JOURNAL OF VIROLOGY, Aug. 1977, p. 394-411

Vol. 23, No. 2
Printed in U.S.A.

Anatomy of Herpes Simplex Virus DNA

VIII. Properties of the Replicating DNA

ROBERT J. JACOB AND BERNARD ROIZMAN*

Marjorie B. Kovler Viral Oncology Laboratories, University of Chicago, Chicago, Illinois 60637

Received for publication 9 February 1977

This paper concerns the properties of herpes simplex virus 1 DNA replicating in HEp-2 and human embryonic lung cells. The results were as follows. (i) Only a small fraction of input viral DNA entered the replicative pool. The bulk of the input viral DNA cosedimented with marker viral DNA and did not appear to be degraded or dissociated into L and S components. (ii) Nascent DNA sedimented faster and banded at a higher density than that of mature viral DNA extracted from virions. Pulse-chase experiments indicated that nascent DNA acquires the sedimentation rate and buoyant density of viral DNA within 30 to 40 min after its synthesis. (iii) Electron microscopic studies indicated that the DNA extracted from cells replicating viral DNA and banding at the density of viral DNA contained: (a) linear, full-size molecules with internal gaps and single-stranded regions at termini; (b) molecules with lariats, consisting of a linear segment up to 2× the size of mature DNA and a ring ranging from 0.5×10^6 to 100×10^6 in molecular weight, showing continuous and discontinuous forks; (c) circular, double-stranded molecules, both full-size and multiples of 18×10^6 in molecular weight, but without forks or loops; (d) molecules showing "eye" and "D" loops at or near one end of the DNA; (e) large, tangled masses of DNA, similar to those observed for T4 and pseudorabies virus replicating DNAs, containing loops and continuous and discontinuous forks. The electron micrographs are consistent with the hypothesis that the single-stranded ends on the DNA anneal to form a hairpin, that the DNA synthesis is initiated at or near that end and proceeds bidirectionally to form a lariat, and that resulting progeny derived by semiconservative replication are "head-to-head" and "tail-to-tail" dimers.

Herpes simplex virus 1 (human herpesvirus 1; HSV-1) DNA has been shown to be $(96 \pm 6) \times 10^6$ in molecular weight and 44.5 ± 2.8 μm in length (16, 26) and to consist of two covalently linked components, designated L and S, containing 82 and 18% of the total DNA, respectively (22, 26). The L components consist of a non-reiterated sequence, U_l, corresponding to 70% of the total DNA, bracketed by the sequence ab (6% of the total) and its inverted repeat, $b'a'$. Similarly, the S component contains a non-reiterated sequence, U_s, corresponding to 9% of the total DNA, bracketed by the sequence ac and its inverted repeat, $c'a'$ (18, 19, 26). The L and S components share only the sequence a, corresponding to 0.25 to 0.5% of the total DNA; the a sequence has been shown to contain a sequence designated d and its inverted repeat, d' (13, 25). A characteristic feature of HSV DNA is that the L and S components appear to orient themselves randomly relative to each other (8, 18, 19, 27). In consequence, viral DNA extracted from virions derived from cloned stocks invariably contains four populations of molecules in equal proportions, differing only with respect to the orientation of the L and S components relative to each other. The mechanism generating the four populations of molecules is not known. One possibility that initially led to the studies described in this article is that after infection the L and S components dissociate and replicate separately. The studies described in this article deal, therefore, with the properties of parental viral DNA infecting cells and with the characterization and properties of viral DNA made in the course of viral replication.

Previous studies on the replication of herpesvirus DNA may be summarized as follows.

(i) Initiation of viral DNA replication requires prior synthesis of virus-directed polypeptides (15, 20).

(ii) The amount of input viral DNA entering the replicative pool is reported to vary from very small, in the case of HSV (9), to relatively large, in the case of pseudorabies virus (2).

(iii) Both pseudorabies virus DNA (15) and HSV DNA (17) replicate in a semiconservative

fashion. Newly replicated DNAs contain relatively small oligonucleotide fragments, which are repaired and/or ligated on maturation (1, 3, 7). Replication of HSV DNA in isolated nuclei is accompanied by extensive repair synthesis (17).

(iv) Frenkel et al. (5, 6) concluded that at least one site of initiation of viral DNA synthesis occurs at or near the terminus of the *S* component. This conclusion was based on the observation that defective HSV DNA consisted of tandem repeats of a small sequence arising from one terminus of the *S* component. Shlomai et al. (23) concluded from electron microscopic studies indicate that newly replicated pseudorabies virus DNA is longer than the mature, presence of replicating loops, and proceeds bidirectionally, resulting in the formation of branched, "Y"-shaped replicating molecules. On the other hand, Jean and Ben-Porat (14) reported that, prior to the onset of DNA synthesis, pseudorabies virus DNA acquires single-stranded ends, presumably as a consequence of digestion by an exonuclease, and that these molecules anneal to form circles and concatemers. DNA synthesis is initiated in an internal site, and replicative loops were observed in both circular and linear molecules.

(v) Hydrodynamic and electron microscopic studies indicate that newly replicated pseudorabies virus DNA is longer than the mature unit-size DNA, and it has been suggested that the newly synthesized DNA is a concatemeric intermediate (1). Electron microscopic studies have also shown the presence of large tangles, similar to those seen in T4-infected cells (12).

(Preliminary reports of the data reported in this article were presented at the meetings on DNA insertions [May 1976] and herpesviruses [September 1976] at Cold Spring Harbor, N.Y.)

MATERIALS AND METHODS

Cell cultures, viruses, and infection. The procedures for the propagation of HEp-2 cells were described elsewhere (21). Human embryonic lung (HEL) cells, originally obtained from Flow Laboratories, Inc., Rockville, Md., were propagated in the same fashion. The isolation and properties of HSV-1(F) and HSV-1(MP) virus strains have been described elsewhere (4, 10). The HEp-2 cell cultures were infected at a density of 4×10^6 cells per plastic flask (25 cm^2, Falcon Plastics, Oxnard, Calif.); HEL cells were grown to stationary phase and used 7 days after passage, at a density of 2.0×10^6 cells per flask. The monolayer cultures were infected at a multiplicity of 20 PFU/cell in a 1-ml volume, by rotary shaking at 37°C for 2 h.

Labeling and extraction of DNA. The cells were infected and maintained in medium 199-1, consisting of mixture 199 (International Scientific Industries, Inc., Carey, Ill.) supplemented with 1% heat-inactivated calf serum. The cells were labeled with [*methyl*-^{3}H]thymidine (specific activity, 59 Ci/mmol; New England Nuclear, Boston, Mass.) at 20 μCi/ml and with bromodeoxyuridine (Calbiochem, San Diego, Calif.) at 22 μg/ml, in 3 ml of medium 199-1 lacking thymine. In pulse-chase experiments, the cultures were exhaustively washed after the pulse and then incubated in medium 199-1 containing a 1,000-fold excess of the unlabeled nucleoside. The incorporation of the radioactive nucleoside was routinely followed by measuring acid-precipitable radioactivity as described below.

For DNA extraction, the cell monolayers were washed and scraped in phosphate-buffered saline (21) and then collected by centrifugation for 10 min at 8,000 rpm and 4°C in the JS13 rotor of the Beckman J21 centrifuge (Beckman Instruments, Inc., Chicago, Ill.). The cell pellets (2×10^6 to 4×10^6 cells) were suspended in 1 ml of either TE buffer (0.01 M Tris-hydrochloride, 1 mM EDTA, pH 7.5) or T buffer (1 mM Tris-hydrochloride, pH 7.5). When the DNA was extracted from total infected cell lysates, Sarkosyl, sodium dodecyl sulfate (Matheson, Coleman and Bell, Norwood, Ohio), Pronase (Calbiochem), and NaCl were added at final concentrations of 1%, 2%, 1 mg/ml, and 0.1 M, respectively, to the TE-buffered cell suspensions. The mixture was then incubated at 39°C for from 4 to 18 h. The Pronase was nuclease-free and prepared as described by Hotta and Bassel (11). When DNA was extracted from nuclei, the cells were allowed to swell in T buffer for 15 min at 4°C. The cells were then lysed by the addition of nonionic detergent (Nonidet P-40; BDH Chemical, Pools, England), 0.5% final concentration, and by four strokes in a tight-fitting Dounce homogenizer. The nuclei were pelleted by centrifugation and digested with Pronase in the presence of Sarkosyl and sodium dodecyl sulfate; the DNA was then extracted as described above. After this step, the DNA was never mixed violently or pipetted through less than a 1.5- to 2.0-mm bore.

Equilibrium centrifugation in CsCl density gradients. The DNA obtained after Pronase digestion was centrifuged to equilibrium in CsCl (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.) density gradients. The gradients were prepared by the addition of 6.5 g of solid CsCl to 5.0 ml of DNA solubilized in TE buffer. The isodensity of the CsCl solution produced in this fashion ranged from 1.712 to 1.718 g/cm^3 and allowed the banding of both host (ρ = 1.695 to 1.69 g/cm^3) and viral (ρ = 1.726 g/cm^3) DNAs at about the middle of the gradient. All centrifugations were done in a Beckman type 65 rotor spun at 36,000 rpm and 20°C for 72 h. Before centrifugation the tubes were overlaid with light mineral oil. The fractions, collected from the bottoms of the tubes by displacing the CsCl solution with oil, were uniformly 140 ± 20 μl in volume; they were collected slowly through a hole not less than 2 mm in diameter. Except for preparative purification of DNA for examination by electron microscopy, the CsCl gradients never contained more than 10 to 20% of the yield from a culture containing 2×10^6 to 4×10^6 cells. For measurements of radioactivity, the

DNA, precipitated with 5% trichloroacetic acid, was collected by filtration through nitrocellulose filters. The filters were washed with 2% potassium acetate in 80% ethanol, 80% ethanol, and then air dried before immersion in toluene-based scintillation counting fluid. It should be noted that studies (unpublished) of the load capacity and maximal band resolution between virus- and host-specific DNAs revealed that as much as half of the DNA yield from one such culture could be reproducibly fractionated in one tube when prepared in the manner described above.

^{14}C-labeled T4 marker DNA was obtained from K. Wilcox of the University of Chicago. ^{32}P-labeled marker DNA was prepared as previously described (8) and repurified by equilibrium centrifugation in CsCl density gradients.

Velocity sedimentation in sucrose density gradients. Centrifugations in sucrose density gradients were performed as described previously (16). Briefly, the DNA in TE buffer was layered on 10 to 30% (wt/wt) sucrose density gradients and centrifuged in an SW41 rotor at 40,000 rpm and 20°C for 2.75 h. Fractions (0.3 ml) were collected from the bottom, and the radioactivity was measured as described above.

