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A Noninverting Genome of a Viable Herpes Simplex Virus 1: Presence of Head-to-Tail Linkages in Packaged Genomes and Requirements for Circularization After Infection

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The wild-type herpes simplex virus 1 genome consists of two components, L and S, which invert relative to each other, giving rise to four isomers. Previously we reported the construction of a herpes simplex virus 1 genome, HSV-1(F)I358, from which 15 kilobase pairs of DNA spanning the junction between L and S components were deleted and which no longer inverted (Poffenberger et al., Proc. Natl. Acad. Sci. U.S.A. 80:2690–2694, 1983). Further studies on the structure of HSV-1(F)I358 revealed the presence of two submolar populations among packaged DNA. The first, comprising no more than 10% of total packaged DNA, consisted of defective genomes with a subunit size of 36 kilobase pairs. The results suggest that this population arose by recombination through a directly repeated sequence inserted in place of the deleted L-S junction. The second minor population consisted of HSV-1(F)I358 DNA linked head-to-tail. Analyses of the structure of HSV-1(F)I358 DNA after infection indicated that the fraction of total DNA linked head-to-tail increased to approximately 40 to 50% within 30 min after exposure of cells to virus. The formation of head-to-tail linkages did not require de novo protein synthesis. Our interpretation of the results is that the termini of full-length DNA molecules are held together during packaging, that a small fraction of the termini is covalently linked during or after packaging, and that the remainder is covalently joined after the release of viral DNA from the infecting virus by either host or viral factors introduced into the cell during infection.

In a preceding paper, we reported on the isolation of a herpes simplex virus 1 (HSV-1) recombinant, designated as HSV-1(F)I358 (I358), whose DNA is frozen in a single-sequence arrangement. In this paper, we report on additional properties of I358 DNA and particularly the requirements for the circularization of HSV-1 DNA after infection. Relevant to this report are the following.

(i) HSV-1 DNA, approximately 150 kilobase pairs (kbp) in length, consists of two covalently linked components, L and S, containing 82 and 18% of the total DNA, respectively (29, 36). Each component consists of unique sequences (U_l and U_s) flanked by inverted repeated sequences. The inverted repeats of the L component have been designated *ab* and *b'a'*, and each contain approximately 9 kbp, whereas those of the S component, designated *a'c'* and *ca*, each contain 6.5 kbp (36). The shared sequence *a*, approximately 500 base pairs (bp) in HSV-1 strain F [HSV-1(F)], is present in one copy at the S component terminus and in one to numerous copies at the L component terminus and at the junction between L and S components (16, 37). A characteristic of HSV-1 DNA is that the L and S components invert (2, 7). As a consequence, viral DNA extracted from virions or from infected cells consists of four equimolar populations that differ in the relative orientation of the two components. One specific orientation was designated P (prototype) and the others were designated I_l, I_s, and I_{sl}, corresponding to the inversion of L, S, and both S and L components, respectively (22). Operationally, the inversions in HSV-1 DNA are readily manifest in DNA digests with restriction endonucleases which do not cleave within the reiterated sequences (e.g., *Bgl*II). Such digests contain four terminal fragments (0.5 M each) and four fragments spanning the junction between the S and L components (0.25 M each) with respect to the molarity of the remaining DNA fragments (7).

(ii) Studies on the mechanisms of inversion of the L and S components revealed that the *a* sequence can act as a *cis*-acting, site-specific inversion site in that insertion of *a* sequences in other regions of the genome causes any DNA segment flanked by inverted repeats of the *a* sequence to invert (18, 19, 30). Moreover, inversions appear to be caused by *trans*-acting factors present in infected but not uninfected cells (20). The physiological requirement of the reproductive cycle that is satisfied by the inversion of the L and S components is not known and is not universal for all herpesviruses. Thus, only two of the five known herpesvirus DNA structures contain components which invert (for a review, see reference 27). In a preceding paper, we reported the isolation of a recombinant in which most of the internal inverted repeat including the *a* sequence was replaced by approximately 3.5 kbp of HSV-1 DNA that included the thymidine kinase (TK) gene (23). I358 DNA is frozen in one (P) arrangement; i.e., it contains only two termini. This report describes further studies on its DNA.

(iii) Replicating HSV-1 DNA forms head-to-tail concatemers, or circles, as deduced by the observation that terminal fragments decrease in abundance, whereas the molarity of junction fragments increases relative to that present in packaged DNA (9). Several lines of evidence suggest that HSV-1 DNA circularizes after infection (1, 9, 11, 15) but the requirements for circularization are not known because only a small fraction of viral DNA brought into cells during infection is replicated (10) and all of the head-to-tail junctions that would be formed during circularization are already present in the four-unit-length isomers of HSV-1 DNA brought into the cell during infection. However, because I358 DNA is frozen in one arrangement, it is ideally suited for studies on the requirements for circularization of DNA

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after infection. We report here that a small fraction of I358 DNA may be packaged as circular DNA, that the fraction of circular DNA increases after infection, and that circularization after infection occurs in the absence of de novo protein synthesis.

MATERIALS AND METHODS

Cells and viruses. The procedures for preparation and titration of virus stocks in HEp2 cells and the properties of HSV-1(F) have been described elsewhere (3, 28). HSV-1(F)Δ305 contains a 700-bp deletion in the TK gene (25). I358 virus is a TK^+ derivative of HSV-1(F)Δ305 (23). Standard virus stocks were prepared from cells infected at a multiplicity of 0.01 PFU per cell. To enrich the defective-genome population (5), the I358 virus was passaged five times at a multiplicity of 100 PFU per cell.

