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Replication of Herpesvirus DNA
III. Rate of DNA Elongation

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The rate of pseudorabies virus DNA elongation was measured by three different techniques: density shift experiments, radioautography examined by light microscopy, and radioautography examined by electron microscopy. The rate of the fork movement at 37°C was estimated to be approximately 1 μm/min.

The replicative forms of the DNA of a herpesvirus, pseudorabies (Pr), have been investigated by electron microscopy and by analyzing the sedimentation characteristics of newly synthesized viral DNA (1, 2, 9; T. Ben-Porat and S. A. Tokazewski, submitted for publication; J-h. Jean, M. L. Blankenship, and T. Ben-Porat, submitted for publication). To correlate the results of these analyses, and to understand them, it becomes essential to know the rate of replication of the viral DNA molecules.

The rate of movement of the replicative fork during replication of eukaryotic DNA has been estimated to be approximately 1 μm/min, or an equivalent of 2×10^6 daltons of DNA/min (3, 5, 7, 8, 12). However, the rate of replication of viral DNA in eukaryotic cells has not been determined. Consequently, experiments, the results of which are described in this paper, were designed to determine the rate of replication of Pr viral DNA in rabbit kidney (RK) cells.

MATERIALS AND METHODS

Virus and cell culture. The properties of Pr virus and the cultivation of RK monolayer cultures were described previously (11). All experiments were performed at 37°C.

Media and solutions. These included: Earle saline containing 0.5% lactalbumin hydrolyzate and 5% bovine serum (ELS), Eagle synthetic medium (4) plus 3% dialyzed bovine serum (EDS), ELS plus 20 μg of 5-fluorouracil and 2 μg of thymidine per ml (ELS-FU), EDS plus 2 μg of 5-fluorodeoxyuridine per ml (EDS-FUdR), and 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.4 (saline citrate [SSC]).

Chemicals and radiochemicals: FU and FUdR were purchased from Calbiochem. [*methyl*-^{3}H]thymidine (62 Ci/mmol) was purchased from Schwarz/Mann, and [^{3}H]5-bromodeoxyuridine (BUdR) (23 Ci/mmol) was purchased from New England Nuclear Corp.

Extraction of DNA. Sedimentation of DNA in sucrose gradients, equilibrium sedimentation in CsCl, and preparation of ^{32}P-labeled BUdR and ^{32}P-labeled thymidine viral marker DNA molecules were performed as described previously (2).

Radioautography of DNA: light and electron microscopy. Light microscopy was performed by the method of Hori and Lark (6), with minor modification. After the appropriate labeling period, the cells (4×10^5 cells/ml) were scraped gently into SSC-1% sodium dodecyl sulfate (SDS). A drop was placed on a glass slide, previously coated with 0.5% serum albumin, and was spread over the surface of the slide with a glass rod. After being air-dried, the slides were fixed with cold 10% trichloroacetic acid for 5 min and washed twice more with 10% trichloroacetic acid, once with water, and twice with absolute alcohol. The dried slides were coated with Ilford-L4 track emulsion, diluted 1:1 with water, dried, exposed for 2 months, developed in a D19 developer for 5 min, and fixed for 7 min.

Labeled viral DNA was purified and spread for electron microscopy as described previously (9). The grids were coated with Ilford-L4 nuclear track emulsion diluted 1:3 with water. After an exposure time of approximately 2 months, the grids were developed with D19 for 90 s and fixed.

Shearing of DNA. Gradual shearing of DNA was performed at 0°C in 1× SSC. The DNA solution was passed through a 22-gauge needle, at full thumb force, 5, 10, or 20 times, depending upon the experiment.

RESULTS

Rate of viral DNA replication as determined by density-shift experiments. One approach to determining the rate of replication of viral DNA molecules is to ascertain the buoyant density of the newly synthesized viral DNA labeled with [^{3}H]BUdR for various periods of time. After a short pulse with [^{3}H]BUdR, one should find labeled DNA molecules, with thymidine substituted by BUdR, only in the part of the molecule that has replicated during the labeling period. Depending upon the length of the

labeling period, the partially replicated, radioactively labeled DNA molecules should occupy various positions in CsCl gradients between L-L (nonreplicated DNA) and H-L (DNA molecules that replicated completely, semiconservatively, in the presence of [^{3}H]BUdR; the heavy-light position). The time required for DNA molecules to appear in the H-L position is the time required for complete replication of these molecules.