Preparation and electron microscopy of DNA cytochrome *c* films. The DNAs banding in CsCl density gradients at the density characteristic for viral DNA were routinely examined in an electron microscope by the formamide modification of the Kleinschmidt spreading procedure. The reagents, spreading, shadowing, and microscopy of the DNA have been described elsewhere (26). The procedures for measuring the contour length of the DNA with the Graf Pen (Science Accessories Corp., Southport, Conn.) interfaced to a digital computer (SP 16/45, General Automation, Inc., Anaheim, Calif.) and conversion of contour lengths into molecular weights were also described previously (26).

RESULTS

Analysis of viral DNA introduced into the cell during infection. (i) Buoyant density of input viral parental DNA. To determine some of the hydrodynamic properties of input parental DNA during viral replication in human cells, three series of experiments were done. In the first series, the cells were infected with ^{32}P-labeled virus at a multiplicity of 20 PFU/cell. The cells were harvested at 1.5, 3, and 6.5 h postinfection and fractionated into nuclei and cytoplasm. The DNA from the nuclear fraction was then extracted and centrifuged to equilibrium in CsCl density gradients. Analysis of the distribution of counts showed that approximately 35% of the acid-precipitable ^{32}P counts reached the nucleus and were retained at 6 h postinfection. As shown in a representative figure (Fig. 1A), in all instances we observed that all of the input [^{32}P]DNA banded at the buoyant density of viral DNA, as indicated by the purified [^{3}H]thymidine-labeled viral DNA added to the extracted DNA before centrifugation. The data indicate that the bulk of viral DNA does not become entrapped or linked with macromolecules, such as host DNA, that would alter its characteristic density.

(ii) Fraction of input viral DNA entering the replicative pool. The purpose of the second series of experiments was to estimate the fraction of the input viral DNA that enters the replicative pool. The first experiment was designed to determine whether viral DNA synthesis could take place in the cells infected with ^{32}P-labeled virus. In this experiment, HEp-2

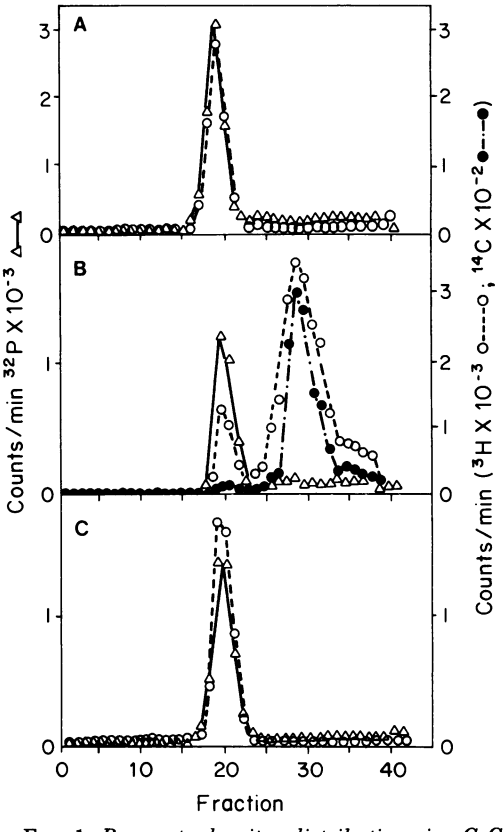

FIG. 1. *Buoyant density distribution in CsCl density gradients of DNA isolated from HEp-2 cells infected with ^{32}P-labeled HSV-1(F) virus. (A) Representative profile of DNA extracted from cells between 1.5 and 6.5 h postinfection. Symbols: △, ^{32}P-labeled input HSV-1 DNA; ○, ^{3}H-labeled HSV-1 marker DNA. (B) DNA was extracted at 6.5 h postinfection from cells labeled with [^{3}H]thymidine (20 μCi/ml) from 5 to 6.5 h. Symbols: △, ^{32}P-labeled input HSV-1 DNA; ●, ^{14}C-labeled T4 phage DNA; ○, [^{3}H]thymidine-labeled DNA. (C) DNA was extracted at 9 h postinfection from cells treated throughout infection (0 to 9 h) with BUdR (22 μg/ml). Symbols: △, ^{32}P-labeled input HSV-1 DNA; ○, ^{3}H-labeled HSV-1 marker DNA. In this and subsequent figures, the bottom of the tube is at the left.*

cells infected with ^{32}P-labeled virus at a multiplicity of 20 PFU/cell were labeled with [^{3}H]thymidine from 5.5 to 6.5 h postinfection. The cells were then harvested; the nuclear DNA was extracted as above, admixed with ^{14}C-labeled T4 DNA, and centrifuged to equilibrium in a CsCl density gradient. As shown in Fig. 1B, [^{3}H]thymidine was incorporated into both viral DNA, which cobanded with input [^{32}P]DNA, and host DNA, whose position is indicated by the ^{14}C-labeled T4 DNA marker. As found reproducibly in several experiments, this ratio of [^{3}H]thymidine in viral and cellular DNA was typical for that observed during a 1-h pulse at this time after infection. The data, therefore, indicated that viral DNA synthesis takes place in cells infected with the ^{32}P-labeled virus.

The second experiment in this series was designed to determine the fraction of input [^{32}P]DNA that enters the replicative pool. In this experiment, HEp-2 cells were infected with ^{32}P-labeled virus and maintained in the presence of BUdR (22 μg/ml). At 9 h postinfection, the cells were harvested and the nuclear DNA was extracted. The results of equilibrium density gradient centrifugation (Fig. 1C) showed no appreciable shift in the buoyant density of [^{32}P]DNA with respect to the [^{3}H]thymidine-labeled viral marker DNA.

In the preceding experiments we found that the bulk of input labeled DNA did not shift in the presence of BUdR, even though the experimental data indicated that viral DNA synthesis was taking place in these cells. The three experiments described below show that: (i) the fraction of viral DNA entering the replicative pool was small and independent of the isotopic marker, the method of virus preparation and storage, or the nature of the cells that were infected with the virus; and (ii) BUdR is incorporated into DNA in these cells.

In the first experiment, HEp-2 cells were exposed from 2 to 9 h postinfection with ^{32}P-labeled virus to a mixture of BUdR (22 μg/ml) and [^{3}H]thymidine (20 μCi/ml). The cells were harvested at 9 h postinfection, and the nuclear DNA was extracted and centrifuged to equilibrium. Figure 2 shows that only a small amount of [^{3}H]thymidine was incorporated into DNA banding at the density of ^{32}P-labeled viral DNA and that the bulk of the [^{3}H]thymidine-labeled DNA banded at a density higher than that of [^{32}P]DNA. As in the previous experiments, the bulk of [^{32}P]DNA did not shift in density. However, the presence of BUdR in the [^{32}P]DNA might be inferred from the asymmetry in the distribution of the ^{32}P label. If this was indeed the case, the amount of [^{32}P]DNA in the light-heavy duplex DNA was probably small and did not exceed 5 to 10% of the total.

The second experiment was designed to determine whether the fraction of viral DNA entering the replicative pool was affected by either the isotope with which the input DNA was labeled, the cell line, or the method of preparation of labeled virus. In this experiment HEL cells were infected with [^{3}H]thymidine-labeled virus as described above. The cells were then incubated in the presence of BUdR (22 μg/ml) from 2 to 9 h postinfection. As shown in Fig. 3A, there was no appreciable shift in the density of [^{3}H]DNA with respect to the ^{32}P-labeled marker DNA. In variations of this experiment, we compared [^{3}H]thymidine-labeled virus that had been stored at −90°C for 1 to 3 months with labeled virus that was used immediately after preparation and had not been stored before use. The results with these stocks were identical to those shown in Fig. 1C and 3A, indicating that the fraction of viral DNA entering the replicative pool was independent of storage, isotopic label, or the cells infected with this virus.

In the third and final experiment of this series we exposed HEL cells infected with unlabeled virus to a mixture of BUdR (22 μg/ml) and [^{3}H]thymidine (20 μCi/ml) for 30 min and 1 h at 8.5 and 8.0 h postinfection, respectively. The density distribution of DNA extracted from these cells at 9 h postinfection is shown in Fig. 3B and C. The significant finding is that [^{3}H]thymidine-labeled DNA banded in three positions, i.e., at the density of BUdR-substituted DNA, at that of unsubstituted viral DNA

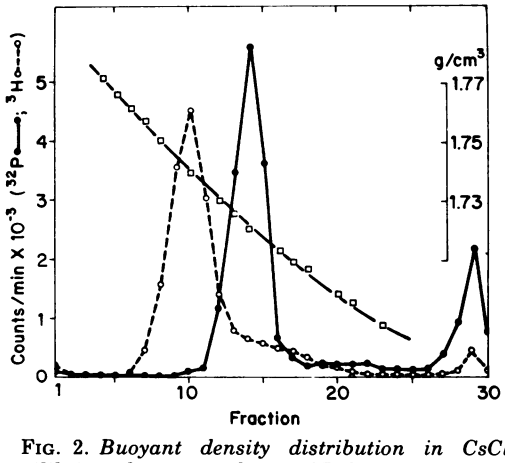

FIG. 2. *Buoyant density distribution in CsCl equilibrium density gradients of DNA extracted at 9 h postinfection from the nuclei of HEp-2 cells infected with ^{32}P-labeled HSV-1(F) virus. The cells were infected and maintained in the presence of BUdR (22 μg/ml) and [^{3}H]thymidine (20 μCi/ml). Symbols: ●, ^{32}P-labeled input HSV-1 DNA; ○, [^{3}H]thymidine-labeled DNA; □, density of CsCl solution, determined from its refractive index.*