Plasmids. The cloned *Bam*HI fragments of HSV-1(F) in pBR322 have been described previously (24). pRB111, pRB114, and pRB124 contain *Bam*HI-E, *Bam*HI-N, and *Bam*HI-X, respectively. pRB209 is a pBR322-derived plasmid carrying HSV-1(F) *Hin*dIII fragment O (23). pRB3119, a pUC9-derived plasmid (34) carrying 550 bp containing a complete HSV-(F) *a* sequence, will be described elsewhere (J. Chou and B. Roizman, manuscript in preparation). pRB408 carries in the pKC7 vector the *Sac*I-*Bgl*II subfragment of *Bam*HI-Q of HSV-1(F). This fragment is contained within the domain of the TK gene and has been deleted from HSV-1(F)Δ305.

Extraction and purification of DNA. The procedures for preparation of plasmid DNAs have been described elsewhere (24).

The procedures for preparation of viral DNA varied according to requirements. Specifically:

(i) For sequence analyses, viral DNA was purified by equilibrium centrifugation in NaI density gradients. At approximately 36 h postinfection with 0.01 PFU per cell, Vero cells were gently disrupted at 4°C by hypotonic swelling in TE buffer (10 mM Tris, 1 mM EDTA) followed by addition of Nonidet P-40 to 0.5%. Nuclei and cytoplasm were separated by low-speed centrifugation. The nuclei were resuspended in TE buffer, and both fractions were separately digested at 45°C for 2 h with 300 to 500 μg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml in the presence of 0.5% sodium dodecyl sulfate. The extracted DNA was centrifuged to equilibrium in NaI density gradients (10, 38).

(ii) Uniformly labeled DNA was obtained from infected Vero cells overlaid with $^{32}P_i$ (New England Nuclear Corp., Boston, Mass.). Flask (25 cm^2) cultures of Vero cells (4×10^6 cells) were infected at either 100 or 0.1 PFU per cell. At 5 h postinfection, the cultures were overlaid with phosphate-free medium containing 30 μCi of $^{32}P_i$ per ml of medium. Cells were harvested at 20 to 24 h postinfection, and cytoplasm and nuclei were separated as described above. DNA was then isolated by proteinase K digestion, phenol extraction, and ethanol precipitation (18).

DNA from nucleocapsids was isolated by digesting nuclei with pancreatic DNase I (100 μg/ml; Worthington Diagnostics, Freehold, N.J.) for 15 min at 37°C before proteinase K-sodium dodecyl sulfate treatment.

(iii) For examination of the amounts and sequence arrangements of viral DNA at times early after infection, flask (25 cm^2) cultures of Vero cells were infected with 40 PFU per cell and harvested at stated times (see Fig. 5) postinfection. Nuclei were separated from the cytoplasm and extracted as described above.

To prevent de novo protein synthesis, cells were pretreated for 2 h before infection and throughout adsorption and postinfection with 50 μg of cycloheximide (Calbiochem-Behring Corp., La Jolla, Calif.) per ml (8). For prevention of viral DNA replication, phosphonoacetate (PAA) (Abbott Laboratories, North Chicago, Ill.) was added throughout adsorption and infection to a final concentration of 300 μg/ml (12, 17).

Hybridization to immobilized restriction fragments. *Bgl*II (New England Biolabs, Beverly, Mass.)-digested DNAs were subjected to electrophoresis on 0.6% agarose gels and transferred to nitrocellulose sheets (31) as described previously (20). Hybridization of labeled probes was done as described previously (24), except nitrocellulose strips containing digests of total cell DNA were preincubated with 100 μg of salmon sperm DNA (Sigma Chemical Co., St. Louis, Mo.) per ml before hybridization with labeled probes. The DNA probes were labeled by nick-translation (26) with [α-^{32}P]dCTP (kit from New England Nuclear Corp.).

When subfragments of plasmids were used as probes, the digested DNAs were separated by electrophoresis on low-melt agarose (FMC Corp., Rockland, Maine), the desired bands were cut out, and DNA was extracted by the glass powder technique (33). Otherwise, the entire plasmid was nick-translated.

RESULTS

Fine structure of I358 DNA: objectives and experimental design. The I358 recombinant virus was selected from the progeny of transfection of rabbit skin cells with intact HSV-1(F)Δ305 DNA and a DNA construct consisting of the coding sequences of the TK gene fused to the promoter regulatory domain of the α4 gene and inserted by blunt-end ligation into the *Bgl*II cleavage site within HSV-1(F) *Hin*dIII fragment O (23). Analyses of I358 DNA revealed that the DNA construct became inserted by recombination through the homologous promoter regulatory domain of the α4 gene. The TK coding sequences and the left portion of *Hin*dIII-O recombined into I358, and, concurrently or sequentially, the internal inverted repeat sequence to the left of the promoter regulatory domain of the α4 gene became deleted (Fig. 1). The sequence to the left of the inverted repeats that was retained in the I358 recombinant is the left terminal region of *Bam*HI-B (23). I358 DNA is frozen in the P arrangement, and, in contrast to wild-type DNA, which contains four termini and four L-S component junctions, it contains only two termini (i.e., *Bgl*II-J and -L) and only one L-S junction, each present in molar concentrations relative to the molarity of the DNA. In a preceding report (23) describing the derivation of the I358 virus, the DNA fragment containing the new molar L-S component junction was designated fragment 2 (i.e., *Bgl*II fragment 2). This section of the results concerns the identity of the submolar fragments present in digests of I358 DNA extracted from capsids accumulating in the cytoplasm of infected cells.

In the preceding report, one of these submolar fragments, designated fragment 1, was observed in digests with all enzymes tested except *Bgl*II. The DNA contained in this band was present at a concentration of approximately 10% of that of the major DNA species and remained at the same relative proportion during serial propagation. Several attempts were made to separate this submolar population from the major population by plaque purification. However, the minor population was present in a stable ratio relative to the major population, even after nine serial passages at low multiplicities of virus isolated from a single plaque. This

virus stock was also used in passages through mouse brain to take advantage of the natural selection systems in this host. Seven passages through mouse brain did not separate the major and minor populations. These observations led to the hypothesis that the minor population represents a defective genome which arises rapidly and is maintained by the helper virus at relatively stable ratios when the virus is passaged serially at low multiplicities.