In all the experiments described in this paper, the cells were incubated for 24 h prior to infection in medium containing 20 μg of FU and 1 μg of thymidine per ml. This procedure suppresses completely cellular DNA synthesis without affecting the subsequent ability of the cells to support viral growth, as determined by the rate of viral DNA synthesis and the production of PFU (10). That viral DNA only is synthesized in FU-pretreated, infected cells is routinely ascertained by the fact that [^{3}H]thymidine is incorporated only into DNA with a buoyant density in CsCl of 1.732 g/cm^3, the density of viral DNA.

RK cells were infected, and after virus adsorption, were washed extensively and incubated in medium containing FUdR up to 2.5 h postinfection. This treatment inhibits the onset of viral DNA synthesis by 75 to 90%, and serves to synchronize partially the first round of parental viral DNA replication as well as to reduce the intracellular pools of thymidine and its phosphorylated derivatives. Replication of parental viral DNA during the first round of DNA replication was followed in all the experiments, because viral DNA synthesized at later times is associated with concatemers (1).

To determine whether density-shift experiments of the type involving the incorporation of BUdR described above would reflect the normal rate of DNA replication, the rates of incorporation into the infected cells of [^{3}H]thymidine and [^{3}H]BUdR were compared.

Under the experimental conditions used, [^{3}H]BUdR was incorporated into viral DNA at approximately the same rate as [^{3}H]thymidine (Fig. 1). Thus, incorporation of BUdR into viral DNA does not affect the rate of viral DNA synthesis. Furthermore, there was no detectable delay in the incorporation of either nucleoside. Incorporation was linear for the first 10 min; thereafter the slopes increased somewhat.

The distribution in CsCl gradients of viral DNA synthesized after various periods of labeling with [^{3}H]BUdR was determined (Fig. 2). After a 10-min labeling period (Fig 2A), most of the radioactive DNA formed a heterogeneous band in the gradient, with buoyant densities

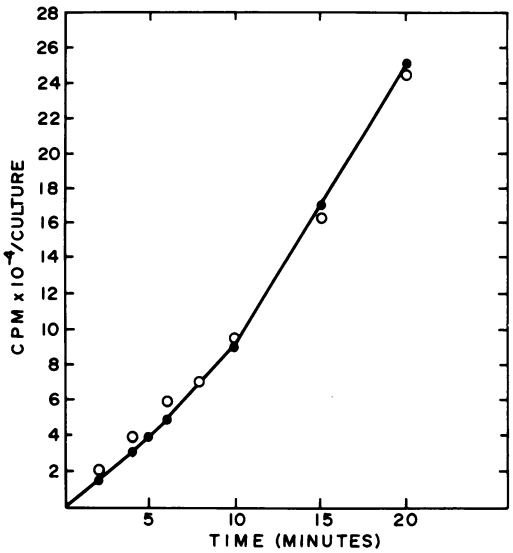

FIG. 1. *Incorporation of [^{3}H]thymidine and [^{3}H]BUdR into viral DNA. RK cells were incubated with ELS-FU for 24 h before the cultures were infected (multiplicity of infection, 20 PFU/cell) and incubated in EDS-FUdR. At 2.5 h postinfection, the cells were incubated with EDS-FUdR containing 30 μCi of either [^{3}H]thymidine (23 Ci/mmol) or [^{3}H]BUdR (23 Ci/mmol) per ml. An aliquot of each sample was precipitated with acid and washed, and the amount of isotope incorporated was determined. Symbols: BUdR (●); thymidine (○).*

intermediate between those of H-L DNA and of L-L DNA, indicating that these DNA molecules had replicated partially to varying degrees during the labeling period. After a 20-min labeling period (Fig. 2B), most of the radioactive label appeared in the semiconservative position. Some labeled DNA appeared also in positions of higher densities. As expected, after a 60-min labeling period (Fig. 2C) most of the labeled DNA occupied the heavy-heavy (H-H) position.