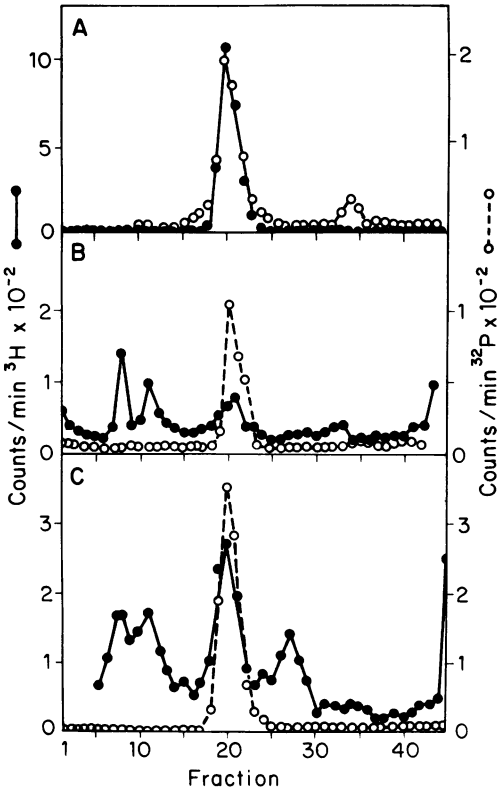

FIG. 3. *Buoyant density distribution in CsCl equilibrium density gradients of DNA isolated from HEL cells infected with HSV-1(MP) virus. (A) Profile of DNA isolated at 9 h after infection with [^{3}H]thymidine-labeled virus. The cells were infected and maintained (0 to 9 h) in the presence of BUdR (20 μg/ml). (B, C) Profiles of DNAs extracted at 9 h postinfection from cells exposed to a mixture of BUdR (22 μg/ml) and [^{3}H]thymidine (20 μCi/ml) for 30 and 60 min, respectively, before extraction. Symbols: ○, ^{32}P-labeled marker HSV-1 DNA; ●, [^{3}H]thymidine-labeled input viral DNA (A) and pulse-labeled DNA (B, C).*

identified by the marker DNA, and at a density expected of host DNA. The presence of [^{3}H]thymidine at the density of unsubstituted viral DNA might be interpreted as indicating an amount of synthesis, such as DNA repair, insufficient to cause a density shift. If this was the case, the data could be interpreted as indicating that, during the 1-h pulse, 40% of the radioactivity was incorporated into replicating DNA and 30% was incorporated into viral DNA undergoing repair.

(iii) Sedimentation velocity analysis of input viral DNA. The purpose of the next series of experiments was to determine whether input labeled DNA fragmented during the early events of replication, as a possible explanation of the results reported in the preceding section. In these experiments HEp-2 cells were infected with ^{32}P-labeled virus (20 PFU/cell). At 2.5 and 6 h postinfection the nuclear DNA was extracted and banded to equilibrium in CsCl density gradients. The viral DNA band was then harvested, concentrated by ethanol precipitation, redissolved in TE buffer, and centrifuged with ^{14}C-labeled T4 marker DNA in a sucrose density gradient (Fig. 4A). In a control experiment done at the same time (Fig. 4B), the ^{32}P-

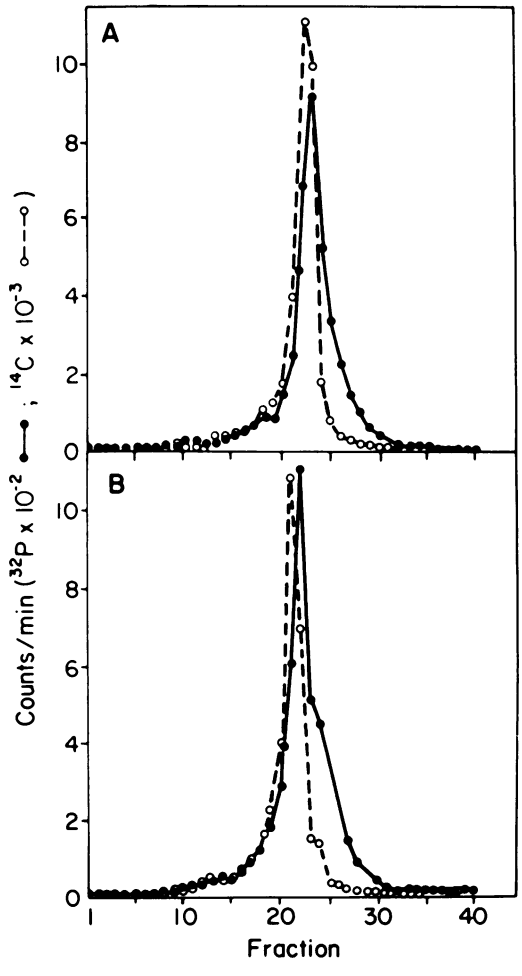

FIG. 4. *Sedimentation profiles in sucrose density gradients of DNA extracted from the nuclei of HEp-2 cells infected with ^{32}P-labeled HSV-1(F) virus. The DNAs were isolated as described in the text and purified by centrifugation in CsCl density gradients. Viral DNA was precipitated with cold ethanol, resuspended in TE buffer, and then cosedimented with a T4 phage DNA marker. (A) Representative profile of DNA extracted up to 6 h postinfection. (B) Profile of DNA extracted from an admixture of cells and labeled virus. Symbols: ●, ^{32}P-labeled input HSV-1 DNA; ○, ^{14}C-labeled T4 phage DNA.*

labeled virus was mixed with uninfected cells; the DNA was then extracted, banded in a CsCl density gradient, collected, and then centrifuged in a sucrose density gradient along with the ^{14}C-labeled T4 marker DNA. The data clearly show that the bulk of the labeled DNA extracted from the nuclei of infected cells was intact and could not be differentiated from the labeled viral DNA extracted from virions with respect to its sedimentation behavior.

Analysis of DNA made during infection. (i) Patterns of synthesis of viral and host DNA in HEp-2 and HEL cells. The purpose of the following experiments was to determine the pattern of incorporation of [^3H]thymidine into viral and cellular DNA in infected HEp-2 and HEL cells. In the first experiments, HEp-2 cells were labeled for 1 h, beginning with 5, 8, and 12 h postinfection and after mock infection. The DNA was extracted immediately after the labeling interval and centrifuged to equilibrium in CsCl density gradients. The results (Fig. 5A through D) were similar to those obtained previously (20) early in infection (Fig. 5B). [^3H]thymidine was incorporated primarily into host DNA, identified on the basis of the banding of DNA extracted from mock-infected cells (Fig. 5A). The fraction of [^3H]thymidine incorporated into viral DNA increased during subsequent labeling intervals (Fig. 5C and D), but in this series of experiments the total incorporated into viral DNA did not exceed that incorporated into host DNA.

In the second experiment we analyzed in a similar fashion the DNA labeled in HEL cells with [^3H]thymidine. The results (Fig. 5E

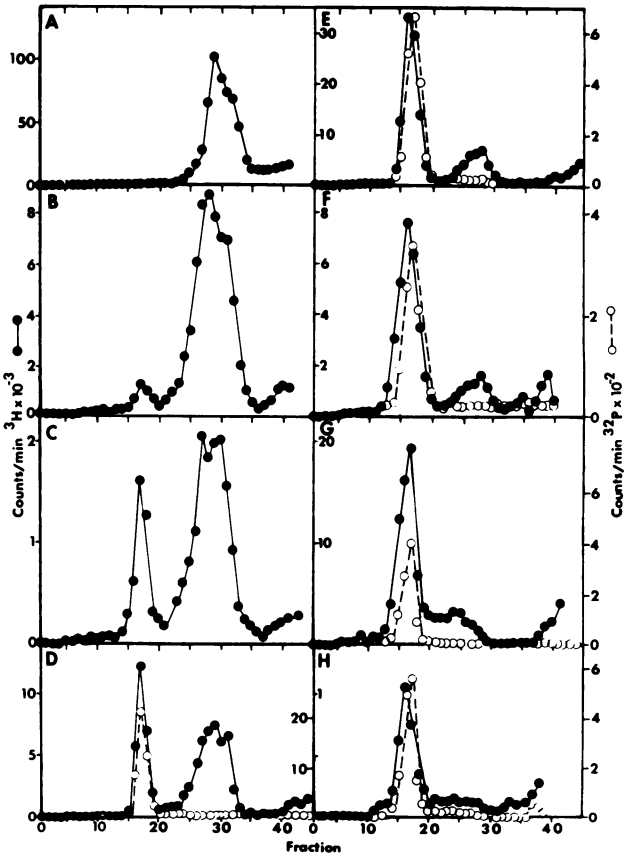

FIG. 5. *Buoyant density profile in CsCl equilibrium density gradients of [^3H]thymidine (20 μCi/ml)-labeled DNA extracted from cells at different times postinfection with HSV-1(F). (A–D) DNA extracted from HEp-2 cells. (E–H) DNA extracted from HEL cells. (A) DNA from HEp-2 cells continuously labeled for 9 h post-mock infection. (B–D) DNAs isolated after a 1-h labeling interval at 5, 8, and 12 h postinfection, respectively. (E) DNA extracted at 9 h postinfection from cells labeled continuously from the time of infection. (F, G) DNA extracted from cells labeled for 1 h at 5 and 8 h postinfection, respectively. (H) DNA extracted from cells immediately after a 10-min labeling interval at 8 h postinfection. Symbols: ●, [^3H]thymidine-labeled DNA; ○, ^{32}P-labeled marker HSV-1 DNA.*

through H) may be summarized as follows. (i) In infected HEL cells, the bulk of [^{3}H]thymidine was incorporated into viral DNA, identified by ^{32}P-labeled viral marker DNA. The fraction of [^{3}H]thymidine incorporated into host DNA appeared to be relatively constant in cells labeled from 2 to 9 h (Fig. 5E), 5 to 6 h (Fig. 5F), and 8 to 9 h (Fig. 5G) postinfection. (ii) Newly synthesized [^{3}H]thymidine-labeled DNA appeared to band at a higher density relative to the ^{32}P-labeled marker DNA. This difference was particularly striking in DNA labeled for 10 min at 8 h postinfection (Fig. 5H) but could be readily seen in the buoyant density profiles of DNA labeled for 1 h (Fig. 5F and G).

(ii) Hydrodynamic properties of nascent and pulse-chased labeled DNA. The results of experiments reported in the preceding section and in Fig. 5E through H indicate that at least a fraction of the viral DNA newly synthesized in HEL cells had a buoyant density higher than that of the labeled viral marker DNA. The experiments reported in this section show that in both HEp-2 and HEL infected cells the nascent DNA had a higher buoyant density than the mature labeled marker DNA and that after continued incubation after removal of [^{3}H]thymidine, the density of labeled viral DNA decreased to that of the marker DNA. The first series of experiments demonstrated that the [^{3}H]thymidine label could be effectively chased in both HEp-2 and HEL cells. In these experiments one set of replicate cell cultures was labeled continuously with [^{3}H]thymidine (20 μCi/ml), for up to 60 min, beginning at 8 h postinfection. At the time intervals indicated in Fig. 6, cells were harvested and the amount of labeled acid-insoluble radioactivity was measured. The second set of replicate cell cultures was labeled for 10 min at 8 h postinfection, washed several times with warm phosphate-buffered saline, and reincubated in 10 ml of medium 199-1. As shown in Fig. 6A, the amount of [^{3}H]thymidine incorporated into the DNA extracted from infected HEp-2 cells labeled for 10 min and chased for 60 min was 2- to 2.2-fold higher than that observed immediately after the pulse. This compares favorably to the observation that during continuous labeling the amount of [^{3}H]thymidine incorporated during 60 min was more than 10-fold higher than that observed after a 10-min pulse. Similar results were obtained in infected HEL cells (Fig. 6B). In both HEp-2 and HEL cells, most of the incorporation of [^{3}H]thymidine during the chase occurred in the first 10 min.