The second minor population became apparent in digests with enzymes that do not cleave wild-type DNA within the inverted repeat sequences. Analyses of this fragment led to the hypothesis that it consists of a junction of the two termini of I358 DNA, i.e., that a small fraction of I358 DNA packaged in virions is joined head-to-tail.

To test these hypotheses, we probed I358 and parental virus [HSV-1(F) and HSV-1(F)Δ305] DNAs in a series of Southern hybridizations with several ^{32}P-labeled HSV-1 DNA fragments. The probes (Fig. 2 and Table 1) were selected to identify the regions of the I358 genome represented in the putative defective genome and to differentiate between these sequences and those of fragment 2 containing the unique L-S component junction of I358 DNA.

Structure of submolar fragment 1. Our hypothesis is that the submolar fragments, designated below as 1a and 1b, are derived from a defective genome which arose by recombination-deletion through the duplicated portions of *Hin*dIII-O DNA (Fig. 1). As previously reported, the molar *Bgl*II fragment spanning the new L-S junction in I358 DNA (fragment 2, 24 kbp in length) hybridizes with *Bam*HI-Q (probe B) and with *Hin*dIII-O (probe F) but does not contain *a* sequences and therefore does not hybridize with probe C. The schematic representation of the putative defective genome (Fig. 1) predicts three *Bgl*II fragments of 27 (fragment 1b), 36 (fragment 1a), and 9 kbp in length. The 27-kbp fragment would be the left terminal fragment of the defective genome monomer, the 36-kbp fragment would span two monomers, and the 9-kbp fragment would be identical in sequence to *Bgl*II-L and therefore could not be differentiated from the corresponding fragment in I358 standard DNA. Fragments 1a and 1b would be predicted to be unique both in size and sequence composition and to hybridize to sequences encoding the TK gene (probe B), sequences comprising the left *Bgl*II portion of *Hin*dIII-O (probe G), sequences at the terminus of the L component (probe A), the *a* sequence (probe C), and the S component sequences adjacent to the deleted portion of the internal inverted repeat (probe D). They could be differentiated only with probes containing sequences from the terminus of the S component (probe E).

The results (Fig. 3) indicate the following.

(i) The bands designated 1a and 1b reacted with probes A (lanes 16 and 17), B (lane 2), C (lanes 12 and 14), F (lanes 4 and 6), and D (lane 10), but not with probe G (lane 8). The diagram shown in Fig. 1 predicts that if the defective genome arises by recombination-deletion through the directly repeated left portions of *Hin*dIII-O, the left portion of *Hin*dIII-O (probe F) would be present and the right portion of *Hin*dIII-O (probe G) would be absent, as has been observed. Previous studies (23) on the common submolar *Kpn*I subfragment of fragments 1a and 1b containing the TK

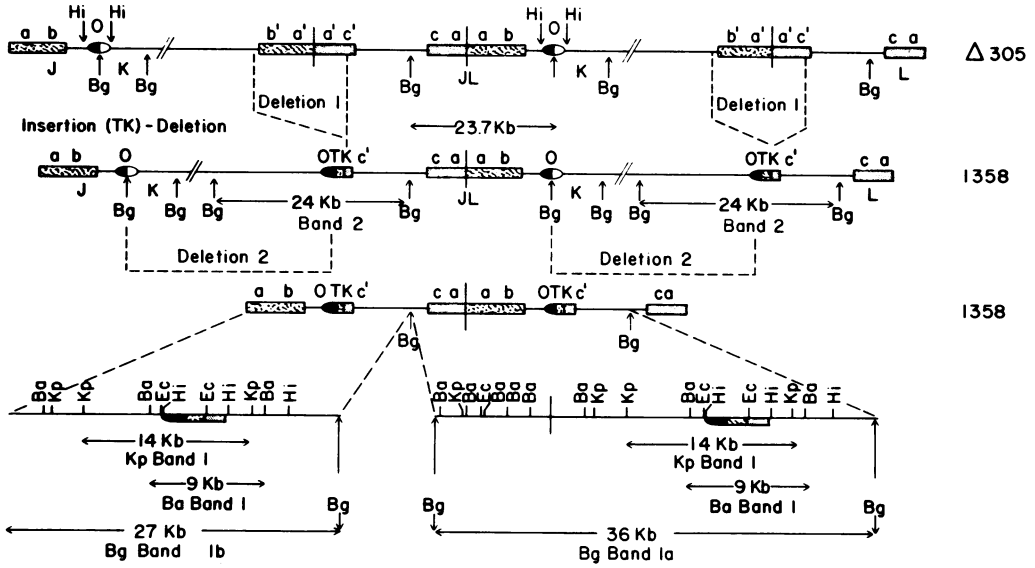

FIG. 1. Schematic representations of the parent HSV-1(F)Δ305 genome and the I358 standard and defective genomes. To facilitate the representation of head-to-tail junctions in I358 DNA and of the putative derivation and structure of I358 defective genomes, parental HSV-1(F)Δ305 DNA (top line) and I358 DNA (second line) are shown as head-to-tail dimers. The dashed lines connecting the first and second lines indicate the portions of parental DNA replaced in HSV-1(F)Δ305 DNA with the left portion of *Hin*dIII-O (filled half oval) fused to the TK gene. The right portion of the *Hin*dIII fragment is shown as a half-open oval. The portion of I358 DNA which is deleted from the defective genome shown in the third line is indicated by the dashed lines. The bottom line is a restriction endonuclease map of the defective genome. The top three lines are drawn to scale relative to each other. The relevant *Bgl*II cleavage sites are shown, and the *Bgl*II terminal fragments J and L are identified. *Bgl*II-K is next to *Bgl*II-J and contains the right 1.2 kbp of DNA from *Hin*dIII-O. *Bgl*II fragment 2 spans the new L-S junction in I358. *Bgl*II-JL represents the head-to-tail junction of the prototype arrangement of parental DNA and the unique head-to-tail arrangement of I358 DNA. The bottom two lines show the two novel *Bgl*II fragments contributed by the defective genome and restriction sites within their sequences. The novel *Bam*HI (fragment 1) and *Kpn*I (fragment 1) fragments spanning the L-S junction and containing the TK gene in I358 DNA were previously described (23). Ba, *Bam*HI; Kp, *Kpn*I; Ec, *Eco*RI; Hi, *Hin*dIII; Bg, *Bgl*II; c', portion of the *c* sequence of the reiterated sequences of the S component (approximately 1,100 of a total of 6,500 bp) remaining at the junction between the L and S components. The c' sequence contains at its left terminus the α4 promoter-regulatory domain fused to the TK gene.

