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Appearance *in vivo* of single-stranded complementary ends on parental herpesvirus DNA*

(single-stranded ends *in vivo*/replicative loops/circular and concatemeric molecules)

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ABSTRACT **Intracellular forms of pseudorabies virus parental DNA were examined before and after the onset of viral DNA synthesis. Before initiation of synthesis, parental viral DNA acquires single-stranded ends. Circular and concatemeric molecules are also observed, indicating that the single-stranded ends are complementary.**

Viral DNA replication is initiated at an internal site within the DNA molecule, giving rise to characteristic replicative loops with single-stranded regions in the *trans* position. Such replicative loops were seen in unit-size (and smaller than unit-size) linear molecules as well as in circular and concatemeric molecules. These results show that the parental viral DNA molecules that acquire single-stranded ends, and consequently are able to form circles and concatemers, proceed to replicate.

The genome of pseudorabies (Pr) virus, one of the herpesviruses, consists of a linear, double-stranded DNA molecule with a molecular weight of approximately 90×10^6. Pr viral DNA is asymmetric with respect to its G+C content and gives rise to characteristic partial denaturation maps, indicating that it is not circularly permuted (1). Mature Pr viral DNA also has single-strand interruptions (is nicked) (2, 3). In all these respects, Pr viral DNA is similar to that of herpes simplex virus, type 1 (HSV-1), a closely related virus (4–7).

Information about the replication of herpesvirus DNA is relatively limited. It is known that replication is semiconservative (8), and that viral DNA forms an intracellular pool from which it is withdrawn at random to be encapsidated into viral particles (9). Despite the presence of nicks, parental viral DNA retains its integrity after replication; consequently, the nicks in the parental viral DNA are probably ligated prior to replication (10). Viral DNA synthesis is discontinuous; the newly synthesized DNA strands consist of short segments (6, 10) to which RNA is covalently linked (11).

Herpes simplex virus DNA is terminally redundant (12–14), which should allow it to circularize and to form concatemers. Indeed, late replication of Pr viral DNA involves the formation of large, concatemeric linear molecules and of tangles (3) similar to those described for bacteriophage T_4 (15). These structures are too complex to be interpreted easily. Consequently, we have undertaken a study of the replication of parental Pr DNA molecules at early stages of infection. A part of these studies is described in this paper.

MATERIALS AND METHODS

Virus and Cell Culture. Recently plaque-purified virus (two passages at low multiplicities of infection) was used. The properties of Pr virus and the cultivation of rabbit kidney (RK) cells have been described (16).

Media and Solution. *Eagle's medium:* Eagle's synthetic medium (17) plus 5% dialyzed bovine serum. *Thymidine medium:* Eagle's medium containing 3% dialyzed serum, thymidine (50 μg/ml) and deoxycytidine (5 μg/ml). *RSB-1% Sarkosyl:* 0.01 M Tris·HCl, pH 7.4; 0.01 M KCl; 0.0015 M $MgCl_2$ (18); plus 1% sodium lauryl Sarkosinate-97. *Pronase solution:* 0.2 M NaCl; 0.02 M Tris, pH 7.3; and self-digested (nuclease-free) Pronase (5 mg/ml). *Saline-citrate:* 0.15 M NaCl; 0.015 M Na citrate, pH 7.4. 6 × saline–citrate is six times these concentrations.

Purification of Virions. Pr virus was purified using the cells as a "purifying" agent, as described previously (10).

Equilibrium Sedimentation in CsCl. The infected monolayers were scraped into RSB-1% Sarkosyl and heated at 45° for 15 min. An aliquot was mixed with an equal volume of Pronase solution and incubated for 2 hr at 37°. [^{14}C]Thymidine-labeled RK cellular DNA was added. The samples were mixed with CsCl and centrifuged to equilibrium in CsCl gradients, as described previously (19). (Less than 30 μg of DNA was centrifuged in a 5 ml gradient.) Samples were collected dropwise (using a flow rate controlling device) from a large hole pierced in the bottom of the tube. Drops were allowed to slide along the side of the tubes.

Extraction of DNA. DNA was extracted as described previously (3).

Retention of DNA by Nitrocellulose Filter. Five milliliters of DNA (0.2 μg/ml) extracted from cells infected [^{3}H] thymidine-labeled virus, in 6 × saline citrate, were passed slowly (less than 1 ml/min) through HAWP Millipore filters which were then washed with 50 ml of 6 × saline citrate. The amount of ^{3}H-labeled DNA retained by the filters was determined.