In the second series of experiments we examined the buoyant density of viral DNA labeled and then chased for different time intervals. In

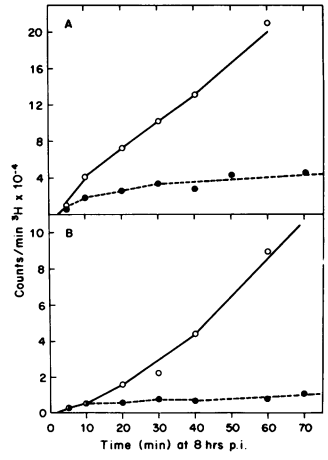

FIG. 6. *The pattern of incorporation of [^{3}H]thymidine at 8 h postinfection into HSV-1(F)-infected HEp-2 (A) and HEL (B) cells. Replicate infected cell cultures (2 × 10^6 cells) in one set were each exposed to 3 ml of labeling medium containing [^{3}H]thymidine (20 μCi/ml; specific activity, 59 Ci/mM) for intervals up to 1 h. Another set of replicate infected cell cultures was pulse-labeled for 10 min, washed extensively, and then reincubated in medium containing excess unlabeled thymidine (1,000×) for varying time intervals of up to 1 h. Symbols: ○, acid-precipitable radioactivity in DNA labeled continuously; ●, acid-precipitable radioactivity in DNA pulse-labeled and then chased.*

these experiments, replicate cultures of HEp-2 and HEL cells were pulse-labeled for 10 min at 8 h postinfection and then chased as described above for 20, 30, and 40 min. The buoyant density profiles of the pulse-chased DNA are shown in Fig. 7.

The data show that in both HEp-2 (Fig. 7A and B) and HEL (Fig. 7D and E) infected cells the peak of nascent viral DNA, labeled for 10 min and chased for 20 to 30 min, had a demonstrably higher density than the peak of the marker DNA band. In both HEp-2 and HEL infected cells (Fig. 7C and F, respectively) the difference in buoyant density was minimal or had largely disappeared after the 40-min chase. This is evident from the observation that the peaks of the [^{3}H]thymidine-labeled DNAs coincided with those of ^{32}P-labeled marker DNAs.

(iii) Velocity sedimentation analyses of replicating viral DNA. Two series of experiments were done to determine the sedimentation properties of nascent and maturing DNA. In the first series, HEp-2 cells were pulse-labeled for 10 min with [^{3}H]thymidine at 8 h postinfection, incubated in the absence of the precursor, and harvested at different time intervals after the initiation of the chase. The DNA was ex-

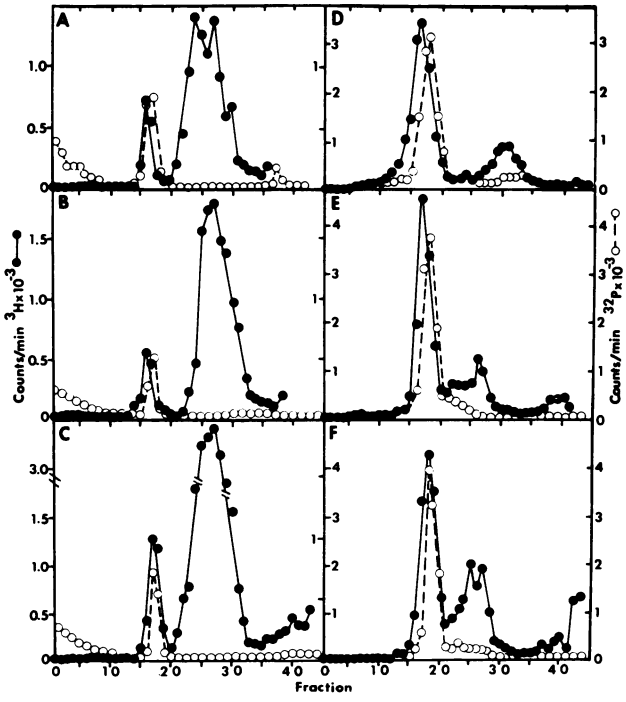

FIG. 7. *Buoyant density distribution in CsCl equilibrium density gradients of nascent viral DNA. Infected cells were labeled with [^{3}H]thymidine for 10 min at 8 h postinfection and chased as described in the legend to Fig. 6. The profiles shown are DNAs isolated from infected HEp-2 (A–C) and HEL (D–F) cells labeled and then chased for 20 (A, D), 30 (B, E), and 40 (C, F) min, respectively. Symbols: ●, [^{3}H]thymidine-labeled DNA; ○, ^{32}P-labeled marker HSV-1 DNA.*

tracted and sedimented in 10 to 30% (wt/wt) sucrose density gradients as described in the legend to Fig. 8. The profiles of the DNA extracted and centrifuged after the pulse and after 20-, 30-, and 40-min chases are shown in Fig. 8.

During the chase, the incorporation of [^{3}H]thymidine continued (Fig. 6), but at a much reduced rate. The amounts of [^{3}H]thymidine incorporated into DNA after a 20-, 30-, and 40-min chases were, respectively, 1.9-, 2.0-, and 2.1-fold higher than the amount incorporated during the 10-min pulse. The data of interest in this experiment were the relative amounts of acid-precipitable label sedimenting to the bottom of the tube and at the position of marker HSV DNA. Immediately after the pulse, there was no discrete band of DNA sedimenting at the position of mature viral DNA, and approximately 19% of the total sedimented to the bottom of the tube. After the 20-, 30-, and 40-min chase intervals the fractions of DNA banding at the position of viral DNA were 12, 13, and 25% of the total, respectively, whereas the fractions of DNA sedimenting to the bottom of the tube were 69, 71, and 23% of the total, respectively. The analyses of these results were complicated by the fact that the shutoff of [^{3}H]thymidine incorporation did not actually occur until the 20-min chase; moreover, these cells incorporated radioactivity into both viral and cellular DNA. However, the data do suggest that on a prolonged chase there was a redistribution of radioactive DNA from the rapidly sedimenting material that reached the bottom to the less rapidly sedimenting DNA that included material cosedimenting with viral marker DNA.

The purpose of the second series of experiments was to analyze the redistribution of radioactive DNA after the chase in HEL cells because, as shown in preceding sections of this paper, after infection of HEL cells, [^{3}H]thymidine is incorporated predominantly into viral DNA. In this experiment cell cultures were again pulse-labeled for 10 min at 8 h postinfection and then chased. The DNA was extracted from the infected cells immediately after the pulse and at 20-, 30-, and 60-min chase intervals and centrifuged in a 10 to 50% (wt/wt) sucrose density gradient, as described in the legend to Fig. 9. The sedimentation profiles of the DNA after the pulse and after the 20-, 30-, and 60-min chases are shown in Fig. 9A

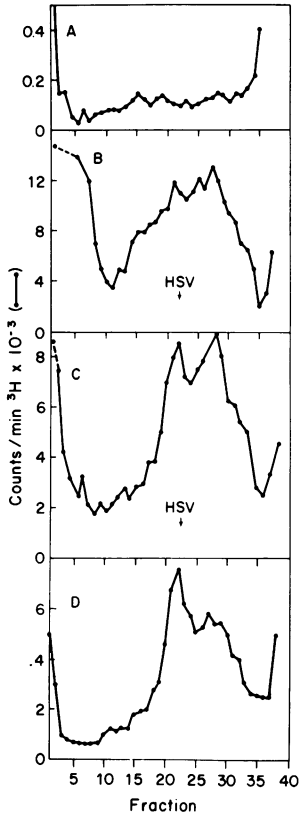

FIG. 8. *Sedimentation profiles in 10 to 30% (wt/wt) sucrose density gradients of DNA extracted from HEp-2 cells labeled at 8 h postinfection with HSV-1(F) and then chased, as described in Fig. 6, for 0 (A), 20 (B), 30 (C), and 40 (D) min. The gradients were centrifuged for 2.75 h, at 20°C and 40,000 rpm in an SW41 rotor. The arrow shows the position of marker HSV DNA.*

through D, respectively. The results may be summarized as follows.

(i) The incorporation of [³H]thymidine during the chase was minimal, and the ratios of acid-precipitable counts per minute at 20-, 30-, and 60-min chases to that observed immediately after the pulse were 1.6, 1.6, and 2.0, respectively (see Fig. 6). (ii) Equilibrium density gradient centrifugation indicated that only 7% of the total radioactivity was incorporated into host DNA throughout the experiment. (iii) The conditions of sedimentation were such (see legend to Fig. 9) that the rapidly sedimenting material formed a band near the bottom but did not actually sediment to the bottom of the tube. However, because of the short sedimentation time, the DNA marker (T4 DNA) barely entered the gradient, and, therefore, we do not have precise estimates of the amounts of mature viral DNA formed during the pulse and subsequent chase. Nevertheless, the data show that the amounts of radioactivity in the rapidly sedimenting material after the pulse and after the 20-, 30-, and 60-min chase intervals were 23, 36, 20, and 13% of the total, respectively. Although the DNA band sedimenting with T4 marker DNA was poorly defined in the sample extracted immediately after the pulse, the data clearly indicate that the amount of DNA cosedimenting with marker DNA increased as the rapidly sedimenting material decreased from the 20- to 60-min chase interval. (iv) We estimated that the sedimentation constant of the rapidly sedimenting DNA was greater than 230S.

Electron microscopic studies of viral DNA. As shown diagrammatically in Table 1, infected cells contained at least five classes of viral DNA molecules, differing from mature viral DNA in structural features.