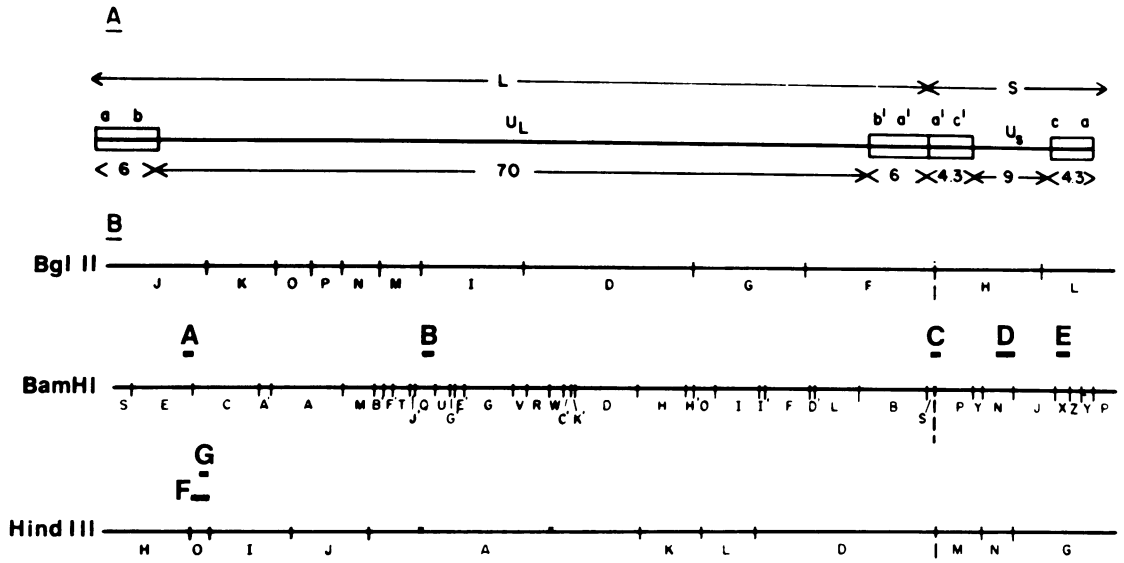

FIG. 2. Schematic diagram of the HSV-1(F) genome in P arrangement showing relevant restriction endonuclease maps and the location in the genome of the viral DNA sequences used as probes in Southern hybridization studies. All HSV sequences used as probes were cloned from HSV-1(F). Note that the head-to-tail *Hin*dIII and *Bgl*II junctions of DNA in P arrangement are HG and JL, respectively. (A) L and S components showing the *ab* and *ca* reiterated sequences; (B) *Bgl*II, *Bam*HI, and *Hin*dIII restriction endonuclease maps. The probe domains are described in the text and in Table 1.

gene sequences (Fig. 1) have shown that it does not contain the left terminus of *Bam*HI-B.

These results indicate that fragment 1 DNAs contained sequences from the left end of the standard DNA (probe A), the left *Hin*dIII-O (probe F), a novel TK L-S junction (probe B), and sequences from the S component (probe D). The absence of sequences from the right portion of *Hin*dIII-O (probe G) and from the left terminus of *Bam*HI-B located to the left of the TK gene in I358 standard DNA suggests that the sequences between the two directly repeated left portions of *Hin*dIII-O, i.e., between that present at its natural location and that inserted at the junction in I358, had been deleted. As predicted by the representation in Fig. 1, *Bgl*II-1a reacted with probe E (*Bam*HI-X), whereas *Bgl*II-1b did not (Fig. 3, lane 19).

(iii) If the submolar *Bgl*II fragments 1a and 1b were part of a defective genome, the monomer size would be 36 kbp. The packaged unit-length molecule would be predicted to consist of one left terminal, 27-kbp, *Bgl*II fragment 1b, three 36-kbp *Bgl*II fragments 1a spanning the junction between two monomers, and one right terminal fragment of 9 kbp in length (*Bgl*II-L), for a total of approximately 150 kbp, i.e., approximately the estimated size of HSV-1 DNA (13). This model predicts that fragment 1a would be three times more abundant than fragment 1b in packaged DNA. Because in nuclei the nonpackaged viral DNA is present largely in the form of head-to-tail concatemers, the ratio of fragments 1a to 1b should be even greater. Another prediction of the model is that the relative abundance of defective genomes should increase upon high-multiplicity passage (5). The results presented in Fig. 4 are consistent with these predictions. Specifically, *Bgl*II fragment 1a was much more prominent than fragment 1b in uniformly ^{32}P-labeled DNA prepared in cells infected with standard, low-multiplicity-passaged virus and extracted from either cytoplasmic virions or nuclear capsids after DNase treatment (Fig. 4, lanes 7 and 8). In the same preparations, fragment 1a was particularly prominent in nuclear DNA untreated with DNase and containing both the packaged and nonpackaged DNA.

