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Anatomy of Herpes Simplex Virus DNA

XII. Accumulation of Head-to-Tail Concatemers in Nuclei of Infected Cells and Their Role in the Generation of the Four Isomeric Arrangements of Viral DNA

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Previous reports (H. Delius and J. B. Clements, J. Gen. Virol. **33**:125-134, 1976; G. S. Hayward, R. J. Jacob, S. C. Wadsworth, and B. Roizman, Proc. Natl. Acad. Sci. U.S.A. **72**:4243-4247, 1975; B. Roizman, G. S. Hayward, R. Jacob, S. W. Wadsworth, and R. W. Honess, Excerpta Med. Int. Congr. Ser. **2**:188-198, 1974) have shown that herpes simplex virus DNA extracted from virions accumulating in the cytoplasm of infected cells consists of four populations of linear molecules differing in the orientation of the covalently linked large (L) and small (S) components relative to each other. Together, these four isomeric arrangements of viral DNA display four different termini and four different L-S component junctions. In the studies reported in this paper, we analyzed with restriction endonucleases the newly replicated viral DNA shortly after the onset of viral DNA synthesis, the progeny DNA accumulating in the nuclei late in infection, and rapidly sedimenting DNA present in nuclei of infected cells at 8 h after infection. In each instance the nuclear viral DNA contained a decreased concentration of all four terminal fragments and an increase in the concentration of fragments spanning the junction of L and S components relative to the concentration of other DNA fragments. The results are consistent with the hypothesis that the viral DNA accumulating in the nuclei consists of head-to-tail concatemers arising from the replication of DNA by a rolling-circle mechanism. A model is presented for generation of all four isomeric arrangements of herpes simplex virus DNA from one arrangement based on excision and repair of unit length DNA from head-to-tail concatemers and known features of the sequence arrangement of viral DNA.

This report concerns the mode of replication of herpes simplex virus type 1 (human herpesvirus 1, HSV-1) DNA. We report that in newly replicated DNA the ends of the molecule are joined head to tail. Pertinent to the purpose and significance of the work are the following data.

(i) HSV-1 DNA, 96×10^6 molecular weight (1, 19, 27), consists of two convalently linked components, L and S, each consisting of unique sequences U_L and U_S bracketed by inverted repeats (25, 27). The inverted repeats bracketing the L component are designated *ab* and *b'a'*, whereas the inverted repeats of the S components are designated *a'c'* and *ca* (27). The *a* sequences, common to both L and S components, are arranged in the same orientation at the ends of the molecule, as evidenced by the fact that HSV-1 DNA circularizes after digestion with a processive exonuclease (8, 25, 28). Of major significance to this study is the evidence that HSV-1 DNA consists of four equimolar populations of molecules differing solely in the orientations of L and S components relative to each other (3, 9, 22). These populations have been designated as P (prototype), I_S (inversion of S component), I_L (inversion of L component), I_{SL} (inversion of L and S component) (9, 22). A consequence of these inversions of L and S components illustrated in Fig. 1 is that restriction endonucleases that do not cleave within the reiterated regions generate three classes of fragments. The first class (e.g., *Bgl* II fragments K, O, P, N, M, I, D, and G; Fig. 1) contains fragments corresponding in concentration to the molarity of intact DNA and located between the first and the last cleavage within the U_L and U_S regions, respectively. The second class contains the four terminal fragments (e.g., *Bgl* II J, F, H, and L; Fig. 1), each present in 0.5 M concentration relative to the molarity of the intact DNA. The third class comprises four fragments spanning the junction between the L and S compo-

nents (e.g., *Bgl* II fragments FH, JH, FL, and JL). These fragments are present in 0.25 M concentration relative to the molarity of the intact DNA.

(ii) The core of the virion has sufficient space to accommodate only one molecule of HSV DNA (7). In plaque assays, the number of plaques is strictly proportional to virus concentration (14). Moreover, analysis of viral DNA extracted from cloned virus showed that all four arrangements of HSV DNA are invariably produced in the course of viral replication (21). These results imply that a single virion can be sufficient to initiate infection and that all four arrangements of HSV DNA can arise from one arrangement.

(iii) Analyses of DNAs extracted from intertypic (HSV-1 × HSV-2) recombinants (21) suggested that two arrangements of the DNA, i.e., I_L and I_{SL}, were not capable of forming recombinants that are subsequently amplified. Because of the paucity of suitable markers and recombinational events in the S component, these experiments could not discriminate between the hypothesis that both P and I_S arrangements are capable of producing recombinants that are amplified and the alternative hypothesis that only one of these was capable of doing so. We interpreted these results to indicate either that P and I_S forms of recombinant DNAs replicated more efficiently or that the orientation of the L component relative to the S component is fixed in the recombinant DNA molecules capable of initiating and completing the reproductive cycle. Because all four arrangements of HSV DNA are present in equimolar concentrations,