Full- or unit-size linear molecules with extended single-stranded regions both at the termini and at internal positions (Fig. 10A) comprised 17% of the total molecules, i.e., circular

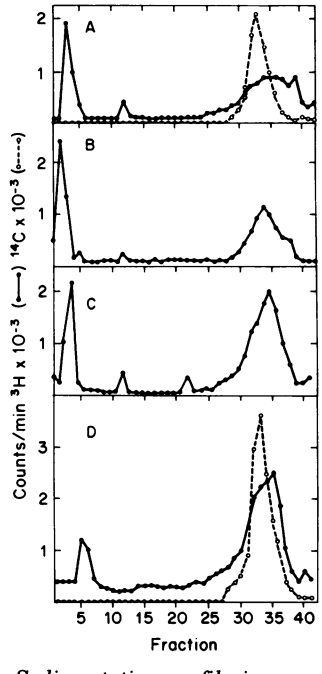

FIG. 9. *Sedimentation profile in sucrose density gradients of DNA extracted from HSV-1(F)-infected HEL cells. The cells were pulse-labeled for 10 min at 8 h postinfection and then chased for 0 (A), 20 (B), 30 (C), and 60 (D) min. The 10 to 50% (wt/wt) sucrose density gradients were overlaid on top of 0.4-ml cushions of saturated CsCl and centrifuged at 40,000 rpm and 20°C for 1.25 h in an SW41 rotor. Symbols: ●, [³H]thymidine-labeled DNA; ○, ¹⁴C-labeled T4 phage marker DNA.*

and linear forms, unit-length or greater, banding at the density of viral DNA. The position of the internal single-stranded region was not random, but rather it appeared to center at approximately 18% of the length of the molecule from one end. One possible interpretation, supported in part by attempts to fill the gap by repair synthesis (T. Buchman and B. Roizman, unpublished studies), is that the wide gap is located at the junction of the L and S components. Although the single-stranded ends and gaps were seen throughout infection, they were particularly prevalent early in the reproductive cycle (6 h postinfection).

Circular molecules, the second class represented in the diagram, were relatively rare (4 to 5% of the total). They contained no visible forks or single-stranded regions. Contour length measurements indicated that they could be subdivided into two classes. The first, represented by a few molecules, corresponded in length to mature, full-size linear DNA molecules (Fig. 11). The second corresponded in length to the S component (18×10^6 in molecular weight) and multiples of that length (Fig. 12).

Molecules shaped like lariats comprised the second largest class, 11% of DNA molecules unit length or greater banding at the density of viral DNA. We have defined lariat molecules as consisting of a linear, double-stranded segment covalently linked to a ring that is, at least partially, double stranded (Fig. 10B, 13). The ring of the lariat ranged from barely detectable (0.25 μm, 0.6×10^6 in molecular weight) to greater than mature DNA in size (45 μm, 96×10^6 in molecular weight). The linear, double-stranded segment of the lariat approached, in some instances, twice the length of mature viral DNA. A characteristic feature of the lariat molecules was the presence of continuous

TABLE 1. *Size and conformation of HSV DNA during replication*[a]

STRUCTURES	MOL. WEIGHT RANGE ($\times 10^6$)	% POPULATION
CIRCLES		
FULL SIZE	100	1-2
S COMPONENT (1x	17-20	
SIZE (2x	33-42	3
SINGLE STRANDED REGIONS		
TERMINAL	NA	17
INTERNAL	NA	9
LARIATS	0.6-100	11
LOOPS		
EYE LOOPS	0.15-0.4	2
D LOOPS	NA	2
LARGE REPLICATING FORMS	>1000	NA

[a] Major classes of viral DNA molecules differing from mature HSV-1 DNA extracted from virions and present in infected cells during viral DNA replication. The large, tangled masses of viral DNA were not included into the count of total molecules. The circular, full-size molecules were too infrequent to be estimated accurately. The frequencies of the remaining classes of molecules were determined by examining four different samples, each containing 50 to 60 DNA molecules greater than 70 to 80% of unit length.

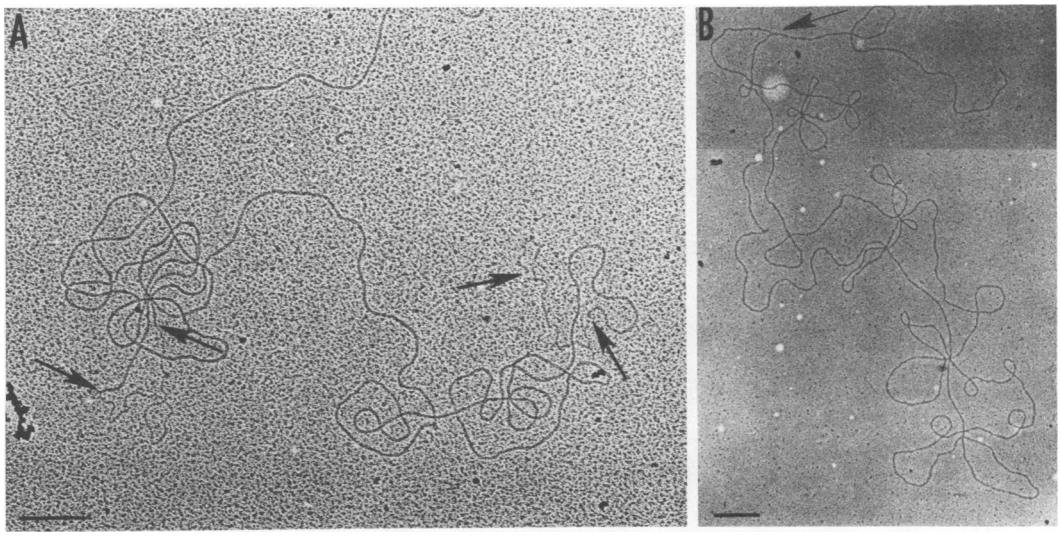

FIG. 10. *Electron micrographs of DNA isolated at the virus-specific density from a CsCl equilibrium gradient. The DNA was extracted at 6.0 h postinfection from HEL cells infected with HSV-1(F) virus. The arrows indicate single-stranded or forked regions seen in the molecules. The bars represent 0.25 μm. (B) A lariat molecule with a ring approximately 9.3 μm in length (20×10^6 in molecular weight).*

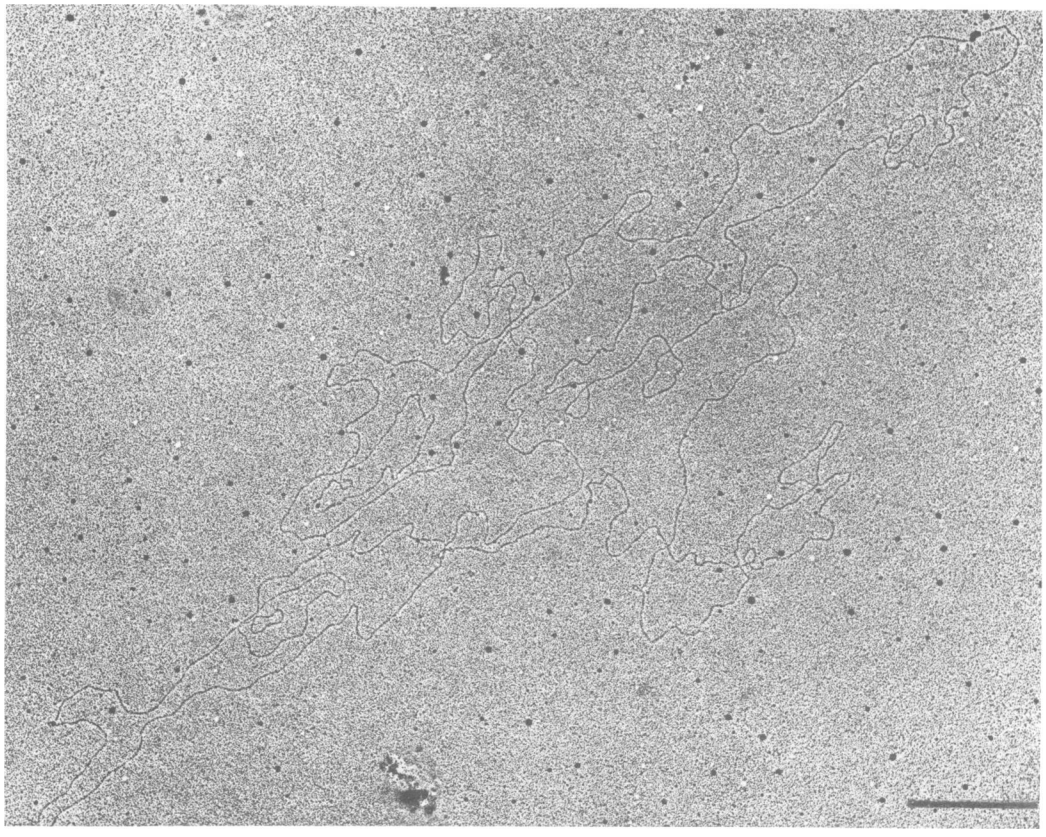

FIG. 11. *Electron micrograph of a circular DNA molecule extracted from infected cells at 2.5 h postinfection and purified by CsCl density gradient centrifugation. This molecule corresponds in size to that of a mature viral DNA. Bar = 1 μm.*

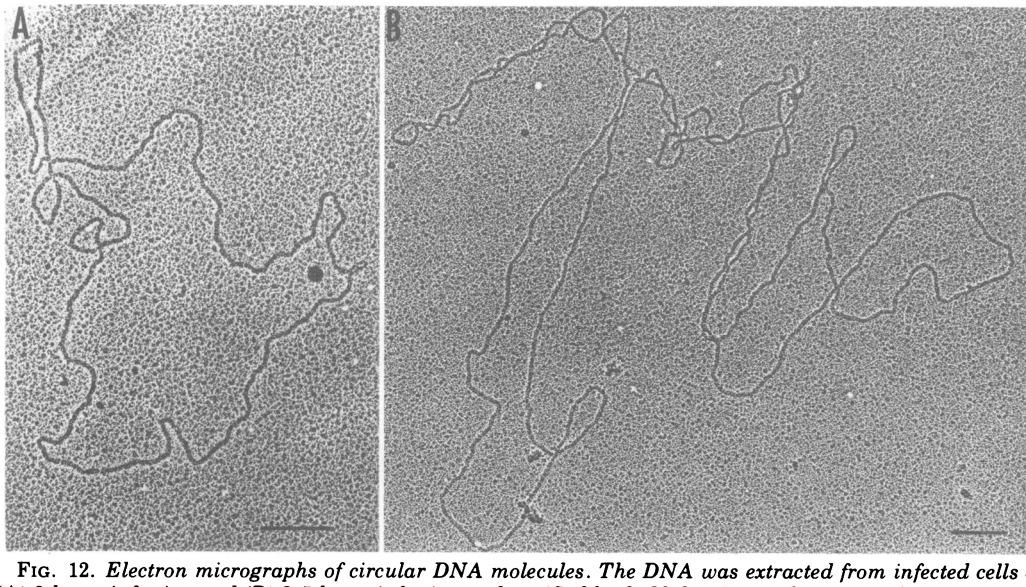

FIG. 12. *Electron micrographs of circular DNA molecules. The DNA was extracted from infected cells at (A) 9 h postinfection and (B) 2.5 h postinfection and purified by CsCl density gradient centrifugation. (A) A circular molecule, corresponding in size to 20 × 10^6 in molecular weight and isolated from the gradients shown in Fig. 3. (B) A circular molecule approximately twice the size of the S component. The bars represent 0.25 μm.*