As indicated above, this type of experiment should allow one to estimate the time required for the completion of the viral genome, provided that the molecules analyzed in the gradients are intact, i.e., are unit size, and neither break nor become part of concatemeric structures. However, after centrifugation in a CsCl gradient, most of the radioactive DNA (Fig. 2A and B, region X) sedimented heterogeneously, with an average value of approximately 30S (mature viral DNA has a sedimentation value of 54S). The low S value of newly synthesized Pr viral DNA is the result of breakage of the replicating molecules during centrifugation in CsCl (Ben-Porat and Tokazewski, submitted for publication). Although it is possible to minimize this

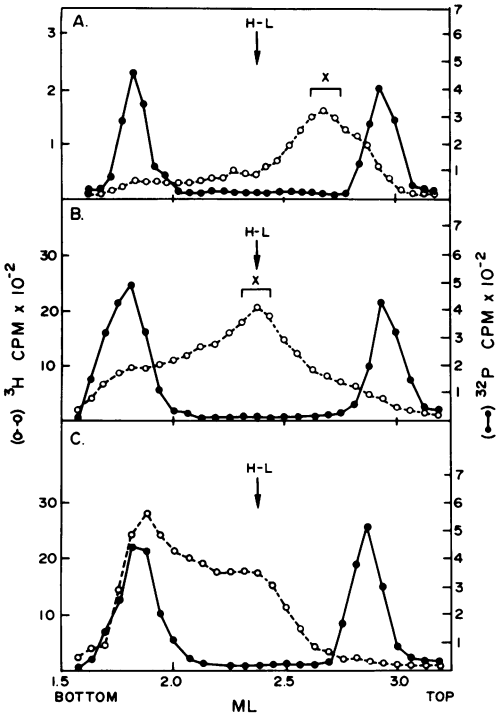

FIG. 2. *Buoyant density of viral DNA synthesized during various periods of labeling with [^{3}H]BUdR. RK cells were incubated for 24 h in ELS-FU before being infected (multiplicity of infection, 20 PFU/cell). The infected cells were incubated in EDS-FUdR up to 2.5 h postinfection when the medium was changed to EDS-FUdR containing [^{3}H]BUdR (30 μCi/ml; 23 Ci/mmol). At various times thereafter, the culture medium was removed, and the cells were scraped into SSC containing 2% Sarkosyl. The DNA was extracted and banded in CsCl with H-H and L-L ^{32}P-labeled viral DNA markers as described in Materials and Methods. Samples were collected by drops, and an aliquot was taken to determine the distribution of the labeled DNA in the gradients. Labeling periods: (A) 10 min; (B) 30 min; (C) 60 min. The material in the regions of the gradient marked X was diluted, and the S value of the DNA molecules was determined in sucrose gradients.*

breakage by varying the conditions of centrifugation, we have been unable to avoid it completely (Ben-Porat and Tokazewski, submitted for publication).

Despite the fact that replicating viral DNA does not remain intact during centrifugation in CsCl, the following conclusions may be drawn from the experiment illustrated in Fig. 2 concerning the higher limits of the rate of viral DNA replication. (i) Since, after a 10-min labeling period, most of the radioactive DNA banded approximately midway between H-L and L-L DNA, and since most of these molecules were smaller than 1/2-unit size, it is likely that more than 20 min are required for the synthesis of a unit-size viral DNA molecule. (ii) Since, after a 30-min pulse, a relatively small amount of newly synthesized DNA banded at a buoyant density greater than H-L, only a small part of the DNA molecules completed one round of replication and initiated a second one during the labeling period. This indicates that most of the viral DNA requires more than 30 min to complete one round of replication. (DNA with a buoyant density greater than H-L could also arise from reinitiation in still uncompleted molecules.)

The experiment in Fig. 2 allows one to estimate the higher limits of the rate of DNA replication. However, because of the difficulty in obtaining unit-size, newly synthesized, viral DNA, it was necessary to resort to a modification of the experiments illustrated in Fig. 2 to determine the rate more exactly. In these experiments, viral DNA was labeled with [^{3}H]-BUdR for increasing periods of time; the DNA was extracted and sheared to various sizes. The *S* values of the fragments were determined, and their molecular weights were calculated on the assumption that most replicative branches and loops break during shearing and that most of the DNA fragments consist of linear, double-stranded molecules. (Less than 5% of the newly synthesized DNA was found to be sensitive to S_1 nuclease either before or after shearing and isopycnic centrifugation in CsCl.)