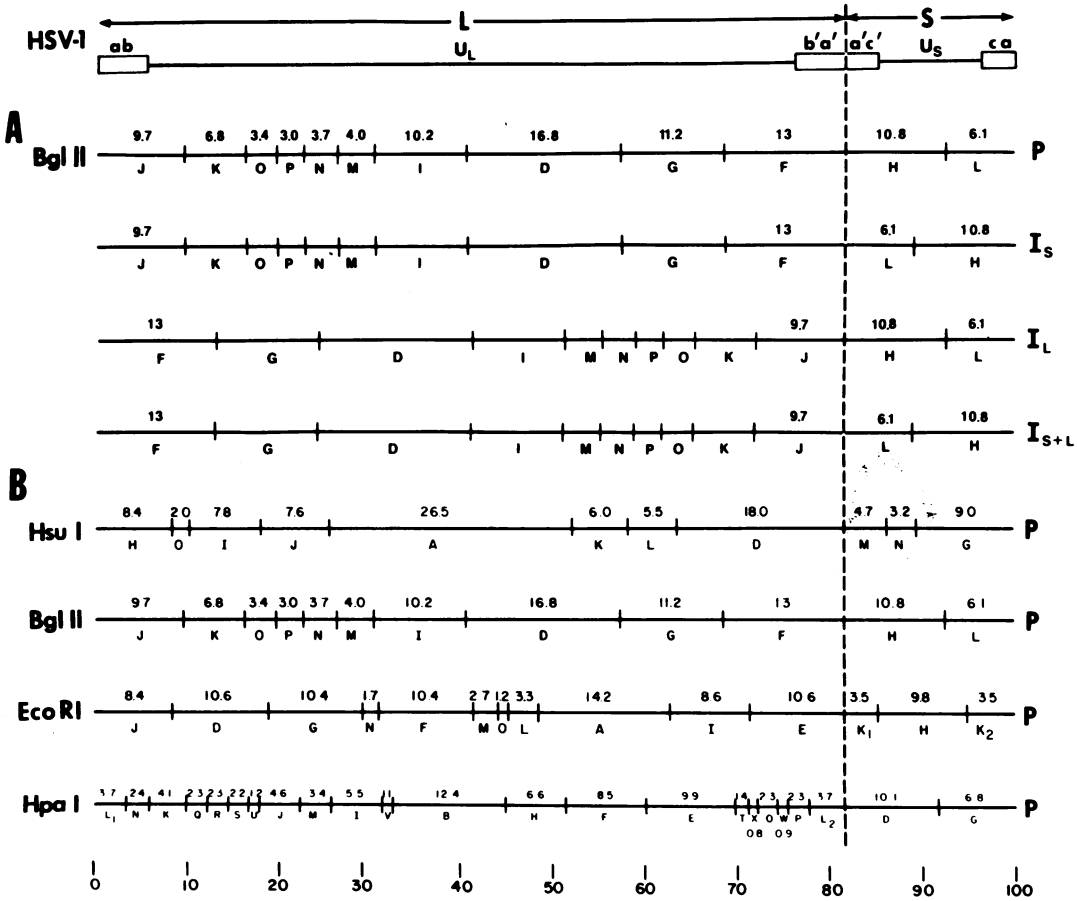

FIG. 1. *Topology of the HSV-1 DNA. (A) Bgl II restriction endonuclease maps of the four arrangements of HSV-1 DNA. P, Prototype arrangement (21); I_S, I_L, and I_{SL} correspond to inversion of S, inversion of L, and inversion of both S and L arrangements. Fragment sizes are given in millions molecular weight. (B) Hsu I, Bgl II, EcoRI, and Hpa I restriction endonuclease maps of HSV-1 DNA in the P arrangement. The other arrangements can be generated by inverting L, S, or both components.*

the inversions must arise in the course of viral replication as a consequence of some obligatory event.

(iv) Published reports on herpesvirus DNA synthesis do not provide a uniform picture. Viral DNA has been reported to begin at our near termini and at a distance from termini (11, 26). Branched molecules, circles, lariats, and multiunit length molecules have been reported to be present in lysates of infected cells (2, 6, 11, 12, 15, 16, 26). In a preceding study, we showed the presence of circles and lariats as well as molecules sedimenting at rates consistent with their being multiunit concatemers in nuclei of infected cells containing replicating HSV DNA (15).

The purpose of the studies reported here was to determine the arrangement of HSV DNA in the concatemers.

(A preliminary report of these results was presented at the XLIII Cold Spring Harbor Symposium on DNA: Replication and Recombination, May–June, 1978.)

MATERIALS AND METHODS

Viruses and cells. The procedures for the propogation and infection of Vero cells and the isolation and properties of HSV-1 (MP) virus strain used in the studies have been reported elsewhere (4, 13, 24). Roller bottle cultures of Vero cells at a density of 2×10^8 were infected at a multiplicity of 5 PFU per cell.