The predicted amplification of the defective genomes is readily seen in uniformly ^{32}P-labeled DNA digests (Fig. 4, lanes 3, 4, and 5). Whereas the abundance of *Bgl*II fragments M, N, and O was the same or slightly lower than that in the low-passage DNA (Fig. 4, lanes 7 and 8), that of fragments 1a and 1b was very much higher. The conclusion that bands 1a and 1b are amplified by high-multiplicity passage is reinforced by the observation that, in packaged DNA (Fig. 4, lane 7) from stock derived by low-multiplicity passage, band 1a was barely discernible and band 1b was undetectable, whereas in packaged DNA (Fig. 4, lane 4) from stocks derived by high-multiplicity passage, both bands were prominent. It should be noted that others have observed that defective genomes consisting of large monomers tend to evolve upon high-multiplicity serial passage to defective genomes consisting of smaller monomers (14). This observation may well account for the numerous diffuse bands which form a grey background both in DNA extracted from cytoplasmic virions (Fig. 4, lane 3) and in DNA extracted from nuclear capsids after DNase digestion of unpackaged DNA (Fig. 4, lane 4). We conclude that the results of these

TABLE 1. Description of DNA probes used in analysis of I358 DNA

Probe	Description	Plasmid designation
A	Right *Xba*I/*Bam* subfragment of *Bam*HI-E (pRB111)	—[a]
B	*Sac*I-*Bgl*II fragment of *Bam*HI-Q	pRB408
C	550-bp fragment containing an *a* sequence	pRB3119
D	Right *Hin*dIII subfragment of *Bam*HI-N (pRB114)	—
E	*Bam*HI-X	pRB124
F	*Hin*dIII-O	pRB209
G	Right *Bgl*II-*Hin*dIII fragment of *Hin*dIII-O (pRB209)	—

[a] —, Purified by gel electrophoresis.

studies are consistent with the model (Fig. 1) predicting that the submolar DNA populations represented by *Bgl*II-1a and -1b represent a defective DNA population.

Presence of submolar head-to-tail junctions in I358 DNA. The evidence supporting the presence of submolar amounts of head-to-tail junctions in preparations of I358 DNA is as follows. A submolar band, designated *Bgl*II-JL, was detected in all preparations of I358 DNA extracted from cytoplasmic or nuclear capsids (Fig. 3, lanes 2, 6, 14, 16, 17, and 19; Fig. 5, lanes 1 and 9). This band comigrated with authentic *Bgl*II-JL formed by head-to-tail concatemers in nuclear DNA and with *Bgl*II-JL of the parent virus HSV-1(F)Δ305 DNA (e.g., Fig. 3, lanes 15, 16, and 17) and hybridized with probes C (*a* sequence), A (left terminus of the L component), and E (*Bam*HI-X) containing a sequence from the inverted repeats of wild-type DNA present only in a single copy at the S terminus in I358 DNA. As would be predicted, submolar *Bgl*II-JL of I358 DNA did not hybridize with probe G (right *Bgl*II subfragment of *Hin*dIII-O) or with probe B (TK gene). Although hybridization to the TK gene is difficult to interpret because of overlap with band 2, which does hybridize with TK, the previously published (23) hybridizations to *Kpn*I digests show that the submolar head-to-tail linked fragments do not hybridize with the TK probe. This JL band therefore contains sequences present at both termini of the standard I358 DNA.

Circularization of HSV-1 DNA after infection: requirements for formation of head-to-tail junctions in nuclei after infection. Consistent with the behavior of wild-type DNA, it would be expected that I358 DNA would form a head-to-tail junction before or during viral DNA synthesis. This is in fact the case, as seen from the presence of increased amounts of head-to-tail junctions (*Hin*dIII-HG, *Bgl*II-JL) in uniformly ^{32}P-labeled DNA extracted from nuclei of cells infected with low-multiplicity-passaged stocks (Fig. 4, lanes 1 and 6). The purpose of this series of experiments was to determine the requirements for the formation of head-to-tail junctions after infection. In these experiments, Vero cells were exposed to 40 PFU of I358 virus per cell in the presence or absence of cycloheximide or in the presence or absence of PAA, as described in Materials and Methods. At 30 min postexposure to the virus and at intervals thereafter, the cells were harvested and the DNA was extracted from the nuclear fractions, limit digested with *Bgl*II, electrophoretically separated in agarose gels, transferred to nitrocellulose sheets, and hybridized with probe A. The results were as follows.

(i) At 30 min postexposure of cells to I358, the amount of *Bgl*II-JL representing head-to-tail linkage of I358 DNA hybridizing to probe A was only slightly less than that of the *Bgl*II-J terminal fragment detected by the same probe (Fig. 5, lanes 3 and 11). The ratio of *Bgl*II-JL to *Bgl*II-J in the nuclear fraction at 30 min postexposure was significantly higher than that of the corresponding fragments in DNA extracted from cytoplasmic virions harvested at the end of a productive infection (Fig. 5, lanes 1 and 9) and corresponding to the infecting virus.

(ii) Cycloheximide had no effect on the ratio of *Bgl*II-JL to *Bgl*II-J at 30 min (lanes 2 and 10), 2 h (lane 4), or 8 h (not