S_1 Nuclease Digestion. S_1 nuclease was prepared and reacted with DNA as described by Vogt (20). Sensitivity of the DNA to the enzyme was estimated by determining the amount of radioactive material that became acid-soluble after treatment with the enzyme.

Electron Microscopic Observation. These techniques have been described previously (1).

RESULTS

Intracellular forms of Pr viral DNA prior to the initiation of viral DNA synthesis

Parental viral DNA present in infected cells before the onset of viral DNA synthesis was examined in the electron microscope. In these experiments, the cells were infected with [^{3}H]thymidine-labeled virions and at various times after infection the cells were harvested and the DNA was extracted and mixed with [^{14}C]thymidine-labeled cellular DNA. Viral DNA was separated from cellular DNA by equilibrium sedimentation in CsCl. The lack of contamination of the band of viral DNA with cellular DNA was demonstrated by the fol-

Abbreviations: Pr, pseudorabies; RK, rabbit kidney.

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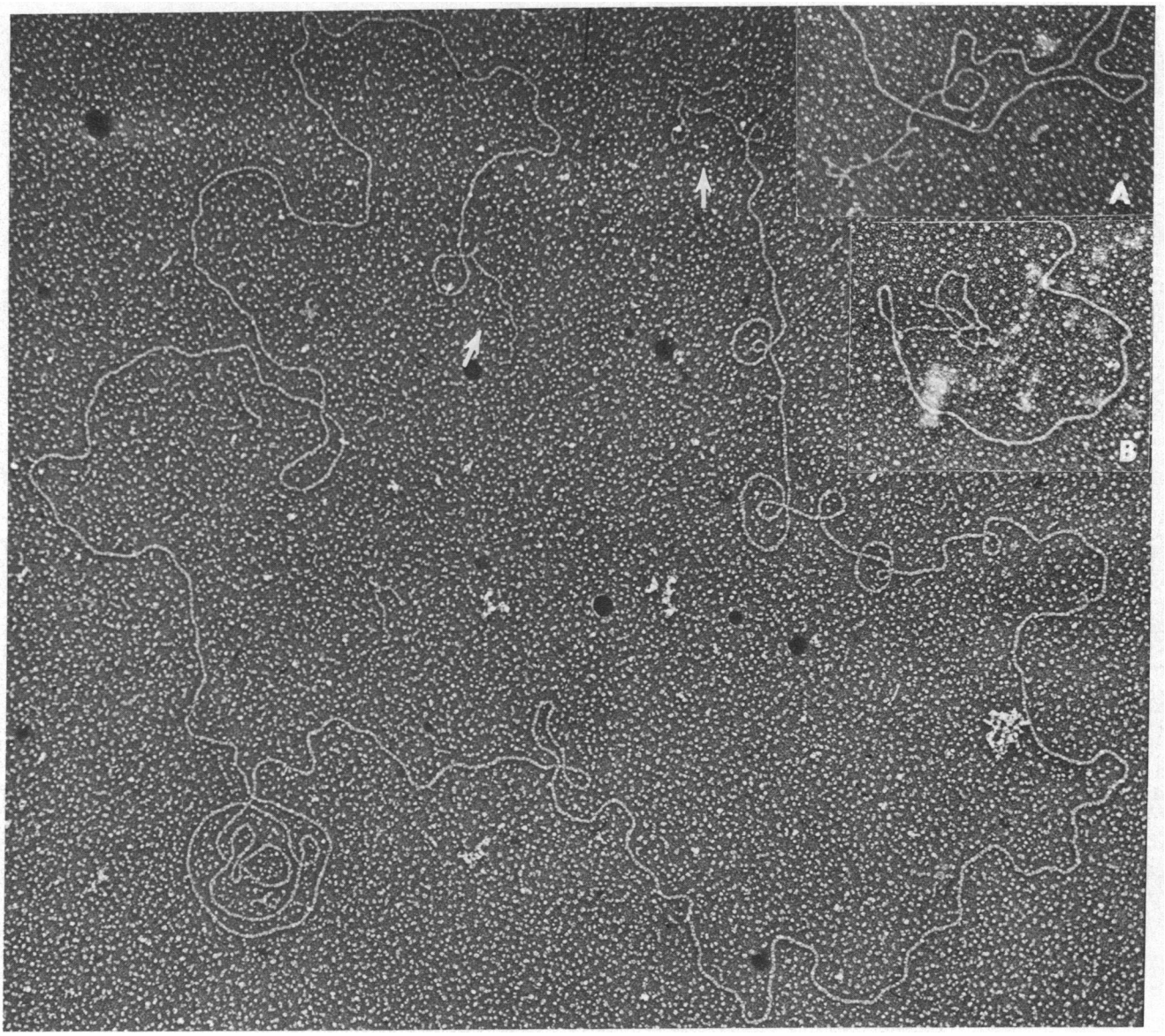