During the fragmentation of the DNA, breaks are introduced into the molecules at random sites. Consequently, some of the fragments will contain intact segments of newly synthesized DNA, whereas other fragments, arising from the breaks introduced within the newly synthesized regions, will consist of different fractions of newly synthesized DNA. Fragments arising as a result of breaks introduced within the newly synthesized part of the molecules band at a lower buoyant density and contain less radioactivity than fragments that arise from breakage at other sites containing an intact region of newly synthesized DNA. If the newly synthesized region is relatively small compared with the size of the DNA fragments analyzed, the peaks of radioactive DNA in CsCl gradients can be considered as containing those molecules in which the newly synthesized region of DNA has remained intact. The fractional content of newly synthesized DNA in the fragments present in these peaks can be ascertained from their position in the gradient, because the relative buoyant density in CsCl of the DNA is related linearly to the degree of

substitution of BUdR for thymidine. Thus, in principle, peaks should be obtained that are skewed toward the L-L position because of the presence of fragments which arose as a result of breaks introduced into the region of newly synthesized, BUdR-containing DNA.

A representative experiment is illustrated in Fig. 3, in which the DNA had been sheared prior to centrifugation into fragments with an average molecular weight of 13.5 × 10^6. After centrifugation in CsCl, the DNA in regions X and Y (Fig. 3A and B) were sedimented again in sucrose gradients, and their molecular weights were calculated. Both regions X and Y sedimented as fragments with molecular weights of 12 × 10^6 to 15 × 10^6 (average, 13.5 × 10^6).

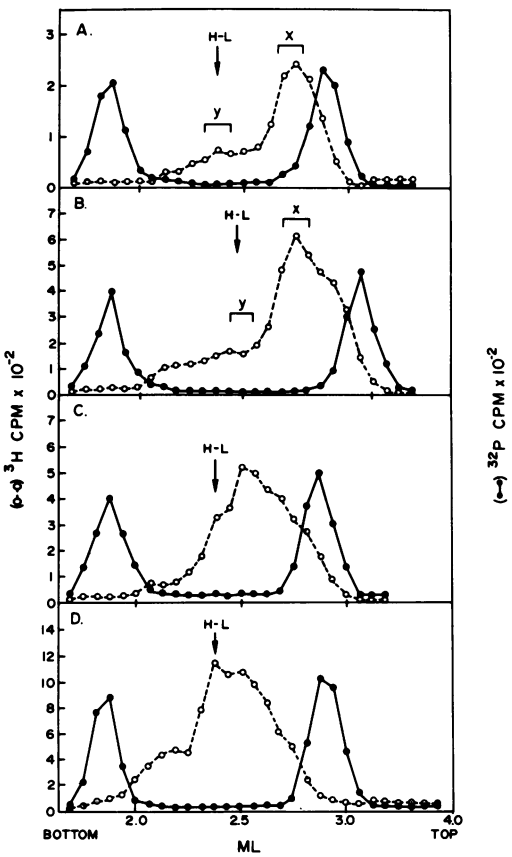

FIG. 3. *Buoyant density in CsCl of fragmented DNA labeled for various labeling periods with [^{3}H]BUdR. The experiment was performed as described in the legend in Fig. 2, but, prior to isopycnic centrifugation in CsCl, the DNA was sheared, as described in Materials and Methods, to an average molecular weight of 13.5 × 10^6. Labeling periods: (A) 2 min; (B) 4 min; (C) 6 min; (D) 8 min. The material in the regions of the gradients marked X and Y was diluted, and the S value of the DNA molecules was determined in sucrose gradients.*