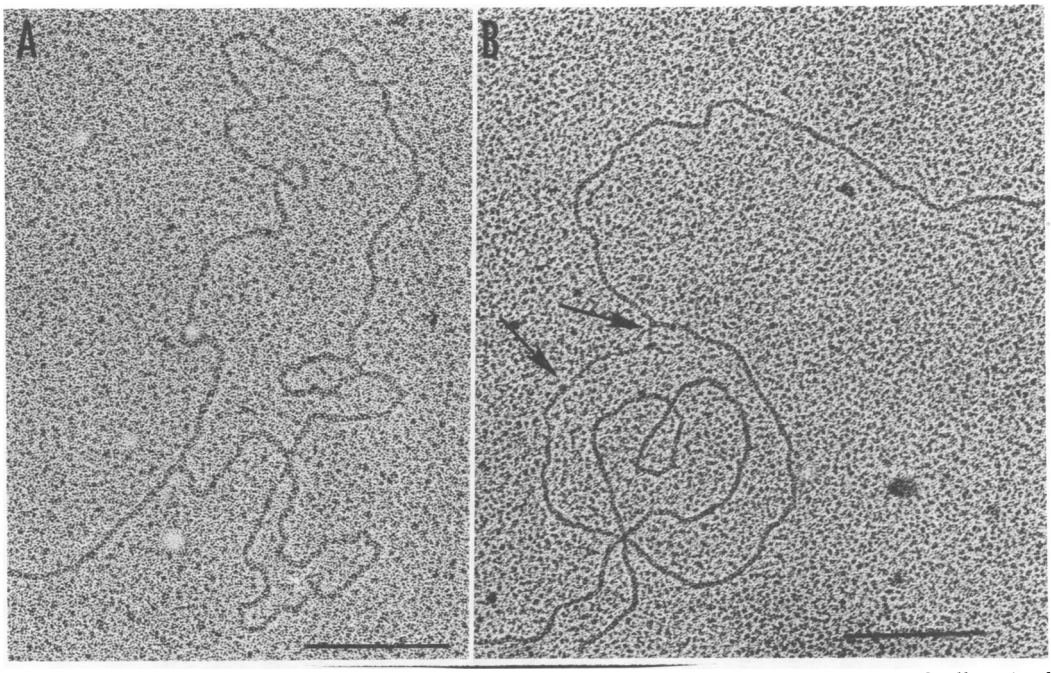

FIG. 13. *Electron micrographs of viral DNA molecules. The DNA was extracted from infected cells at 2.5 h postinfection and purified by CsCl density gradient centrifugation. (A) A lariat with a 3.49-μm (7.5 × 10⁶ in molecular weight) ring containing a continuous fork. (B) A lariat with a 1.40-μm (3.0 × 10⁶ in molecular weight) ring containing a discontinuous fork. Arrows indicate single-stranded regions. The bars represent 0.25 μm.*

(Figs. 10B and 13A) and discontinuous (Fig. 13B) forks. Possibly related to the lariats were two other classes of DNA molecule not shown in Table 1. The first of these consisted of mature-length DNA molecules with aberrant ends. In some instances (Fig. 14) these appeared as loops 0.25 μm or less in size, either entirely single stranded or double stranded and giving the impression that the end had folded upon itself to form a hairpin structure. These were similar to the loops observed in vitro after processive exonuclease digestion (13, 25). In the second class were mature-size or greater-than-mature-length molecules with single continuous (Fig. 15B) and discontinuous (Fig. 15A) forks and with unequal-size branches. These were the only molecules seen in this study that resembled the branched molecules reported by Shlomai et al. (23). It is not clear at present whether these are molecules that replicate differently from the lariats or merely lariats whose rings were broken during extraction and purification.

Another significant (2% of the total) class was linear, unit-length molecules with replicating loops. Figure 16A shows a "D" loop, i.e., a loop double stranded on one side and single stranded on the opposite side; D loops were seen infrequently, possibly because of the spreading technique used in our studies. Figures 16B through D are examples of "eye" loops, in which both sides are double stranded. Eye loops are considered to be indicative of bidirectional replication. The small, less than 10^6 in molecular weight, loops were usually at or near one end of the molecule.

The large masses of DNA, similar to those reported for replicating T4 (12) and pseudorabies virus (1) DNA, were especially frequent late (9 h postinfection) in the replication of viral DNA (Fig. 17 and 18). We have estimated that these masses were at least 10 times the unit lengths of mature DNA. Because we frequently could not differentiate the individual "tangles," these were not included in the count of molecules banding at the density of viral DNA. Forks and single-stranded regions were frequently noted in such masses. These forms were present in the bottom fractions of sucrose gradients, as shown in Fig. 8 and 9.

DISCUSSION

The salient features of the data presented in this paper may be summarized as follows.

(i) Only a small fraction of input HSV DNA entered the replicative pool. The size of this fraction appeared to be independent of cell line, virus storage, and the isotope with which the viral DNA was labeled. HSV thus appears to

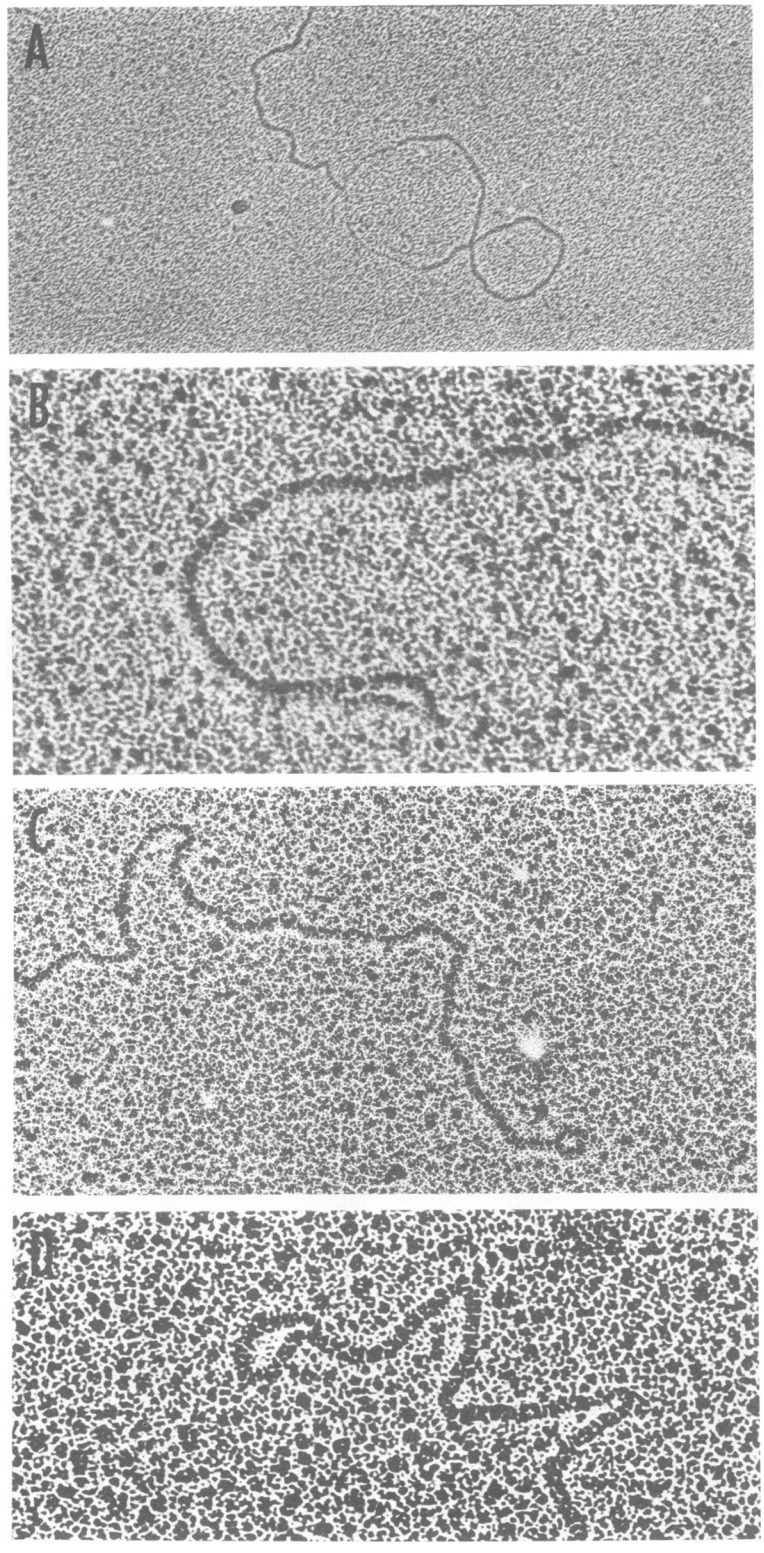

FIG. 14. *Electron micrographs of virus-specific DNAs with aberrant ends. The DNA was extracted from infected cells at 2.5 h postinfection and purified by CsCl density gradient centrifugation. (A, B) Molecules with ends that appear to contain single-stranded sequences. (C, D) Molecules with ends that appear to be primarily double stranded. The "looped ends" range from 0.19 μm (C) to 0.93 μm (A) in size.*