Labeling, extraction, and purification of virus-specific DNA. The infected cell cultures were labeled with [*methyl*-^{3}H]thymidine (specific activity, 59 Ci/mmol, New England Nuclear Corp., Boston, Mass.), at times and for intervals stated in the text. The infected cell monolayers were harvested and separated into nuclear and cytoplasmic fractions, and the DNA was extracted and purified by procedures previously reported (15) except for two modifications. Specifically, the duration of Pronase digestion was reduced to 4 h. In addition, the DNA was banded in CsCl equilibrium gradients formed in a Beckman Vti 50 rotor, centrifuged for 18 h at 40,000 rpm and 20°C. In the experiments that required the purified DNA to be high molecular weight, the total nuclear DNA was purified first by centrifugation in a 10 to 30% (wt/wt) sucrose gradient overlaid on a 1-ml cushion of saturated CsCl. These gradients were centrifuged in a Beckman Vti 50 rotor for 1 h at 40,000 rpm and 20°C. The bottom fractions were pooled, dialyzed against TEN (0.01 M Tris-hydrochloride, pH 7.5–0.01 M EDTA–0.1 M NaCl) buffer, and then purified by CsCl equilibrium centrifugation as described above. These modifications were made to reduce damage to replicating molecules.

Analysis of HSV DNA with restriction endonucleases. The purification of restriction endonucleases, the conditions for digestion and electrophoresis of DNA fragments on 0.4% agarose gels, ethidium bromide staining, autoradiography, and the procedures for measuring the radioactivity contained in the DNA fragments were described elsewhere (9, 20, 21). The designation of the restriction endonuclease fragments follows the rules adapted at the Third Herpesvirus Workshop, Cold Spring Harbor, N.Y., September 1976, and described by Jones et al. (17).

RESULTS

The experimental design employed in these studies was based on the expectation that HSV DNA in concatemers would be linked head to tail or head to head and, therefore, in restriction endonuclease digests of concatemeric DNA the individual terminal fragments should be replaced by new fragments arising from fusion of the terminal fragments. Three series of experiments were done to test these expectations.

In the first series we examined the DNA accumulating in nuclei and cytoplasm of infected cells within a short interval after the onset of viral DNA synthesis at approximately 3 h postinfection (23). In these experiments Vero cells were labeled with [^{3}H]thymidine (100 μCi/ml) from 2 to 4 and from 2 to 6 h postinfection. The nuclear and cytoplasmic DNAs were then purified as described above, digested with *Bgl* II restriction endonuclease, and then subjected to electrophoresis on agarose gels. The results, shown in part in Fig. 2, were as follows. (i) The amount of labeled viral DNA recovered from the cytoplasm of cells labeled from 2 to 4 h postinfection was too low to permit adequate resolution. This is to be expected, inasmuch as previous studies have shown that viral DNA synthesis is initiated about 3 h postinfection and that at least 1 h elapses between the completion of viral DNA synthesis and its appearance in a mature virion in the cytoplasm. Labeled viral DNA was recovered from cytoplasm of cells incubated in [^{3}H]thymidine from 2 to 6 h postinfection, and the distribution of radioactivity among the electrophoretically separated restriction endonuclease fragments (Fig. 2C) indicated that all fragments were present in amounts expected for unit length, mature viral DNA (9). (ii) The distribution of radioactivity among the electrophoretically separated fragments from viral DNA recovered from nuclei labeled from 2 to 4 and 2 to 6 h postinfection (Fig. 2A and B), revealed a decrease or absence of terminal fragments (*Bgl* II F, H, J, and L). However, no new fragments, absent from the digest of unit length DNA extracted from the cytoplasm, could be detected.

The objective of the second series of experiments was to quantitate the relative amounts of the fragments present in nuclear viral DNA. In the first experiment, designed to determine whether concatemers accumulate late in infection, viral nuclear and cytoplasmic DNAs were extracted from 18-h infected cells, digested with

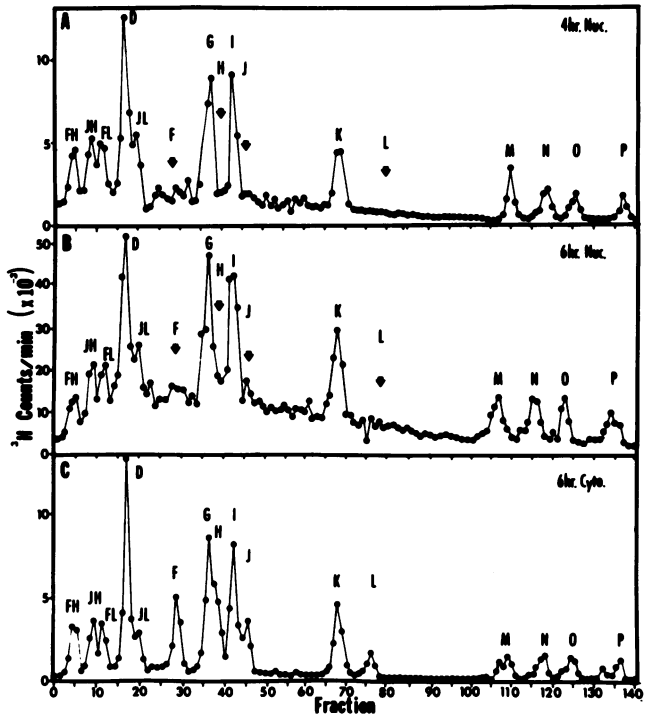

FIG. 2. *Radioactivity profiles of electrophoretically separated DNA fragments from Bgl II digests of viral DNA. (A) Viral DNA extracted from nuclei of Vero cells labeled with [³H]thymidine from 2 to 4 h postinfection. (B) Viral DNA extracted from nuclei of Vero cells labeled with [³H]thymidine from 2 to 6 h postinfection. (C) Viral DNA extracted from cytoplasm of Vero cells labeled with [³H]thymidine from 2 to 6 h postinfection. The arrows point to the position of the bands containing the 0.5 M terminal fragments.*

