
5. Britten, R. J., and D. E. Kohne. 1967. Nucleotide sequence repetition in DNA. Carnegie Inst. Washington Yearb. 65:78–106.
6. Britten, R. J., and J. Smith. 1970. A bovine genome. Carnegie Inst. Washington Yearb. 68:378–386.
7. Brutlag, D., R. Scherkman, and A. Kornberg. 1971. A possible role for DNA polymerase in the initiation of M13 DNA synthesis. Proc. Nat. Acad. Sci. U.S.A. 68:2826–2829.
8. Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. Biophys. J. 3:309–321.
9. Frenkel, N., and B. Roizman. 1971. Herpes simplex virus: genome size and redundancy studied by renaturation kinetics. J. Virol. 8:591–593.
10. Hayward, S. G., and M. G. Smith. 1972. The chromosome of bacteriophage T5. I. Analysis of the single stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. 63:383–395.
11. Horowitz, M. S. 1971. Intermediates in the synthesis of type 2 adenovirus deoxyribonucleic acid. J. Virol. 8:675–683.
12. Kieff, E. D., S. L. Bachenheimer, and B. Roizman. 1971. Size, composition, and structure of the deoxyribonucleic acid of subtypes 1 and 2 herpes simplex virus. J. Virol. 8:125–132.
13. Kieff, E. D., B. Hoyer, S. Bachenheimer, and B. Roizman. 1972. Genetic relatedness of type 1 and type 2 herpes simplex viruses. J. Virol. 9:738–745.
14. Lee, L., E. D. Kieff, S. L. Bachenheimer, B. Roizman, P. G. Spear, B. R. Burmester, and K. Nazerian. 1971. The size and composition of Marek's disease virus DNA. J. Virol. 7:289–294.
15. Nonoyama, M., and J. S. Pagano. 1971. Detection of Epstein-Barr viral genome in nonproductive cells. Nature N. Biol. 233:103–106.
16. Roizman, B. 1969. The herpesvirus—a biochemical definition of the group. Curr. Top. Microbiol. Immunol. 49:1–79.
17. Roizman, B., and N. Frenkel. 1972. Herpesvirus DNA: structure, synthesis and transcription. Proc. Symposium on Molecular Studies in Viral Neoplasia of the M. D. Anderson Hospital and Tumor Institute, Houston, Tex., 8–10 March 1972, *in press*.
18. Roizman, B., and P. R. Roane, Jr. 1964. The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp-2 cells. Virology 22:262–269.
19. Roizman, B., and P. G. Spear. 1968. Preparation of herpes simplex virus of high titer. J. Virol. 2:83–84.
20. Studier, F. 1965. Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373–390.