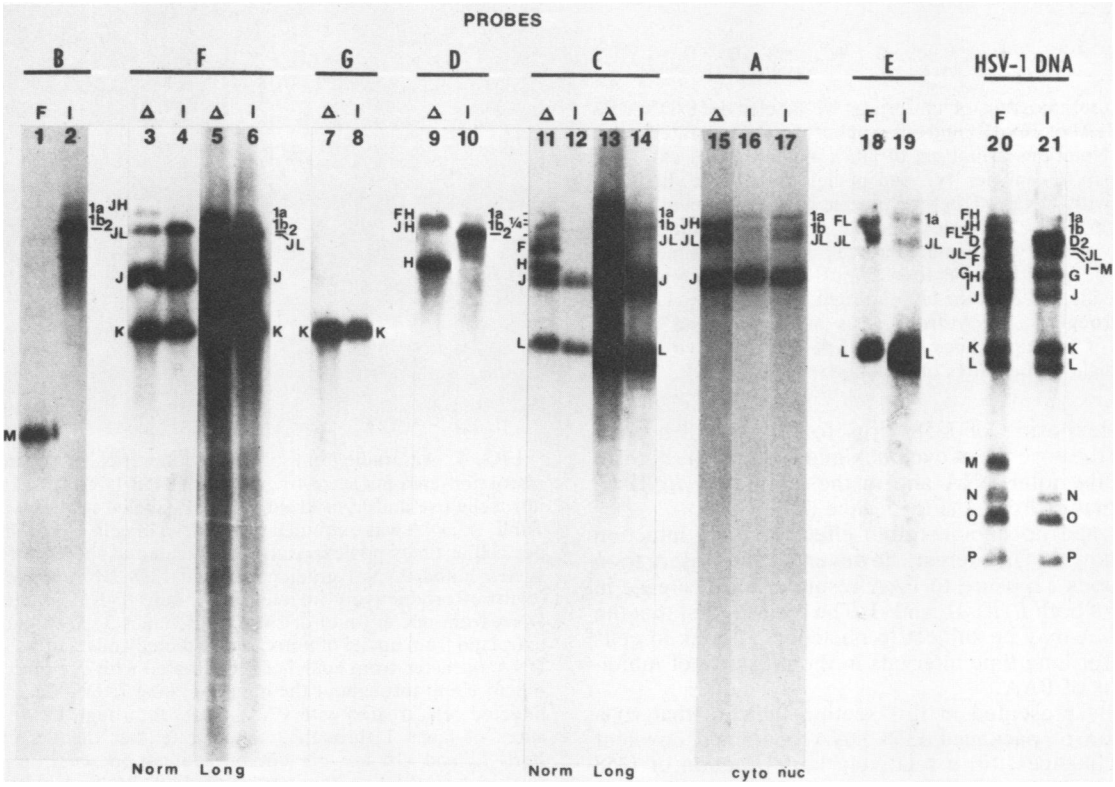

FIG. 3. Autoradiograms of *Bgl*II digests of HSV-1(F) (F), I358 (I), and HSV-1(F)Δ305 (Δ) DNAs electrophoretically separated on 0.6% agarose gels, transferred to a nitrocellulose sheet, and hybridized with probes A through G as described in the text and in Table 1 (lanes 1 through 19) or with a probe consisting of whole nick-translated HSV-1(F) DNA (lanes 20 and 21). Norm and Long, Normal and long-term autoradiographic exposures of the blots. All DNAs were from cytoplasm, except that in lanes 16 and 17, which show a direct comparison of DNAs extracted from cytoplasm (cyto) and nuclei (nuc) of the same infected cells.

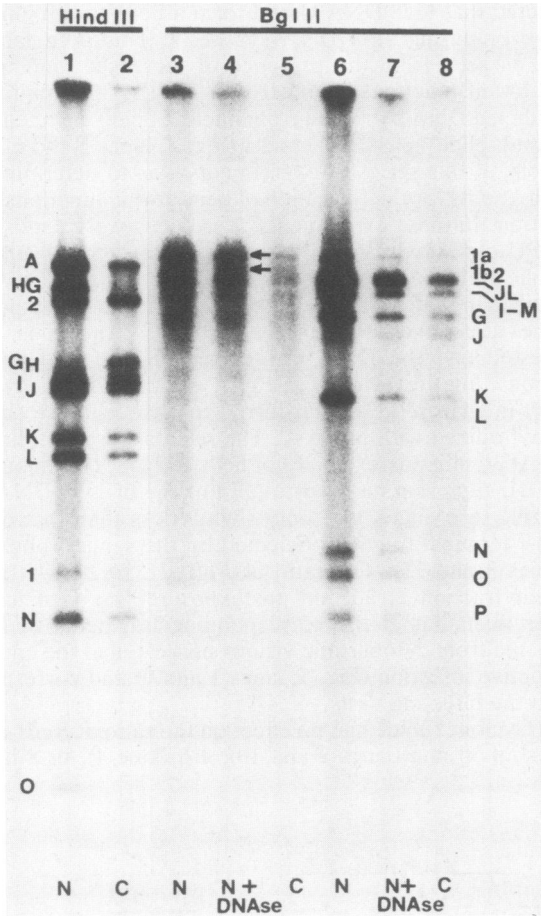

FIG. 4. Autoradiogram of uniformly ^{32}P-labeled I358 DNA digested with *Bgl*II or *Hin*dIII and electrophoretically separated on an agarose gel. Notations at bottom of lanes indicate DNA extracted from cytoplasm (C), nuclei (N), and nuclei which were disrupted and treated with DNase I before proteinase K treatment (N + DNase) to hydrolyze all DNA except that protected within capsids. Viral DNAs were prepared from Vero cells infected and incubated with $^{32}P_i$ as described in the text. *Hin*dIII and *Bgl*II restriction fragments are identified on the left and right, respectively. Lanes 1, 2, and 6 through 8, DNA from virus stock passaged at low multiplicities of infection; lanes 3 through 5, DNA from virus stock passaged at high multiplicities of infection.

shown) postexposure of I358 virus to cells. At 8 h postexposure in the absence of cycloheximide, there was both an increase in the total DNA and in the amount of *Bgl*II-JL relative to that of *Bgl*II-J (Fig. 5, lane 6).

(iii) PAA had no demonstrable effect early in infection (data not shown). Of interest, however, is the observation that continuous exposure to PAA resulted in a decrease in the amount of both *Bgl*II-JL and -J. The data suggest that the infecting DNA may be subject to nucleolytic attack in cells maintained for long time intervals in the presence of inhibitory amounts of PAA.