After a 2-min pulse with [^{3}H]BUdR (Fig. 3A), the peak of radioactive DNA occupied a position in the CsCl gradient with a fractional increase in density of 0.3 (the increase in density between L-L and H-L is considered 1.0), indicating that these fragments consisted of 30% newly replicated H-L DNA. Since the average molecular weight of the DNA fragments is 13.5 × 10^6, the results indicate that the equivalent of a segment of DNA with a molecular weight of 4.1 × 10^6 (30% of 13.5 × 10^6) had replicated during the 2-min labeling period, i.e., DNA with an approximate molecular weight of 2.0 × 10^6 (or 1.0 μm in length) replicated per min. Figure 3B shows that after a 4-min pulse, the peak of labeled DNA occupied a position which indicates that this DNA consists of 52% H-L DNA. Consequently, an equivalent of a segment with a molecular weight of 7.0 × 10^6 replicated during the 4-min labeling period, and replication occurred at a rate of 0.88 μm/min. The results of this and similar experiments, in which the DNA was sheared either to somewhat smaller or to larger fragments, are summarized in Table 1. These results indicate that a segment of molecular weight between 2.0 × 10^6 and 1.5 × 10^6 replicated per minute, and that viral DNA proceeds at a rate of about 1 μm/min.

The estimate of the rate of DNA replication from the results of the different experiments varies by as much as 25%. This is probably due to the method's lack of sensitivity. That the estimated rate of replication in a 5-min pulse is similar to that in a 2-min pulse indicates that extensive recombination within the replicating pool of viral DNA probably does not occur and does not affect the estimate of the rate of replication of the viral DNA.

In all experiments, a relatively small amount of radioactive DNA banded in a position of

TABLE 1. *Rate of DNA replication as determined by buoyant-density-shift experiments*[a]

Expt	Length of labeling period (min)	Avg mol wt of fragment × 10^6	Fractional BUdR content	Mol wt of DNA segment replicated/min × 10^6
A	2	13.5	0.30	2.0
	4	13.5	0.52	1.7
B	2	12.0	0.30	1.8
	4	12.0	0.30	1.5
C	5	22.5	0.42	1.9

[a] See legend to Fig. 3 and text for details.

higher density than did the peak of radioactive DNA (Fig. 3). This DNA consisted of fragments of molecular weight similar to that of the DNA fragments in the peak, and it is likely that a small proportion of the viral DNA replicates more rapidly than the bulk of the DNA. The significance of this finding will be discussed below. The data in Fig. 3 also show that even after a very short labeling period a small proportion of labeled DNA bands at a greater buoyant density than H-L DNA. This DNA could possibly be the product of reinitiation of molecules in the process of replication. It constitutes, however, only a very small part of the total labeled DNA and does not affect the estimation of the rate of replication of the bulk of the viral DNA.

Rate of replication of viral DNA as determined by radioautography. In these experiments, cells were infected and incubated up to 2.5 h postinfection in EDS-FUdR, as described above. Thereafter, the cultures were incubated with [^{3}H]thymidine for various periods of time. The cells were then lysed with SDS, and the lysates were spread directly onto slides which were then covered with radioautographic emulsion, as described in Materials and Methods. After an appropriate exposure time, the radioautograms were developed and examined by light microscopy. A characteristic microscopic field of a radioautogram is illustrated in Fig. 4. As a control, [^{3}H]thymidine-labeled DNA present in a preparation of purified virions was also spread and examined. The length of the tracks of silver grains was 40 to 50 μm, indicating that the length of the tracks of silver grains provides a good estimate of the length of the DNA (Pr viral DNA molecules measure approximately 45 μm).

Since, in these experiments, the cells were lysed with SDS immediately after the labeling period and nonpurified DNA was spread (only viral DNA is labeled under the conditions of infection that were used), it is unlikely that the replicating DNA molecules broke, as is the case after purification of viral DNA by isopycnic centrifugation in CsCl. Therefore, the length of the tracks of silver grains observed after various labeling periods should reflect the length of the newly synthesized stretches of DNA. Although the method is relatively insensitive and subject to error, a reasonable estimate of the rate of replication may still be obtained. Replicative structures such as forks or loops could, however, not be distinguished reliably.