Bgl II, *Hpa* I, and *Hsu* I restriction endonucleases, and subjected to electrophoresis in agarose gels. The photograph of the electrophoretically separated fragments stained with ethidium bromide is shown in Fig. 3. The salient feature of the results is the apparent decrease in the concentration of all terminal 0.5 M fragments in the digests of nuclear DNA as compared with those of cytoplasmic DNA.

The second experiment was designed to determine precisely the relative concentrations of the fragments in nuclear and cytoplasmic viral DNA. In this experiment infected Vero cells were labeled with [³H]thymidine (20 μCi/ml) from 2 to 18 h postinfection. The viral DNA was then extracted and digested with *Bgl* II restriction endonuclease. The electrophoretic profiles of the fragments contained in the cytoplasmic DNA and nuclear digests are shown in Fig. 4A and B, respectively. The molar ratios of the fragments in cytoplasm and nuclei calculated from known molecular weights of fragments and relative amounts of [³H]thymidine in each band are shown in Table 1. The data indicate that all four 0.5 M terminal fragments were decreased in concentration in nuclear DNA as compared with

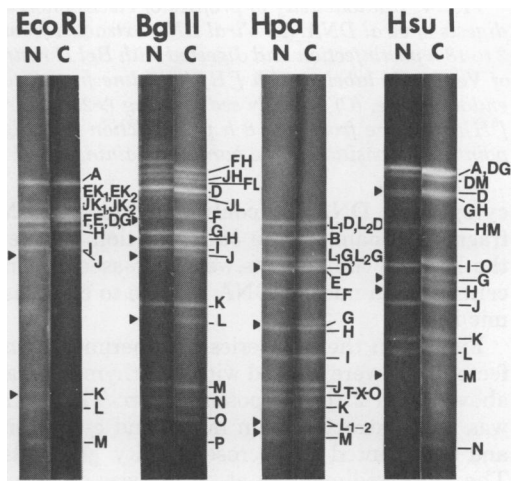

FIG. 3. *Photograph of ethidium bromide-stained restriction endonuclease DNA fragments electrophoretically separated in 0.4% agarose gels. N, Viral DNA extracted and purified from infected cell nuclei; C, viral DNA extracted and purified from infected cell cytoplasm. Solid triangles mark underrepresented or missing 0.5 M terminal fragments in DNA extracted from nuclei.*

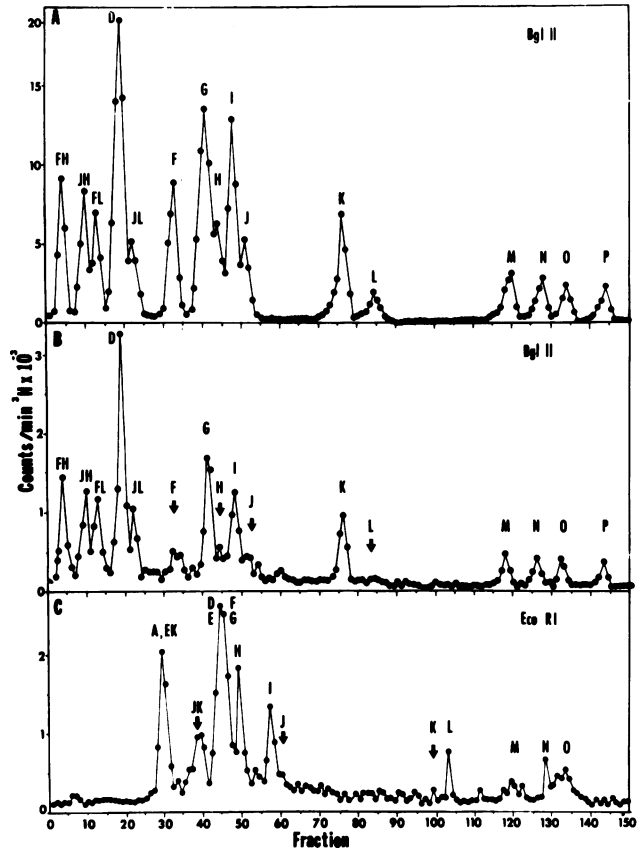

FIG. 4. *Radioactivity of profiles of electrophoretically separated DNA fragments from Bgl II and EcoRI digests of viral DNA. (A) Viral DNA extracted from cytoplasm of Vero cells labeled with [³H]thymidine from 2 to 18 h postinfection and digested with Bgl II restriction endonuclease. (B) Viral DNA extracted from nuclei of Vero cells labeled with [³H]thymidine from 2 to 18 h postinfection and digested with Bgl II restriction endonuclease. (C) Rapidly sedimenting (>230S) viral DNA extracted from nuclei of Vero cells labeled with [³H]thymidine from 2 to 8 h postinfection and digested with EcoRI restriction endonuclease. The arrows point to the position of the bands containing 0.5 M terminal fragments in digests of nuclear viral DNA.*

cytoplasmic DNA. By contrast, all four 0.25 M fragments spanning the *baac* junction between the L and S components were increased in concentration in nuclear DNA relative to cytoplasmic DNA.