The results presented in this section indicate that (i) a small fraction of packaged I358 DNA contained covalent head-to-tail linkages, (ii) a relatively large fraction of I358 DNA formed head-to-tail linkages very early after infection, (iii) the ratio of covalently linked to unlinked DNA remained relatively stable for several hours, i.e., most of the linkages occurred immediately after infection, and (iv) the formation of these linkages did not require de novo protein synthesis.

DISCUSSION

The studies described in this report centered on the characterization of the submolar populations in I358 DNA and on the formation of head-to-tail linkages by I358 DNA after infection.

Defective genomes. The submolar population identified in these studies as fragments 1a and 1b was predicted to arise from recombination-deletion of all I358 DNA sequences located between the directly repeated portions of *Hin*dIII-O DNA. Consistent with these predictions, *Bgl*II fragments 1a and 1b hybridized with a spectrum of probes located to the left of *Hin*dIII-O at its natural location and to the right of the inserted, directly repeated portion of *Hin*dIII-O at the new junction between the L and S components. Thus, the predicted terminal fragment 1b contained sequences from *Bgl*II-J, including the *a* sequence, the left portion of *Hin*dIII-O, the TK gene inserted at the new L-S junction, and the unique sequences within *Bam*HI-N, whereas fragment 1a, predicted to span two defective genome monomers, contained, in addition, sequences from the terminal portion

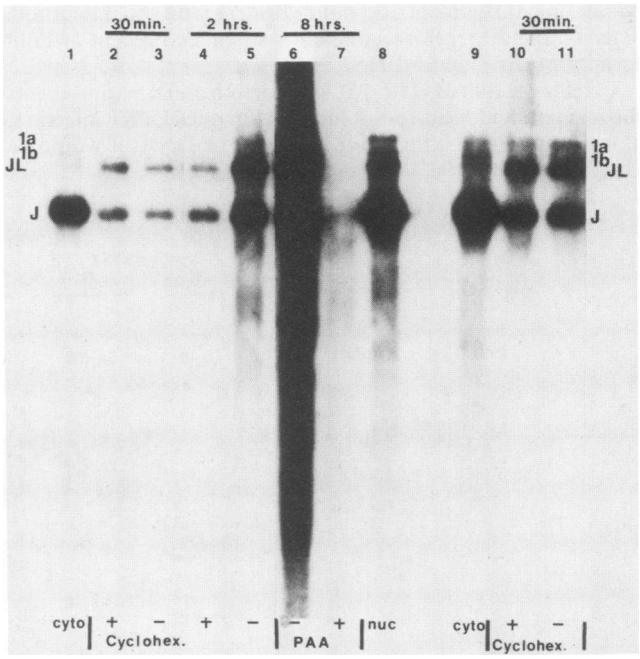

FIG. 5. Autoradiogram of electrophoretically separated *Bgl*II restriction endonuclease fragments of I358 DNAs immobilized on nitrocellulose and hybridized with ^{32}P-labeled probe A (Fig. 2 and Table 1). DNA was extracted from nuclei of cells exposed to 50 PFU per cell at times postexposure as indicated at the top of the figure. Lanes 1 and 9, NaI gradient-purified I358 DNA from cytoplasm (cyto) of productively infected cells; lane 8, NaI gradient-purified DNA from nuclei (nuc) of same cells; lanes 3, 5, 6, and 11, DNA extracted from nuclei of untreated, infected cells; lanes 2, 4, and 10, DNA extracted from nuclei of cells treated with cycloheximide for 2 h before and throughout the infection; lane 7, DNA extracted from infected cells treated with PAA. Lanes 9 through 11 are overexposures of lanes 1 through 3 to indicate that the defective bands *Bgl*II-1a and -1b are present but do not increase in abundance relative to *Bgl*II-J at 30 min postinfection. Probe A hybridized only with DNA fragments carrying the unique sequences immediately flanking the *ab* reiterated sequences at the terminus of the L component. Thus, only the *Bgl*II-J terminal fragment, *Bgl*II fragments 1a and 1b of the defective species, and the head-to-tail fragment (*Bgl*II-JL) of I358 DNA hybridized to probe A.

of the S component (*Bam*HI-X). Neither fragment 1a nor 1b hybridizes with the right portion of *Hin*dIII-O, as shown in this study, or to the left terminus of *Bam*HI-B, as previously reported. Consistent with the predictions, fragment 1a was presented in a higher abundance than fragment 1b, and both fragments increased in abundance relative to standard DNA upon serial passage at high multiplicities of infection.

Generation of defective genomes through recombination-deletion of directly repeated sequences has been observed previously. Smiley et al. (30) reported generation of defective genomes from HSV-1 DNA into which additional *a* sequences were inserted. The defective genomes appear to have arisen through recombination deletion of DNA flanked by direct repeats of the *a* sequence (30). The general rule appears to be that such defective genomes emerge in an abundance sufficient to be detected only if they are packaged, and therefore the monomer size must be an integral divisor of the approximate size of the unit length of HSV DNA. The size of the defective genome monomer of I358 DNA is 36 kbp; it would contain two origins of viral DNA replication from the S component (21), and the predicted size of the packaged defective genome would be close to that of the standard HSV-1 DNA (150 kbp). A significantly larger monomer would yield a unit-length molecule which would not package and hence would not be readily detected in stocks passaged serially at relatively low multiplicities. This prediction was, in fact, verified in stocks of an I358 derivative virus in which the TK gene was replaced by the genomic chicken ovalbumen gene (K. Poffenberger, M. Arsenakis, and B. Roizman, manuscript in preparation). The virus stocks prepared in exactly the same fashion as those of I358 virus lack the submolar fragments corresponding to *Bgl*II-1a and -1b.

Presence of head-to-tail junctions in I358 DNA and requirements for their formation after infection. The significance of the finding of head-to-tail linkages in I358 DNA and of the observation that these linkages form rapidly and in the absence of de novo protein synthesis centers on several considerations.