The distribution of the length of silver grain

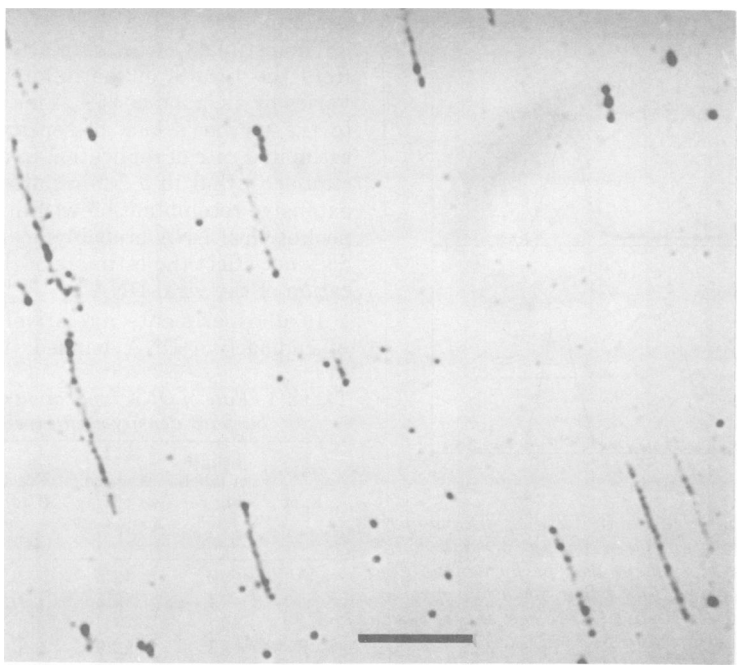

FIG. 4. *Radioautography of viral DNA molecules as observed in the light microscope. RK cells were infected as described in the legend to Fig. 2. At 2.5 h postinfection, the medium was changed to EDS-FUdR containing [^{3}H]thymidine (50 μCi/ml, 62 Ci/mmol) for 10 min. The cells were collected and the DNA was radioautographed as described in Materials and Methods. The bar represents 10 μm.*

tracks obtained after labeling periods of 10 and 20 min is illustrated in Fig. 5. Only tracks longer than 10 μm could be measured with any degree of accuracy, and only these tracks are included in this figure. After 10 min, the tracks observed ranged between 10 and 20 μm in length. Most of them clustered between 13 and 17 μm. One can estimate therefore that replication had occurred at a rate of 1.5 μm/min. After a 20-min labeling period, most of the tracks were between 20 and 30 μm in length, a length consistent with a rate of replication of approximately 1.5 μm/min. However, some of the silver tracks were twice as long.

A similar experiment was performed using electron microscopic radioautography. In these experiments, the infected cells were labeled as described in the legend to Fig. 4, but after lysis by SDS, the DNA was extracted and viral DNA was separated from cellular DNA by isopycnic centrifugation in CsCl. In this case, therefore, many of the molecules in the process of replication were broken (see above). However, by measuring only stretches of DNA covered with silver grains, which are bound by two stretches of DNA devoid of grains, one can arrive at an estimation (although a rough one) of the length of the DNA segment that replicated during the labeling period. An example of an electron microscopic radioautogram is illustrated in Fig. 6. (Because the resolution of autoradiograms is poor, and the molecule would not be clearly visible at low magnification, only half of the important part of the molecule is shown.) Table 2 summarizes the length distribution of the regions of DNA with grains after labeling periods of 10 to 20 min. The size of the longest grain tracks indicates that the DNA had replicated at approximately 1.2 μm/min. Probably because of the high degree of breakage of newly synthesized DNA, which occurs during the purification of the viral DNA required prior to electron microscopy, as well as because of the relatively small number of molecules measured, the longer stretches of newly synthesized DNA seen in the light microscope after a 20-min label were not observed. Forks or loops expected to be associated with replicating DNA were also not seen, again, probably because of breakage of replicating DNA as well as the low resolution obtained when one examines DNA preparations covered with nuclear track emulsion.