Finally, in the last series of experiments, infected cells were labeled with [³H]thymidine, as above, from 2 to 8 h postinfection. The DNA was then extracted from nuclei and cytoplasm and sedimented in sucrose density gradients. The DNA sedimenting at >230S was collected and centrifuged to equilibrium in CsCl density gradients. The DNA banding at the density of HSV DNA was then collected and digested with *Eco*RI restriction endonuclease. The profile of the radioactivity in the electrophoretically separated fragments are shown in Fig. 4C. Again, the results show that in fast sedimenting DNA the concentrations of the terminal fragments *Eco*RI J and K_1 and K_2 (Fig. 1) are decreased in concentration relative to the concentration of fragments in cytoplasmic DNA. It should be noted that the terminal fragment E in this digest cannot be resolved from 1.0 M fragments D, F, and G. Of the 0.25 M, only JK_1 and JK_2 are clearly resolved, and in the fast sedimenting DNA these are increased in concentration relative to the concentration of these fragments in cytoplasmic DNA.

DISCUSSION

The salient feature of the results is that the HSV DNA accumulating in the nuclei of infected Vero cells yielded upon digestion with restriction endonuclease a decreased concentration of 0.5 M terminal fragments and a corresponding increased concentration of 0.25 M fragments relative to the 1.0 M fragments in the

same DNA and relative to the fragments in unit length mature DNA extracted from cytoplasmic virions. In unit length DNA the 0.25 M fragments span the junction of L and S components (9). Therefore, in nuclear viral DNA the number of such junctions must be increased at the expense of terminal fragments. The results are therefore consistent with the hypothesis that viral DNA accumulating in nuclei is largely linked, L component to S component, and contains a decreased number of free ends relative to unit length DNA. The arrangements of DNA consistent with such results are unit length circles, and concatemers in which the unit length molecules are arranged head to tail. Although we observed a few circular forms of unit length molecules 100×10^6 in molecular weight, the sedimentation studies indicated the presence of DNA sedimenting at a rate greater than 230S, which is consistent with multiunit concatemers (15). The rapidly sedimenting DNA (>230S) analyzed in this study (Fig. 4C) also shows a decrease in terminal fragments and an increase in junction fragments.

The presence of head-to-tail, i.e., L-component- to S-component-linked, concatamers both early and late in infection, coupled with the observation (15) that during a short pulse labeled thymidine becomes incorporated predominantly into rapidly sedimenting DNA rather than unit length, 56S DNA (19), suggests that HSV DNA might be made by a rolling-circle type of replication.

A rolling-circle type of replication predicts continuous synthesis of one strand and discontinuous synthesis of the other. Single-stranded DNA larger than unit length DNA was observed (R. J. Jacob, L. S. Morse, and B. Roizman, manuscript in preparation). In addition, if the direction of continuous-strand synthesis is unique and all four arrangements of the DNA enter the replicative pool, it would be predicted that the intact strands would be complementary and capable of annealing to each other. If only one arrangement were capable of entering the replicative pool, it would be predicted that the sequences within the continuously synthesized strand would be unique and capable of self-annealing only at the reiterated sequences. It is of interest to note that an earlier study reported that the intact strands from virion DNA showed minimal self-annealing compared with total DNA (5).

The results presented in this paper exclude several alternative models of replication of HSV DNA. For example, if L and S components were replicated independently, it would be predicted that two concatemers would be formed, and that upon digestion with restriction endonuclease the L-component concatemer would yield a novel fragment consisting of joined L-terminal fragments (*Bgl* II J-F, 22.7×10^6 predicted molecular weight), whereas the S-component concatamer would yield a novel fragment consisting of joined S-terminal fragments (*Bgl* II H-L, 16.7×10^6 in predicted molecular weight). Alternatively, if DNA synthesis began at one end and extended to the other, as the finding of various size lariats in an earlier study predicted, we would also expect to see head-to-head fragments (*Bgl* I J-J, 19.4×10^6; *Bgl* I F-F, 26×10^6; *Bgl* I H-H, 21.6×10^6; and *Bgl* I L-L, 12.2×10^6). These fragments were not detected either by analyses of the DNA as presented in this study or in hybridizations of ^{32}P-labeled DNA to electrophoretically separated digests of nuclear DNA transferred to nitrocellulose blots (data not shown).