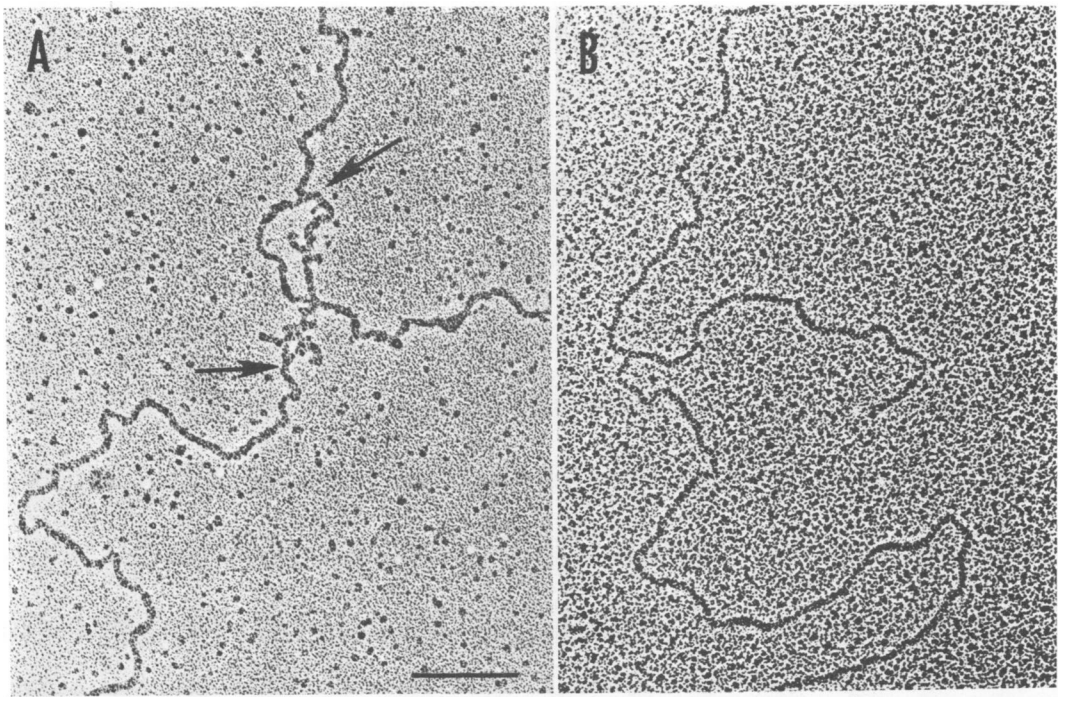

FIG. 15. *Electron micrographs of discontinuous (A) and continuous (B) forks isolated from replicating HSV-1 DNA. The DNA was extracted from infected cells at 9.0 h postinfection and purified by CsCl density gradient centrifugation. The bars represent 0.25 μm.*

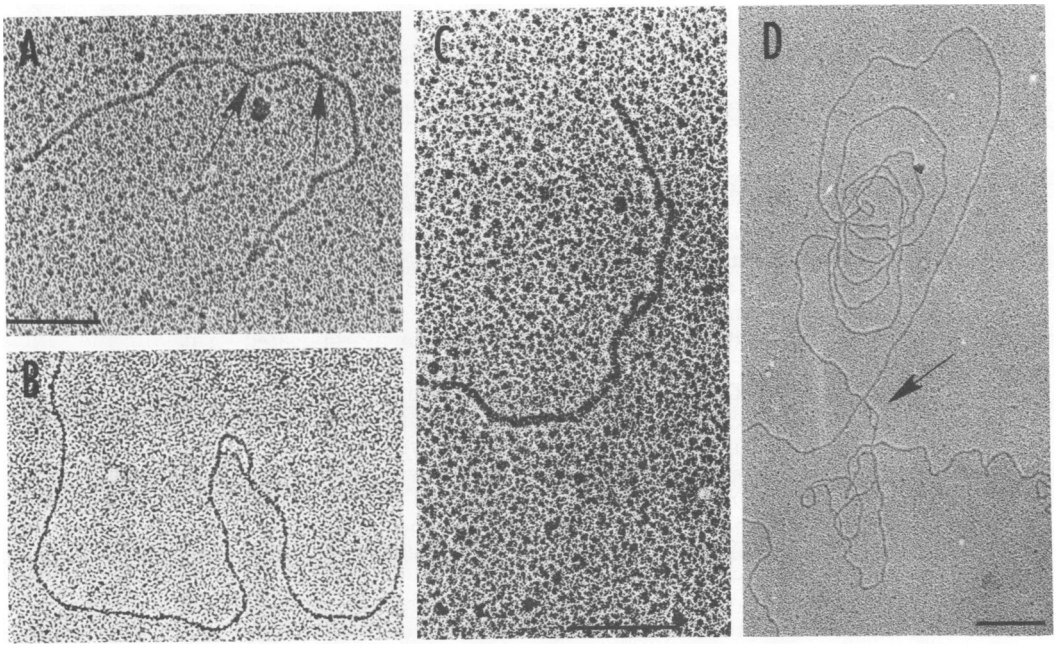

FIG. 16. *Electron micrographs of viral DNA containing replicating loops. The DNA was extracted from infected cells at 2.5 h postinfection and purified by CsCl density gradient centrifugation. (A) D loop; the arrows indicate the single-stranded region. (B–D) Eye loops. The bars represent 0.25 μm.*

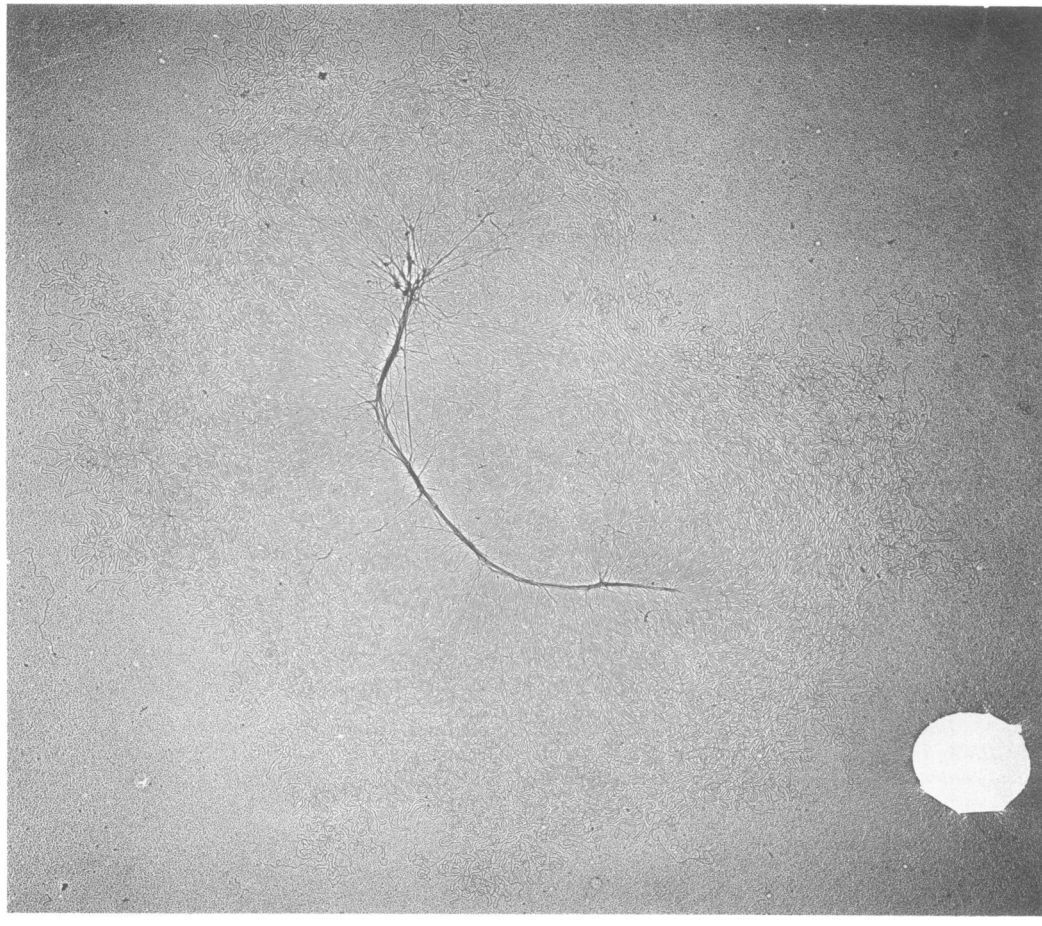

FIG. 17. *Electron micrograph of a large HSV DNA tangled mass. The DNA, extracted from cells at 9 h postinfection, was first banded in CsCl density gradients; the fraction containing viral DNA was then centrifuged in sucrose density gradients. The DNA tangles shown in this electron micrograph were recovered from fractions near the bottom of the tube. Bar = 10 μm.*

differ from pseudorabies virus in this respect. The data also indicate that the bulk of the DNA was not grossly degraded, as evidenced by the fact that it cosedimented with mature marker DNA. We have, at this time, no evidence that input viral DNA dissociates into *L* and *S* components, as would be expected if they were to multiply independently.

(ii) Newly replicated viral DNA sedimented faster than mature viral DNA, consistent with the hypothesis that it consists of concatemers that must be processed into smaller, mature-size molecules, as has been noted for pseudorabies virus DNA (1). We have also noted that newly replicated HSV DNA banded at a slightly higher density, suggestive of extensive single-stranded regions in replicating DNA. The observation that, in the presence of BUdR, [^{3}H]thymidine was incorporated into DNA that bands at the density of mature viral DNA suggests that the accumulated viral DNA was being repaired. Presumably, this is the filling of the nicks and gaps that have been shown to be present in nascent and mature viral DNA (3, 7, 23). Pulse-chase experiments indicate that both the processing of the high-molecular-weight, newly replicated forms and the filling of single-stranded regions signaled by the shift from higher density to the density of mature DNA takes approximately 30 to 40 min from the time of synthesis.