FIG. 1. Viral DNA molecule with single-stranded ends. The viral DNA was isolated from infected cells 90 min after inoculation. The single-stranded ends are indicated by the arrows. This DNA molecule measures 44.5 μm. Inserts A and B are the ends of another DNA molecule.

lowing criteria: (*i*) No detectable ^{14}C label was found in the band of ^{3}H-labeled viral DNA. (*ii*) The DNA of uninfected similarly treated cells was centrifuged in CsCl and the fractions with the density of viral DNA (1.732 g/cm^3) were examined by electron microscopy. No DNA was found in these fractions. (*iii*) DNA from uninfected cells labeled [^{14}C]thymidine was mixed with [^{3}H]thymidine-labeled virion DNA and viral DNA was separated from cellular DNA as described above. The fractions containing viral DNA were examined by electron microscopy. Only linear double-stranded molecules of approximately 45 μm (unit-size viral DNA) or smaller were seen. Preparations of viral DNA extracted from infected cells up to 75 min post infection also contained only linear unit-size molecules. However, in preparations of viral DNA isolated from infected cells at 90 min post infection (in addition to linear, unit-size double-stranded molecules) three types of molecules were observed. These molecules were observed both in infected cells that had been treated with cycloheximide from the time of infection and in untreated infected cells. The distribution of these molecules is summarized in Table 1. The characteristics of these three classes of molecules are as follows.

Molecules with Single-Stranded Ends. These were linear, double-stranded molecules with a single-stranded region at either one or both ends of the molecules. Fig. 1 shows a characteristic molecule with two single-stranded ends. The inserts A and B show the ends of another molecule. The presence of molecules with single-stranded ends indicates that the parental viral DNA is digested by an exonuclease within the infected cells. Most of the single-stranded ends observed at this time after infection (90 min) were approximately 0.5–1.0 μm long. Exact estimations of the lengths of the single-stranded regions were difficult to make because single-stranded DNA does not spread well under the conditions used. That the formation of single-stranded regions at this time after infection was limited to a small part of the genome was confirmed by the lack of sensitivity of the viral DNA to S_1 nuclease (see Table 2).

Circular Molecules. These consisted of closed, double-stranded, unit-size length, circular molecules, an example of