The finding that in nuclear DNA all terminal (0.5 M) fragments were decreased in concentration and that, conversely, all junction (0.25 M) fragments were increased in concentration has significant implications with regard to the probable arrangements of HSV DNA that are capable of producing infectious progeny. Briefly, as illustrated in Fig. 5, if only the P arrangement were able to replicate, and if the inversions of L and S components were a post-DNA synthesis event as suggested by the studies described in

TABLE 1. *Molar ratios of restriction endonuclease fragments in digests of nuclear and cytoplasmic HSV-1 DNA*

Bgl II DNA fragments[a]	Fraction cpm/fraction mol wt		Nuclear/ cytoplasmic DNA
	Nuclear	Cytoplasmic	
FH●	0.34	0.26	1.31
JH●	0.34	0.24	1.42
FL●	0.43	0.28	1.52
D	0.94	1.00	0.94
JL●	0.39	0.31	1.26
F○	0.27	0.57	0.48
G	1.09	1.08	1.01
H○	0.23	0.46	0.50
I	0.81	0.91	0.89
J○	0.33	0.50	0.66
K	1.09	1.00	1.09
L○	0.22	0.50	0.44
M	0.86	0.85	1.01
N	0.82	0.79	1.04
O	0.86	0.05	1.01
P	0.88	0.78	1.03

[a] Open circles, 0.5 M terminal fragments in unit length (cytoplasmic) virion DNA. Closed circles, 0.25 M junction fragments in unit length virion DNA.

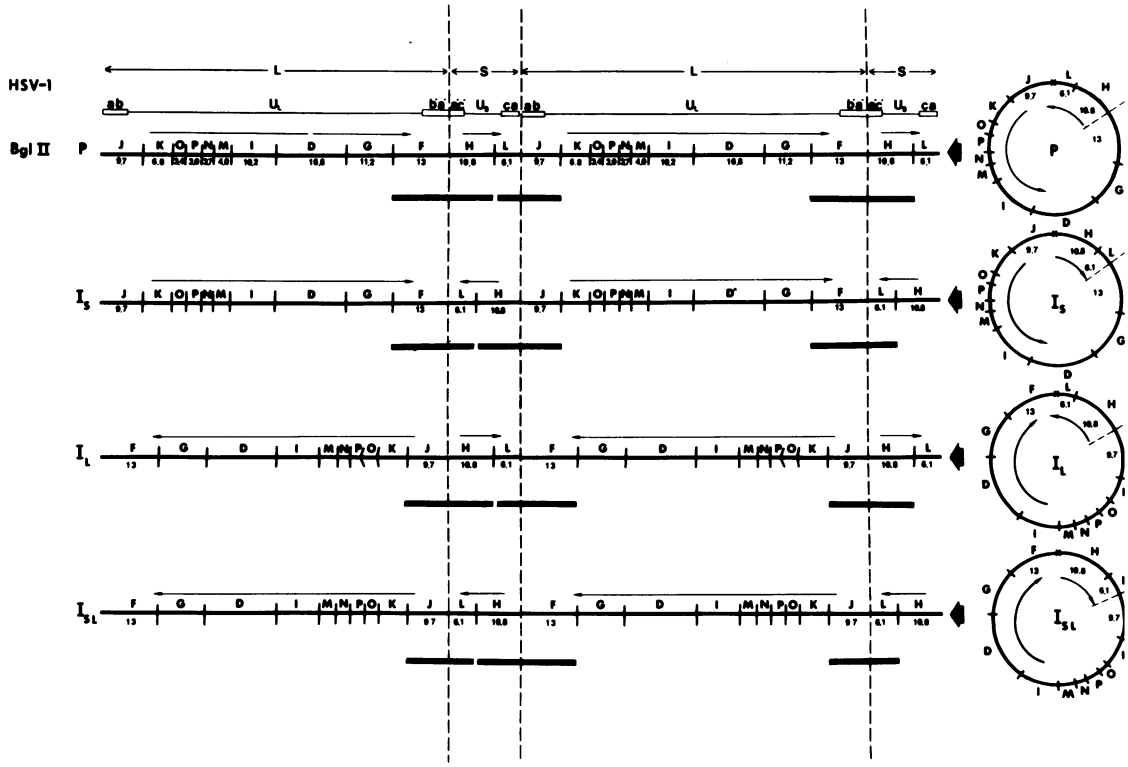

FIG. 5. *Sequence arrangements in HSV-1 DNA circles and concatemers that would be generated by a rolling-circle mechanism of DNA synthesis. The Bgl II restriction endonuclease maps (see Fig. 1) are shown in the P, I_S, I_L, and I_{SL} arrangements of HSV-1 DNA. The thick lines below the maps identify the DNA fragments spanning the junctions of L and S components that are present in concatemers and comigrate with the 0.25 M fragments present in unit length HSV-1 DNA extracted from virions. The diagram shows that each circle and concatemer contains two junction fragments, although the unit length linear molecule from which the circle and concatemer are generated contains only one junction fragment. Concatemers which arose from both the P and I_S arrangements would generate the four observed junction fragments. The diagram shows that at least two arrangements of the DNA (e.g., P and I_S) must enter the replicative pool and generate concatemers to account for the increased relative molar concentration of all four junction fragments in nuclear DNA.*

the introduction, it would be expected that only one type of concatemer would form and that only the junction fragments *Bgl* II F-H and *Bgl* II J-L present in that concatemer would be increased in concentration. The fact that all four junction fragments were increased in concentration implies that at the very least two arrangements can initiate replication and produce infectious progeny inasmuch as the concatemers of at least two arrangements must have been present to yield all four junction fragments. Although the results presented in this study are not inconsistent with the results of the analyses of the structure of recombinant DNAs (21), we have no evidence that only two arrangements of HSV DNA are actually reiterated head to tail in the concatemers.