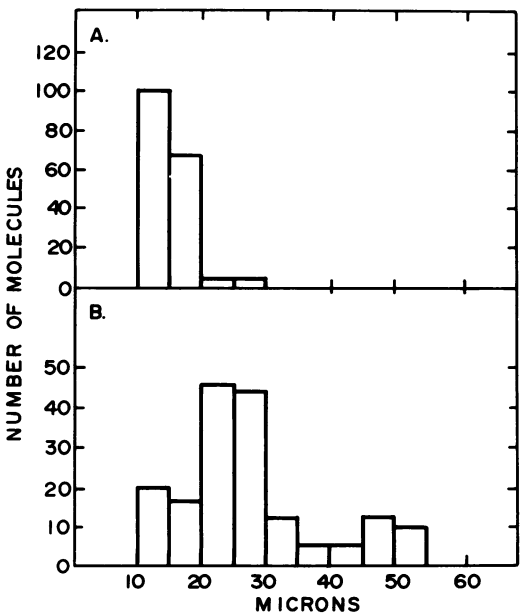

FIG. 5. *Length distribution of tracks after radioautography of DNA as observed in the light microscope. The experiment was performed as described in the legend to Fig. 4. The lengths of the tracks were measured using a calibrated ocular micrometer. Labeling periods: (A) 10 min; (B) 20 min.*

DISCUSSION

By means of three different methods, we have determined that, at early times after infection, Pr viral DNA chain elongation occurs at a rate of approximately 1 μm/min at 37°C. To calculate the time required to complete a molecule the size of mature viral DNA, the number of initiation sites per DNA molecule has to be considered. It is also necessary to know whether the rate of replication measured in these experiments represents the movement of a single fork or represents bidirectional replication.

Our previous results show that at early times after infection, periods corresponding to those during which the experiments described in this paper were performed, initiation of replication occurs predominantly either near (or at) one of the ends of the molecules or at a site situated between 18 and 24 μm from one of the ends of the molecules (Jean, Blankenship, and Ben-Porat, submitted for publication), from where it probably proceeds bidirectionally. Furthermore, initiation of replication occurs in most of the molecules at only one site.

The experiments reported in this paper were performed with infected cells treated with FUdR up to 2.5 h postinfection, when they were pulsed with [^{3}H]BUdR or [^{3}H]thymidine for various periods of time. This procedure will result in at least partial synchronization of DNA replication, once the labeled nucleoside is