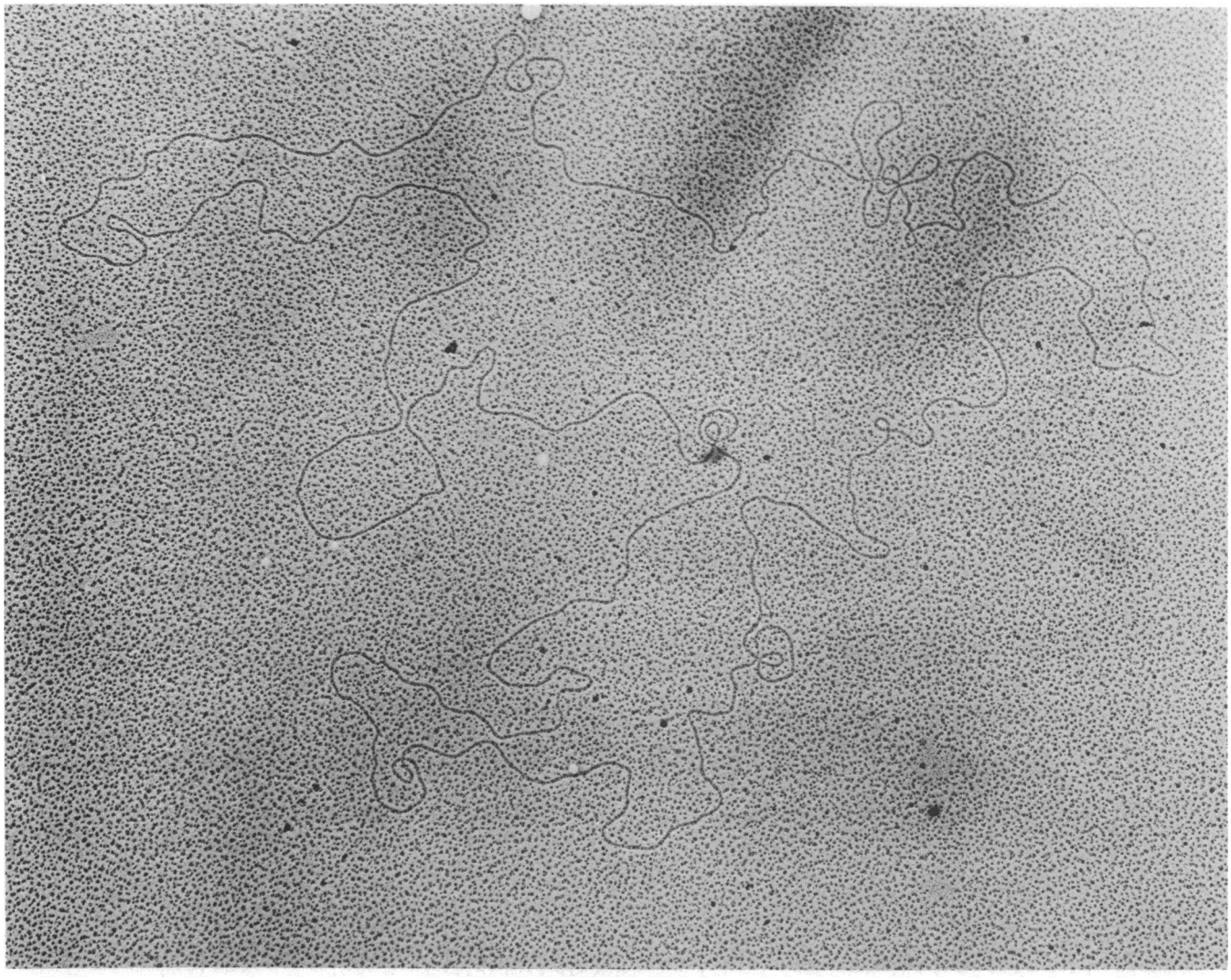

FIG. 2. Circular viral DNA molecule. Viral DNA was isolated from infected cells 90 min after inoculation. This DNA molecule measures 48 μm.

which is illustrated in Fig. 2. These circular molecules were relaxed; no supercoils similar to those reported for Epstein-Barr virus (21) were observed. The fact that circular molecules appear indicates that the single-stranded ends of the viral DNA that are exposed within the infected cells can anneal with each other and that therefore Pr virus DNA is terminally redundant. This finding is not unexpected, since the DNA of herpes simplex virus, a virus closely related to Pr virus, has terminally repetitive regions (12–14).

Molecules Longer Than Unit Size. Circular (one such molecule of 90 μm i.e., two-unit size, was seen) or linear molecules longer than mature Pr virus DNA, with or without single-stranded ends, were observed. The longer than unit-size molecules probably arise as a result of the anneal-

Table 1. Frequency distribution of various intracellular forms of parental Pr viral DNA molecules

Types of DNA molecules	Number (or percentages) of molecules		
	Infected untreated	Infected cycloheximide-treated	Size range (μm)
Unit size linear molecules	147 (64.5%)	152 (84%)	40–49
Unit size molecules with sticky ends*	53 (23.2%)	18 (9.9%)	40–48
Circular molecules	10 (4.4%)	3 (1.7%)	46–49
Molecules longer than unit length (linear or circular)	18 (7.9%)	8 (4.4%)	56–91

RK cells were infected with [^{3}H]thymidine-labeled virions (10 plaque-forming units per cell) in thymidine medium with or without cycloheximide (100 μg/ml). After adsorption, the cells were washed extensively to remove unadsorbed virus and incubated further in the same medium. At one and a half hours after infection, the cells were harvested and the DNA was extracted and centrifuged to equilibrium in a CsCl density gradient to separate viral DNA from cellular DNA. The viral DNA was spread as described in *Materials and Methods* and examined in the electron microscope. Only molecules of unit size or longer were tabulated. These molecules comprised approximately 70% of the DNA in the preparation.

* Either one or both ends of the molecules were single-stranded.