The central feature of interest in the replication of HSV DNA is the mechanism by which one arrangement of HSV DNA generates all four arrangements found in virions. The evidence for the presence of head-to-tail concatemers of viral DNA is a key finding that, together with other data (8, 9, 20, 22, 25, 27, 28) on the structure of mature DNA, paves the way for a critical model that can be tested specifically as follows. (i) The simplest explanation for the finding of head-to-tail concatemers is that viral DNA circularizes and is then replicated by a rolling-circle type of replication. Although HSV DNA could circularize by blunt-end ligation as well as by cohesive-end ligation, the latter mechanism is supported by the presence of *a* sequences reiterated in the same orientation at the ends of the molecule (8, 25, 28) and by the observation that a substantial fraction of unit length DNA extracted from nuclei within the time span of viral DNA synthesis contains single-stranded ends (15, 16). It is con-

venient to assume that cohesive-end circularization in the cell requires the same amount of exposed terminal sequences as that required in vitro. We reported previously that the highest frequency of circularization is obtained when 0.25 to 0.50% of DNA defined as the *a* sequence is digested by 5′ lambda exonuclease (28). If the DNA circularizes in this fashion in the cell, it would follow that the circular molecule would contain two different L-S junctions. Although Wagner and Summers (29) have shown that the sequence arrangement at the L-S junction is heterogeneous in some HSV-1 strains, it is convenient to assume for the sake of simplicity that the unmodified junction present within unit length molecules consists of sequences *baac*, whereas at the closure of the circle the modified sequence is *bac* (Fig. 6). As a consequence, head-to-tail concatemers would contain the modified junction and, by extension, the missing *a* sequence would have to be regenerated during excision and repair of unit length DNA. Parenthetically, blunt-end-ligated circles would produce concatemers of unit length DNA containing two unmodified *baac* junctions. This alternative model requires that the excision of unit length DNA from the concatemer occur at every other *baac* junction. Such discrimination of *baac* junctions could only be accomplished if cleavage were to occur during packaging, but this mechanism would preclude accumulation and reentry of unit length DNA in the replicat-

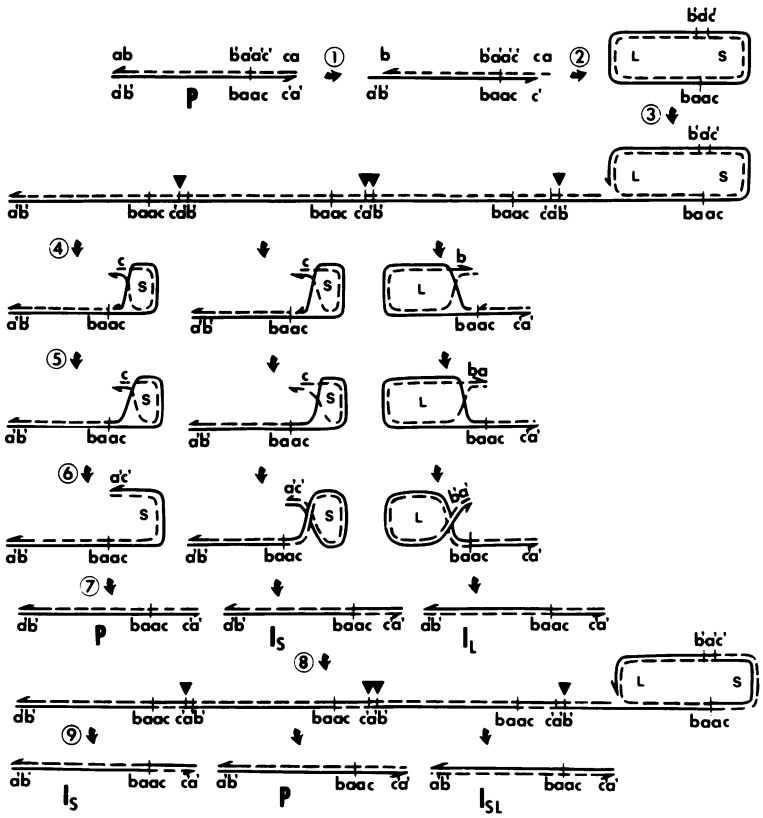

FIG. 6. *Rolling-circle model for the replication of HSV DNA. This model embodies the features discussed in the text and attempts to fulfill the requirements that one arrangement of the DNA gives rise to all four arrangements. The model envisions that after infection HSV DNA is digested by a processive exonuclease (step 1) exposing cohesive single-stranded ends. This enables the DNA to circularize (step 2), forming a modified junction designated as bac which differs from the internal junction b′a′a′c′ in the absence of an a sequence. The DNA is then replicated (step 3) unidirectionally by a rolling-circle mechanism. The resulting concatemer is cleaved into unit size DNA only at the bac junction to either the left or right of the a sequence as indicated by the filled inverted triangles. Steps 4 through 7 are diagrammatic representations of the regeneration of the missing a sequence. These steps are shown in three different columns to demonstrate how the replacement of these sequences could lead to the production of progeny in P (left column), I_S (center column), and I_L (right column) arrangements. Replication of I_S progeny (steps 8 and 9) would lead to the production of progeny I_S, P, and I_{SL} arrangements by the mechanism outlined in steps 4 through 6.*

ing DNA pool. Available data indicate that this is not the case (18).