Comparison of the abundance of *Bgl*II-JL with that of its constituent terminal *Bgl*II-J indicates that, in I358 packaged DNA, the head-to-tail junction comprises 5% or less of the total available terminal fragments. As indicated in the introduction, the small amount of head-to-tail junction fragment observed in I358 DNA would not be detected in wild-type DNA preparations, inasmuch as wild-type DNA contains all of the possible head-to-tail junction fragments in much greater abundancies than that observed in I358 DNA. The problem, in essence, is that the structure of wild-type HSV-1 DNA does not permit authentication of packaging of a small amount of circular DNA. Although the absence of such authentication does not invalidate the observations of head-to-tail junctions in I358 DNA, it raises questions as to whether they are a unique property of I358 DNA imposed by its smaller size and the absence of internal inverted repeats or whether they are generally predictive for standard DNA as well. We cannot resolve these questions with certainty, except to point out that (i) the head-to-tail junction was present in packaged HSV-1 recombinant R3213, which was constructed from I358 by replacement of 500 bp with a 7.5-kb fragment carrying the ovalbumen gene (Poffenberger et al., manuscript in preparation), and (ii) a small amount of circular forms were detected in preparations of packaged varicella-zoster virus DNA (W. Ruyechan, personal communication). The DNA of this virus contains an internal inverted repeat of one of its termini. At least in this instance, the presence of an internal inverted repeat did not obviate the packaging of circular forms. Pending verification, the presence of head-to-tail junctions in I358 DNA may be predictive of small amounts of circular forms in packaged wild-type HSV-1 DNA.

The nearly 10-fold increase in the amount of head-to-tail junctions detected in nuclei of cells infected with I358 virus in both the presence and absence of cycloheximide indicates that the covalent head-to-tail linkage of infecting viral DNA reaches a maximum level in less than 30 min after exposure of the cells to virus and that the process does not require proteins made after the infection. These observations suggest that the termini of the DNA are available for ligation upon entry into the nucleus and that the ligation is carried out by either a host enzyme or by a viral protein brought into the cells during infection. These observations, together with the presence of head-to-tail junctions in packaged I358 DNA, the available information on the structure of HSV-1 termini (21), and the mode of replication and packaging of HSV-1 DNA (9, 10), suggest the following sequence of events.

(i) Late in infection, HSV-1 DNA accumulates as head-to-tail concatemers (9) which must be cleaved within directly repeated *a* sequences located at unit-length distances from each other (21, 35). The unit-length molecules are then packaged into capsids and form torroidal structures within the core (6). Genetic and biochemical studies on the packaging of pseudorabiesvirus DNA suggest that cleavage of the unit length occurs during and is linked to packaging (15). DNA lacking an *a* sequence is not packaged (32; R. R. Spaete and N. Frenkel, Proc. Natl. Acad. Sci. U.S.A., in press). The model predicts, therefore, that packaging is initiated from the concatemer itself or an appropriate free end by recognition of an *a* sequence by a packaging protein and that the viral DNA is drawn into the preformed capsid until it encounters the next directly repeated *a* sequence. In this respect, the packaging of HSV-1 DNA would resemble in its general features that proposed for bacteriophage λ (4).

(ii) Previous studies (21) from this laboratory have shown that the HSV-1(F) *a* sequence has a characteristic structure consisting of a 20-bp direct repeat (DR1), a 64-bp unique sequence (Ub), a 12-bp sequence repeated 19 to 22 times (DR2), a 37-bp sequence repeated 2 to 3 times (DR4), a 59-bp unique sequence (Uc), and a terminal DR1. The S component terminal *a* sequence ends with only 1 bp and a single 3′ nucleotide extension of the DR1, whereas the terminal *a* sequence of the L component ends with 18 bp and a single 3′ nucleotide extension of the DR1 such that when the two portions of the DR1 are placed together, they form a complete 20-bp DR1 sequence. These results suggest that the cleavage of the concatemer to yield a unit-length HSV-1 DNA molecule occurs across two juxtaposed DR1 sequences such that the S component terminus of HSV-1 DNA contains only one *a* sequence, whereas the L component terminus contains at least one *a* sequence (21). Recent studies (N. Frenkel, L. Deiss, and R. R. Spaete, ICN-UCLA Symp. Mol. Cell. Biol., in press; L. Deiss and N. Frenkel, manuscript in preparation) indicate that this requirement necessitates amplification of *a* sequences in the case of defective genome monomers containing single *a* sequences.

(iii) The results presented in this paper suggest that the cleaved termini of packaged HSV-1 DNA are held together. This conclusion hinges on the evidence that *a* sequences are required for packaging of HSV DNA (32; Spaete and Frenkel, in press), i.e., that packaging is not initiated at random sites, and rests on the observations that the head-to-tail

linkages form very rapidly after infection and are already present in small amounts in packaged DNA. Similar conclusions have been reached by Deiss and Frenkel (manuscript in preparation) on the basis of studies on the requirements for packaging of defective genomes. Maintenance of juxtaposed termini very likely requires proteins bound to the *a* sequences. Tightly but noncovalently bound proteins at the termini of HSV-1 DNA have been reported (39). Although the function of these proteins is not known, such proteins could be predicted to hold the termini together to facilitate ligation and protect the DNA from exonucleolytic attack, to which it may be subjected in the infected cell, as has been reported (10, 11).

The contribution of this paper to this model is the demonstration of a small amount of head-to-tail junctions in packaged DNA and in the rapid formation of head-to-tail junctions after infection. These observations would not have been possible on standard DNA, in which the inversion of the L and S components generates all of the possible head-to-tail junctions in abundant amounts. Further studies on the state of the termini and the unique head-to-tail junction generated by I358 DNA may reveal the nature of the linkages between the termini in packaged I358 DNA and help identify the activities that modify the DNA early in infection.

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