Table 2. Acquisition of single-stranded regions by parental viral DNA within the infected cells

Time after infection (min)	Total cpm per sample ($\times 10^{-3}$)	Digested by S_1 nuclease (%)	Retained by nitrocellulose filters (%)	Retained by nitrocellulose filters after S_1 digestion (%)
30	19.3	<1	13.4	0
75	20.1	<1	14.3	0
90	21.2	<1	27.3	0.5
120	17.4	<1	44.8	2.8
180	19.3	2.1	26.8	2.6

Stationary RK cells were infected with [^{3}H]thymidine-labeled Pr virus, as described in *Materials and Methods*. After a 30-min adsorption period, the unadsorbed virus was removed by washing, and the cells were further incubated in thymidine medium for various periods of time, when the cells were harvested and the DNA was extracted. The total amount of ^{3}H-labeled DNA in the samples, as well as the amount sensitive to S_1 nuclease or retained on nitrocellulose filter before and after digestion with S_1 nuclease, was determined.

ing of the cohesive ends of two parental DNA molecules. DNA replication does not play a role in the formation of these molecules since viral DNA replication starts at a later time (2.0 hr post infection). Furthermore, such molecules were also observed in cells that had been treated with cycloheximide from the time of infection (see Table 1), in which viral DNA synthesis does not occur.

Table 2 summarizes the results of an experiment in which the presence of regions of single-strandedness in parental viral DNA was determined by testing its sensitivity to S_1 nuclease, as well as by its retention on nitrocellulose filters. The results show that during early stages of infection the percentage of parental viral DNA that becomes sensitive to the enzyme is small (less than 1%). However, although the parental viral DNA is not detectably sensitive to S_1 nuclease, it nevertheless acquired regions of single-strandedness. Thus, immediately after infection 14% of the parental viral DNA was retained by nitrocellulose filters. Similar results are also obtained when the retention of DNA extracted from mature virions was tested. At 2 hr post infection, 45% was retained. Thereafter, the proportion of parental viral DNA retained by the filters decreased. Whether this is due to repair of single-stranded gaps or ends or whether the regions of single-strandedness anneal with one another (as would be expected from the terminally redundant ends) is not clear. Retention of the DNA by the filters is abolished by treatment of the DNA with S_1 nuclease.

Intracellular forms of viral DNA after initiation of viral DNA synthesis

Examination of viral DNA molecules (more than 2000 were examined) present within the infected cells after viral DNA synthesis had begun (2.75–3 hr post infection) revealed the following. Many of the molecules contained one or more replicative loops, indicating that DNA replication is initiated at internal sites. An example of a typical replicative loop with single-stranded regions in the *trans* position is shown in Fig. 3.

The characteristics of the replicative loops and their location within the molecules will be discussed in detail elsewhere (Jean *et al.*, to be published). Related to the observations described in this paper is the finding that replicative loops were observed in linear molecules of unit size and smaller than unit size, as well as in linear molecules longer than unit size and in some circular, unit-size, molecules. The presence of loops in concatemeric and circular Pr viral DNA