(ii) As illustrated diagrammatically in Fig. 6, the *a* sequence could be regenerated by using the internal inverted repeat as a template. This mechanism was proposed by Heumann (10) for replacement of terminal RNA primer sequences by deoxynucleotide sequences. The necessary steps would be cleavage of the concatemers at the modified junction, introduction of a nick or a gap within the *baac* junction, displacement of the strand on one side of the interruption, branch migration allowing annealing of the residual terminal reiterated sequences in one strand to the complementary copy of the internal inverted repeat on the same strand, and, finally, elongation of the interrupted strand to fill the gap.

The structure of the DNA within this complex after repair would resemble a lariat consisting of a loop with a long and a short double-stranded stem (step 4, Fig. 6). To regenerate the linear form, the *baac* junction would have to be nicked. Branched migration of the repaired strand after nicking would regenerate the same arrangement of L and S components as in the molecule that served as a template for the replication of the DNA. Thus, if a P arrangement of HSV DNA initiated the replication process and the cleavage of the concatemer occurred between the *a* and *c* sequences in the *bac* junction, cleavage and branch migration of the repaired strand at the L-S junction (step 6, left column, Fig. 6) would regenerate the P arrangement. However, cleavage and branch migration of the template strand and branch migration of its complement, as shown diagrammatically (step 6, middle column, Fig. 6), would generate the I_S arrangement. Similarly, cleavage of the concatemer between *b* and *a* sequences at the *bac* junction would ultimately generate the P or the I_L arrangement.

(iii) The generation of the I_{SL} arrangement could follow two pathways. The first assumes, as was discussed in the preceding section, that the I_S arrangement is capable of replication. As illustrated diagrammatically, regeneration of the *a* sequence after cleavage of an I_S concatemer would lead to the production of P, I_S, and I_{SL} arrangements. The second pathway, not shown, would arise if the cleavage of the concatemer would occur within the *a* sequence or on both sides of the *a* sequence within the *bac* junction. If this were the case, all or part of the *a* sequence at both ends of the molecule would have to be regenerated. Simultaneous repair of both termini using the internal *baac* junction as a template would generate a circle with two *baac* junctions. Random cleavage of one *baac* junction within a circle produced from a P-arrangement concatemer would yield either the P or the I_{SL} arrangement.

Three lines of evidence support the hypothesis that the terminal sequences are regenerated by repair-mediated synthesis utilizing the internal inverted repeat as a template. First, the model calls for interruptions of the *baac* junctions. As reported elsewhere (15), single-stranded sequences were observed not only at the termini but also internally at a distance from one end of the molecule corresponding to the position of the *baac* junction. The results of those studies suggest the existence within infected cells of a single-strand-specific nucleolytic enzyme initiating digestion at the *a* sequence but acting only on a double-stranded DNA—a prediction that should be fullfilled if this model is correct. Second, if repair does occur, it would be predicted that the terminal regions of the *a'c'* and *ca* sequences as well as of the *ab* and *b'a'* sequences would be obligatorily identical. As described elsewhere (20), this has been demonstrated in the case of terminal regions of the *a'c'* and *ca* sequences in the sense that nucleotide substitutions or insertions in the distal (nearest to the *a* sequence) portion of one reiterated region of the S component become reflected in the reiterated sequence at the other end. Preliminary evidence suggests that regions of obligatory identity are also present in distal portions of the reiterated sequences of the L component (D. M. Knipe, W. T. Ruyechan, R. W. Honess, and B. Roizman, manuscript in preparation). Lastly, we reported the presence of lariats within nuclei of infected cells (15). It remains to be shown whether the structure of the lariats and the size ranges of the loops correspond to those predicted by this model.

This model does not incorporate features that would discriminate any arrangement of HSV DNA from entering the replicative pool. However, it suggests that the generation of all four HSV DNA arrangements from one arrangement requires generation and reentry of another arrangement of HSV DNA into the replicative pool. Our preference for the pathway illustrated in Fig. 6 for the generation of I_{SL} arrangement starting with the P arrangement stems from two considerations. First, analyses of the restriction endonuclease fragments spanning the *baac* junction are consistent with the results showing that the concatemers consist of head-to-tail concatemers of at least two arrangements of HSV DNA. This hypothesis implies that all four arrangements need not be generated immediately after the first round of replication. Second, generation of the I_{SL} arrangement from the P arrangement

by simultaneous repair of both ends would generate a circle with identical *baac* junctions. To generate the I_{SL} arrangement, it would be necessary to postulate an enzyme that could cleave either *baac* junction but only once within the circle.

This model of viral DNA replication is consistent with many of the known features of viral DNA structure and synthesis, but is probably not unique. Further studies of the many features predicted by this model will determine its heuristic usefulness for the generation and discrimination of alternative models.

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