(iii) The electron microscopic studies indicate that viral DNA exists in a number of forms in cells supporting viral replication. We have focused attention on linear DNA molecules with extensive terminal and internal single-stranded regions, circular DNA molecules lacking forks or replicating loops, lariats with continuous and discontinuous forks, linear molecules with D and eye loops, and large masses

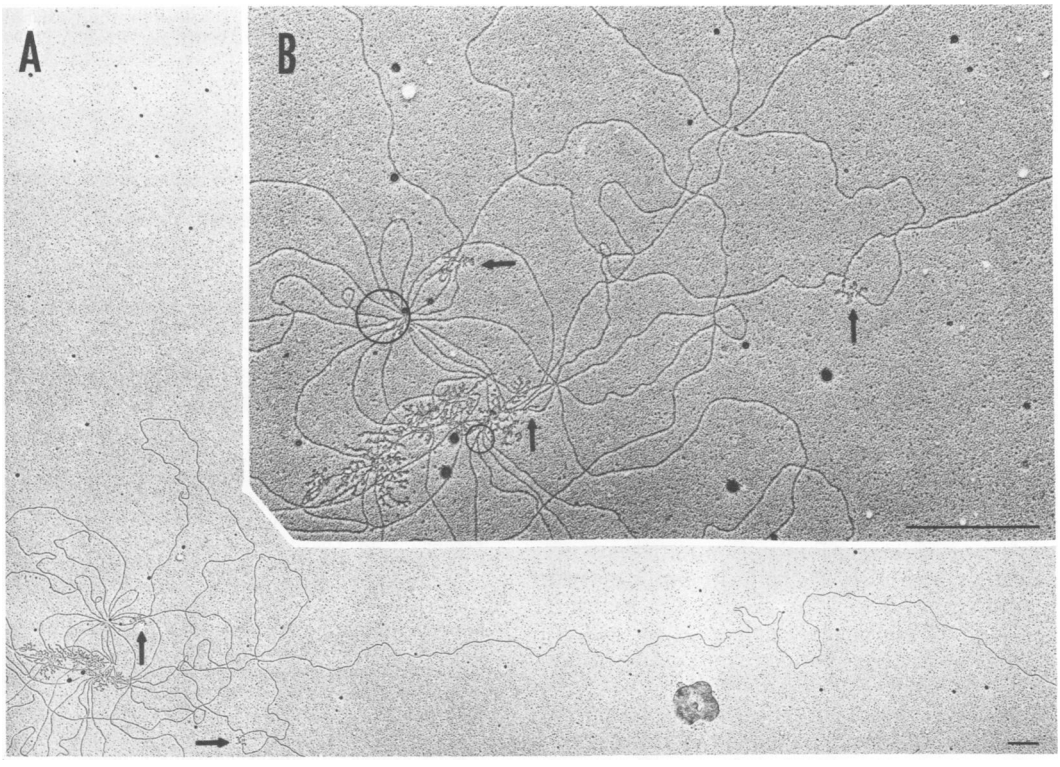

FIG. 18. *Electron micrograph of an edge of an HSV-DNA tangle and mass showing continuous (circles) and discontinuous (arrows) forks. The DNA was extracted from infected cells at 9 h postinfection and was recovered from CsCl density gradients at the density of viral DNA and then centrifuged in a sucrose density gradient. The DNA was spread by the aqueous procedure. The bars represent 1 μm (A) and 0.25 μm (B).*

with forks reminiscent of the T4 DNA replicating complexes. With the exception of the lariat molecules, these forms were also reported in pseudorabies-infected cells (1, 14). It is noteworthy that, with the exception of the linear molecules with extensive single-stranded regions, none of the others were seen in cells in which viral DNA synthesis was blocked by inhibitors of DNA synthesis (manuscript in preparation).

(iv) In principle, the presence of forks and replication loops is indicative and sufficient evidence that DNA is undergoing replication. The presence of molecules with lariats and replicating loops in DNA shifted to a higher density by BUdR reinforces this conclusion. It is interesting, therefore, that we have not seen either forks or loops in circular molecules, although such molecules have been reported in pseudorabies virus-infected cells (14). The observation that circular DNA forms are either full size or multiples of 18×10^6 in molecular weight, i.e., the size of the *S* component, raises the possibility that the entire molecule or components thereof may circularize and replicate in this fashion, although this study failed to show that such molecules actually undergo replication. However, it is possible that the more advanced replicating forms of these circles take the form of large tangles and knots and are so complex that our spreading conditions do not allow us to visualize and resolve them clearly. It should be pointed out that we have focused attention on the small circles as being the size of the *S* component only because one of the objectives of these studies was to determine whether the *L* and *S* components dissociate and occur independently. The paucity of the small circles makes it difficult to identify them precisely as the *S* components, and the failure to detect loops or forks makes it unlikely that they serve as a major source of the *S* component for mature viral DNA progeny.

(v) The predominant replicating molecules that were available for measurement were lariats and linear molecules with replicating loops near one end. As noted in the text, some molecules were larger than, and frequently twice the size of, the mature viral DNA. We suspect that they constitute the bulk of the population

of replicating molecules early in infection. The origin of the lariat, in particular, presents an interesting question that presently has not been totally resolved. One possibility is that viral DNA synthesis is initiated in the form of a loop near one end and extended by bidirectional replication, but that the end nearest to the site of initiation remains covalently linked until the entire molecule is replicated. A more attractive alternative that takes into account most of the observed classes of DNA molecules is that (a) the input parental DNA is digested by a processive host or viral exonuclease, creating single-stranded ends as well as gaps within the DNA. (b) The single-stranded terminus folds upon itself and anneals to an inverted repeat, shown by Hyman et al. (13) and Wadsworth et al. (25) to be present in at least one terminus. These putative molecules might well be the molecules with aberrant ends described in Results and shown in Fig. 14. (c) Replication may start at the end or within the hairpin. In either event, the copying of the DNA around the hairpin should produce a lariat. Replication by such a mechanism should produce first dimers and then quartermers and octamers.

(vi) The presence of concatemeric forms of viral DNA has been established in both pseudorabies virus- (1) and HSV-infected cells. They could arise by a mechanism akin to a rolling circle or by the model proposed in this paper. The two mechanisms, however, give distinctly different concatemers inasmuch as the concatemer produced by a rolling circle will give the arrangement of its monomers as "head to tail," whereas in the concatamer produced by a lariat the arrangement is "head to head" or "tail to tail." The two forms are readily differentiated inasmuch as cleavage of the rolling-circle concatemers by restriction endonucleases should produce fragments the size of the sum of the two termini, whereas cleavage of head-to-head and tail-to-tail concatemers should produce fragments twice the size of each terminus. Observations recorded in this paper would predict a head-to-head arrangement for the unit HSV-1 DNA in concatemers. Preliminary studies in this laboratory of replicating HSV-1 DNA forms digested with restriction endonucleases indicate the presence of new species of fragments corresponding to twice the size of the individual terminal fragments and clearly different from the head-to-tail fragments.

ACKNOWLEDGMENTS

We thank Kent Wilcox for the preparation of ^{14}C T4 DNA and for his participation and advice in some of the experiments and Patricia Wiedner for her assistance in electron microscopy.

These studies were aided by grants from the National Cancer Institute, U. S. Public Health Service (CA 08494 and CA 19264), the American Cancer Society (ACS VC 103L), and the National Science Foundation (PCM76-06254).

LITERATURE CITED

1. **Ben-Porat, T., A. S. Kaplan, B. Stehn, and A. S. Rubenstein.** 1976. Concatemeric forms of intracellular herpesvirus DNA. Virology 69:547-560.
2. **Ben-Porat, T., B. Stehn, and A. S. Kaplan.** 1976. Fate of parental herpesvirus DNA. Virology 71:412-422.
3. **Biswal, N., B. K. Murry, and M. Benyesh-Melnick.** 1974. Ribonucleotides in newly synthesized DNA of herpes simplex virus. Virology 69:87-99.
4. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. 3:357-364.
5. **Frenkel, N., R. J. Jacob, R. W. Honess, G. S. Hayward, H. Locker, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. III. Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. 16:153-167.
6. **Frenkel, N., H. Locker, W. Batterson, G. S. Hayward, and B. Roizman.** 1976. Anatomy of herpes simplex virus DNA. VI. Defective DNA originates from the S component. J. Virol. 20:527-531.
7. **Frenkel, N., and B. Roizman.** 1972. Separation of the herpesvirus deoxyribonucleic acid duplex into unique fragments and intact strands on sedimentation in alkaline gradients. J. Virol. 10:565-572.
8. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. Proc. Natl. Acad. Sci. U.S.A. 72:4243-4247.
9. **Hochberg, E., and Y. Becker.** 1968. Adsorption, penetration and uncoating of herpes simplex virus. J. Gen. Virol. 2:231-241.
10. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. 70:208-219.
11. **Hotta, Y., and A. Bassel.** 1965. Molecular size and circularity of DNA in cells of mammals and higher plants. Proc. Natl. Acad. Sci. U.S.A. 53:356-362.
12. **Huberman, J. A.** 1968. Visualization of replicating mammalian and T4 bacteriophage DNA. Cold Spring Harbor Symp. Quant. Biol. 33:509-524.
13. **Hyman, R. W., S. Burke, and L. Kudler.** 1975. A nearby inverted repeat of the terminal sequence of herpes simplex virus DNA. Biochem. Biophys. Res. Commun. 68:609-615.
14. **Jean, J.-H., and T. Ben-Porat.** 1976. Appearance of single stranded complementary ends on parental herpesvirus DNA. Proc. Natl. Acad. Sci. U.S.A. 73:2674-2678.
15. **Kaplan, A. S., T. Ben-Porat, and C. Coto.** 1967. Studies on the control of infective process in cells infected with pseudorabies, p. 527-545. *In* J. S. Colter and W. Paranchych (ed.), Molecular biology of viruses. Academic Press Inc., New York.
16. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. 8:125-132.
17. **Kolber, A. R.** 1975. In vitro synthesis of DNA in nuclei isolated from human lung cells infected with herpes simplex type II virus. J. Virol. 15:322-331.
18. **Roizman, B., G. S. Hayward, R. J. Jacob, S. C. Wadsworth, N. Frenkel, R. W. Honess, and M. Kozak.**

1975. Human herpesvirus 1: a model for molecular organization and regulation of herpesviruses, p. 3-38. *In* G. de The, M. A. Epstein, and H. Zur-Hausen (ed.), Proceedings of the symposium on herpesviruses and oncogenesis, Nurenberg, October 14-16, 1974. I. A. R. C., Lyon, Lyons.

19. **Roizman, B., G. S. Hayward, R. J. Jacob, S. W. Wadsworth, and R. W. Honess.** 1974. Human herpesvirus 1: a model for molecular organization of herpesvirus virions and their DNA, p. 188-198. Proc. of the XIth Int. Congress on Cancer, Florence. Excerpta Med. Int. Congr. Ser. no. 350. *In* Chemical and viral carcinogenesis, vol. 2.
20. **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.
21. **Roizman, B., and P. G. Spear.** 1968. Preparation of herpes simplex virus of high titer. J. Virol. **2**:83-84.
22. **Sheldrick, P., and N. Berthelot.** 1974. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. **39**:667-678.
23. **Shlomai, J., A. Friedmann, and Y. Becker.** 1976. Replicative intermediate of herpes simplex virus DNA. Virology **69**:647-659.
24. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143-159.
25. **Wadsworth, S., G. S. Hayward, and B. Roizman.** 1976. Anatomy of herpes simplex virus DNA. V. Terminally repetitive sequences. J. Virol. **17**:503-512.
26. **Wadsworth, S., R. J. Jacob, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. II. Size, Composition, and arrangement of inverted terminal repetitions. J. Virol. **15**:1487-1497.
27. **Wilkie, N. M., and R. Cortini.** 1976. Sequence arrangement in herpes simplex virus type 1 DNA: identification of terminal fragments in restriction endonuclease digests and evidence for inversions in redundant and unique sequences. J. Virol. **20**:211-221.