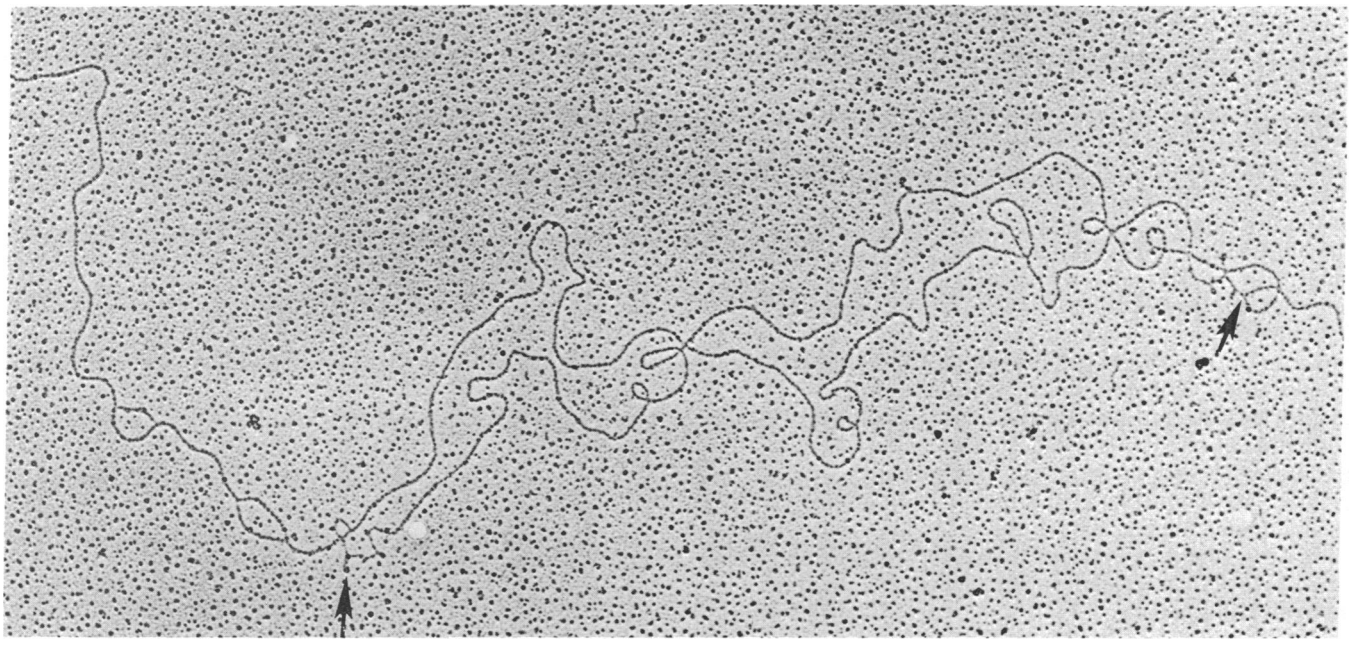

FIG. 3. Replicative loop in viral DNA. The viral DNA was isolated 2.75 hr post infection. This replicative loop measures 10 μm; the entire molecule measures 43 μm. The arrows indicate the regions of the forks and of single-strandedness.

molecules suggests that these structures may be replicative intermediates in viral DNA replication. However, since many replicative loops were also observed in unit-size (or smaller than unit-size) linear molecules, this point is not entirely clear. While the smaller molecules may be breakage products, it is also possible that the circular and concatemeric molecules may be incidental, and may result from the presence of the "sticky ends" on the molecules. The finding that replicative loops are present on circular and concatemeric molecules, however, shows clearly that viral DNA molecules that acquire "sticky ends" proceed to replicate.

DISCUSSION

The results described in this paper show that parental Pr viral DNA is digested within the infected cells by an exonuclease which exposes the terminal sequences of the DNA. The terminal sequences are complementary to each other and anneal intramolecularly to form circles or join end-to-end intermolecularly to form molecules longer than unit size. Complementary terminal sequences of parental viral DNA are also exposed in cells treated with high concentrations of cycloheximide from the time of infection. Although the frequency in the appearance of these molecules is decreased in cycloheximide-treated cells, it is unlikely that the exposure of the single-stranded ends of the molecules is due to the synthesis of a small amount of viral protein, since no detectable increase in the activity of enzymes induced by infection (such as thymidine kinase and DNA polymerase) can be detected under these conditions. We conclude that the enzyme(s) exposing the single-stranded ends is probably not virus-induced.

The transient appearance at early stages in infection of single-stranded regions on parental viral DNA has been confirmed by its retention by nitrocellulose filters. The regions of single-strandedness are, however, small and only a small proportion of the DNA is digested by S_1 nuclease (see Table 2). Whether only terminal regions of the molecules become single-stranded or whether single-stranded internal regions are formed as well, is not clear. Appearance of internal regions of single-strandedness could not be identified by electron microscopy up to 2 hr post infection.

Under the conditions of infection used in these experiments, up to 90% of the parental viral DNA molecules replicate (despite a ratio of 1:10 to 1:20 of infectious to noninfectious particles in the virion population) provided that fresh preparations of virions are used and that each cell is infected with at least one infectious virion (10). The digestion of the parental viral DNA by an exonuclease is therefore not a step in the degradation and elimination of the viral DNA from the infected cell. This is also indicated by the fact that molecules with "sticky ends," which are able to form circles and concatemers, proceed to replicate. The relevance of these structures to the processes of viral DNA replication has not, however, been clearly established because unit-size (and smaller than unit-size) linear molecules with replicative loops were also observed. Furthermore, it is possible that the formation of circular and concatemeric molecules from linear molecules with sticky ends is incidental only.

After the completion of this manuscript, a report (22) appeared indicating that the replication of DNA of herpes simplex virus, type 1, is initiated at an internal site of the molecule, a conclusion which is in agreement with the results described in this paper.

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