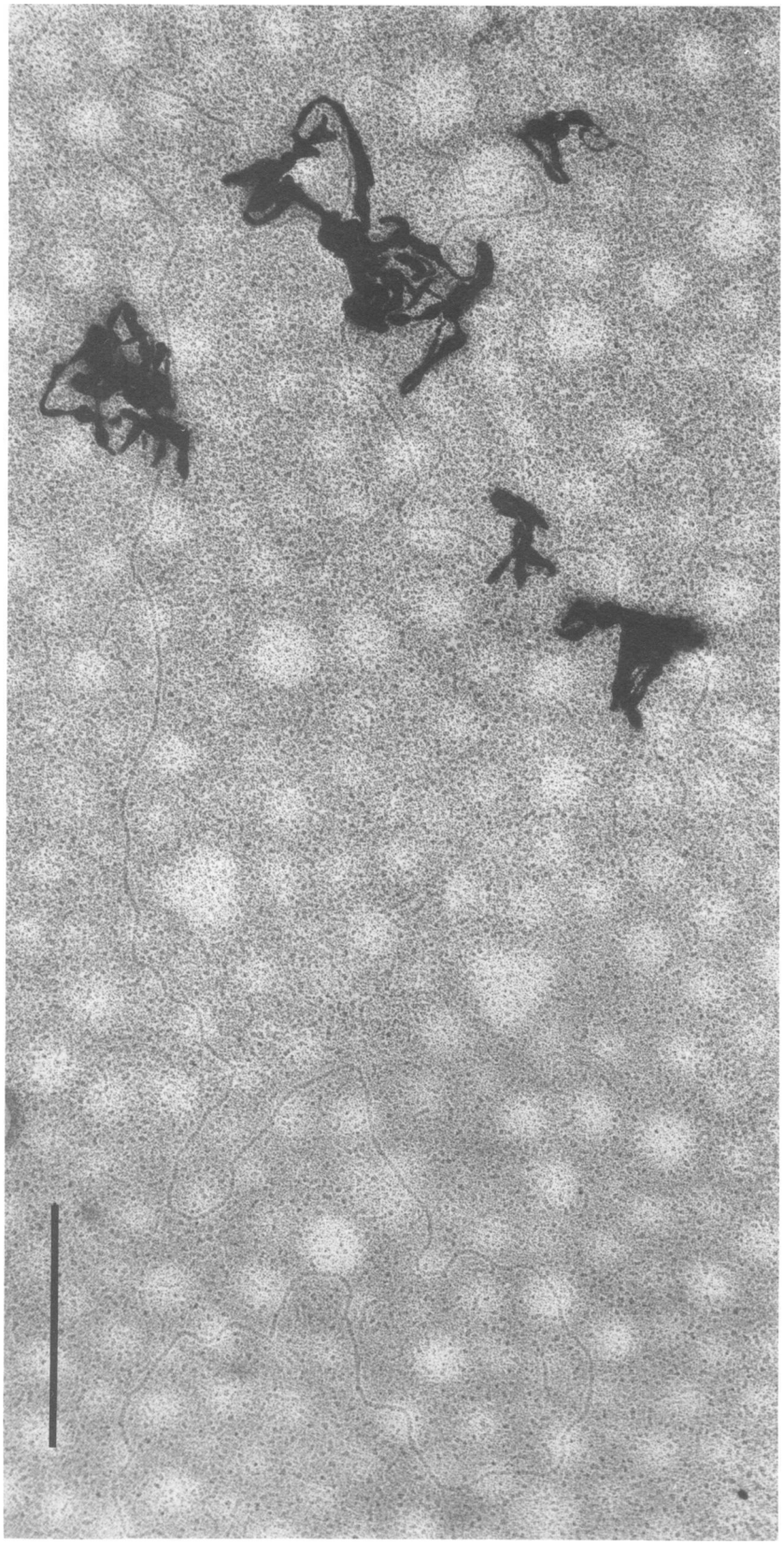

FIG. 6. *Radioautography of viral DNA molecules as observed in the electron microscope. The experiment was performed as described in the legend to Fig. 4. Viral DNA was separated from cellular DNA by isopycnic centrifugation in CsCl, and the viral DNA was spread for electron microscopy and radioautography, as described in Materials and Methods. The bar represents 0.5 μm.*

TABLE 2. *Size distribution of newly synthesized DNA as determined by electron microscopic radioautography*[a]

Labeling period (min)	Length of DNA with silver grains (μm)				
10	6.89	7.95	8.79	8.95	8.97
	10.22	11.26	12.31	12.34	12.70
20	18.60	21.32	24.37	24.49	26.47

[a] See text for details.

supplied to the cells. Indeed, in infected cells treated with FUdR up to 3 h postinfection, no replicative loops or branches were detected by electron microscopy (Jean, Blankenship, Ben-Porat, submitted for publication).

If, indeed, as indicated by our electron microscopic observations, partial synchronization of initiation of replication was achieved by the FUdR treatment, we should have measured the following two rates of replication: (i) the rate of DNA synthesis resulting from bidirectional replication initiated at the internal site; and (ii) the rate of unidirectional replication in either the molecules in which DNA synthesis was initiated at the end of the molecules or in the molecules in which synthesis was initiated internally but in which DNA synthesis was not synchronized. Our results indicate that viral DNA had indeed replicated at two different rates.

The density-shift experiments show that a small amount of DNA replicates more rapidly than the bulk of DNA (Fig. 3). Possibly, the relatively rapid rate of replication of this DNA reflects bidirectional replication, most of the DNA replicating unidirectionally at the rate of approximately 1 μm/min. The data obtained from radioautography (in the light microscope) show also that most of the DNA replicates at the rate of approximately 1 to 1.15 μm/min. After a 20-min pulse, most of the molecules had stretches of silver grains 20 to 30 μm in length. However, molecules with stretches of silver grains approximately 50 μm were also observed. These results are consistent with the interpretation that a single fork moves at a rate of approximately 1 μm/min and that the rate of DNA replication, which we have measured, represents primarily the movement of a single fork. Some molecules of DNA, however, replicated bidirectionally at approximately twice that rate. Thus, Pr viral DNA molecules in which DNA replication is initiated near or at the end of the molecule would require approximately 45 min to replicate. Molecules initiated at a site 20 μm from one of the ends in which replication proceeds bidirectionally would require 25 min for replication.

The rate of movement of the replicative fork in mammalian cells has been estimated to be approximately 1 μm/min per fork (3, 5, 7, 8, 12). Our results indicate that the rate of the fork movement during Pr viral DNA replication is within the same range.

ACKNOWLEDGMENT

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