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Structure of Replicating Herpes Simplex Virus DNA

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We have investigated the molecular anatomy of the herpes simplex virus replicative intermediates by cleavage with the restriction endonuclease *Bgl*II. We find that in populations of multiply infected cells, pulse-labeled replicating herpes simplex virus DNA contains at least two and probably all four sequence isomers. Also, it contains no detectable termini. In pulse-chase experiments, we show that endless replicative intermediates are the precursors to virion DNA and that maturation is a relatively slow process. The results are discussed in terms of their significance to possible models of herpes simplex virus DNA replication.

Herpes simplex virus type 1 (HSV-1) has a linear double-stranded DNA genome with a molecular weight of about 10^8 (18). The peculiar sequence organization of HSV-1 DNA has been extensively reviewed (21, 22) and can be summarized as follows: two unique sequence regions, U_L and U_S, are both bracketed by inverted repeats, labeled ab/b'a' and ac/c'a', respectively, generating the general structure ab U_L b'a'c' U_S ca (9, 23). The short (300- to 400-base-pair) "a" sequence is directly repeated at both ends of the genome (as well as internally), thus allowing its circularization after a limited exonucleolytic digestion (9, 23). The long (L) and short (S) regions of the genome are found in all four possible relative orientations, designated P (prototype), I_S (inversion of S), I_L (inversion of L), and I_{SL} (10). All four isomers are found in approximately equimolar concentrations in DNA extracted from virions, even after a recent plaque purification. Coupled with the observations that HSV plaques with single-hit kinetics (13) and that one viral particle cannot contain more than one DNA molecule (8), this implies that all four isomers can be generated from a single parental form during replication.

Data about viral DNA replication are still scanty. Several electron microscopy studies (7, 11, 16) have provided very little evidence as to the mode of DNA replication, probably due to the large size and fragility of the viral replicative intermediates. The sedimentation rate of replicating viral DNA is drastically different from that of parental genomes: it varies from slightly more rapid at early times p.i. to extremely rapid (>200S) at the peak of DNA synthesis (1, 12, 16). The latter fact led to the suggestion that herpesvirus replicative intermediates are concatemeric (1, 16). From a restriction endonuclease analysis of the viral DNA accumulating in infected cell nuclei, Jacob et al. (15) concluded that the replicative intermediates were head-to-tail concatemers. On the basis of this observation, they proposed a rolling circle model for viral DNA replication.

Additional data relevant to the present study should be mentioned: (i) analysis of the genomes of recombinants formed in HSV-1 × HSV-2 crosses suggested that one or two sequence isomers could preferentially participate in replication or recombination or both (20); (ii) a recent study performed in the pseudorabies virus system strongly indicates that initiation of DNA replication is not limited to one isomer, even under conditions in which no intermolecular complementation could occur (4); (iii) a thorough reassessment of the genetic map of HSV-1 has provided evidence that markers mapping at extreme ends of the genome might be linked, thus generating a circular or concatemeric recombination map (14).

The present study was undertaken with the aim of defining more accurately the molecular anatomy of the viral replicative intermediates, using restriction endonucleases as an analytical tool. More specifically, we sought to answer the following questions. Is there any evidence for one sequence isomer being preferentially replicated? Are the intermediates really concatemers, and, if so, what is their average length at different times after infection? Is there any specificity to the DNA maturation process?

To answer our questions about the anatomy of replicative intermediates, we digested replicating HSV with the restriction enzyme *Bgl*II. This enzyme has the advantage of not cutting

within the repeat sequences, thus generating four different terminal fragments and four different L/S junction fragments, whose combinations would be diagnostic of individual sequence isomers. In addition, these terminal and junction fragments are easily separable by gel electrophoresis (Fig. 1).

Preliminary experiments (data not shown) showed that under our conditions of infection, the peak of viral DNA synthesis occured at about 6 h postinfection (p.i.). Vero cells were infected with HSV-1 (MP) and pulse-labeled for 30 min with [^{3}H]thymidine at 6 h p.i. Viral DNA was isolated by preparative banding in CsCl and analyzed by digestion with *Bgl*II. The most important features of the banding pattern were as follows: (i) The fragments generated from the termini of unit-length DNA from mature virions were present in undetectable amounts, if at all, in pulse-labeled intracellular DNA (Fig. 1, 6- to 6.5-h lane). This result confirms previous observations by Jacob et al. (15) but also extends them in several ways. First, our shorter labeling time (30 min versus 2 h) makes it more likely that we are analyzing the true replicative intermediates. Second, we do not observe any incorporation into termini, implying that the replicative templates are truly endless. (ii) No fragments were detected in the replicating DNA that were not seen in virion DNA. This implies that the new junctions, which cause the disappearance of terminal fragments, are head-to-tail, since head-to-head (or tail-to-tail) junctions would generate new fragments twice the size of the terminal fragments from which they originated. This result confirms previously published reports (2, 15). (iii) All four L/S fragments are labeled in an approximately equimolar fashion. If only one sequence isomer had been engaged in replication, only two of the joint fragments would have been labeled. All four fragments, however, can be generated from only two linear isomers or from both circular isomers. Consequently, this type of analysis can definitely rule out the idea of a single isomer exclusively participating in replication, as had been suggested from the data of Morse et al. (20).

Since our data on late (6-h) replicative intermediates showed that they are endless and heterogeneous in their isomer composition, we decided to analyze replicative forms produced at earlier times p.i. The possibility still existed that only one sequence isomer could enter the first round of replication and that the others would progressively be generated during subsequent rounds. Also, we hoped to find evidence for shorter concatemers, in which end fragments should be detectable.

At no time p.i., even when viral DNA synthesis is barely detectable (2 to 2.5 h p.i.), is there indication of sequence specificity with regard to template DNA, as judged by the presence of all 0.25 M fragments or of replicative intermediates containing termini since the band patterns are identical at all times tested. We interpret this result to mean that at least two sequence isomers can be replicated very shortly p.i. and that the disappearance of termini by circularization or concatemer formation is an obligatory prelude to DNA replication.

Since all the restriction endonuclease cleavage patterns observed so far had been identical, the question remained as to whether we would be able to detect termini by our methods and also whether the forms we were detecting after a pulse-label were the true precursors to mature, unit-length viral DNA molecules. To examine these questions pulse-chase experiments were performed in which the structure of the labeled viral DNA was analyzed by restriction endonuclease cleavage after various times of a cold thymidine chase. The test for the maturation of replicative intermediates was the appearance of terminal fragments in the restriction pattern.

The results of two such experiments are shown in Fig. 2. Although the patterns obtained after a 30-min pulse confirm our previous results, it is shown that the terminal fragments slowly increase in relative concentration with increasing chase times. It is noteworthy that within the chase times used (up to 2 h), DNA does not reach an equilibrium state in which no more processing occurs, and the terminal fragments do not reach the 0.5 M relative concentration, characteristic of mature virions, during this period. From densitometer scans of the fluorograms, we estimate that the half-time for the processing of replicative intermediates into unit-length molecules is about 2 h, as monitored by the increase in the area under the peaks corresponding to terminal fragments. Other data from our laboratory (Y.-F. Roth, unpublished data) indicate that DNA synthesized at early times p.i. (4 h p.i.) is still being processed at very late times (11 to 12 h p.i.), thus supporting the idea of a stochastic process in which virion DNA is withdrawn from the replicating pool at random and with kinetics independent of those of DNA synthesis.

We also noted that all the terminal fragments seem to appear at similar rates. This result would be expected if the encapsidation process is blind to sequence arrangements and is consistent with the fact that isomers are found in equimolar amounts in virion populations.

The results presented support the following

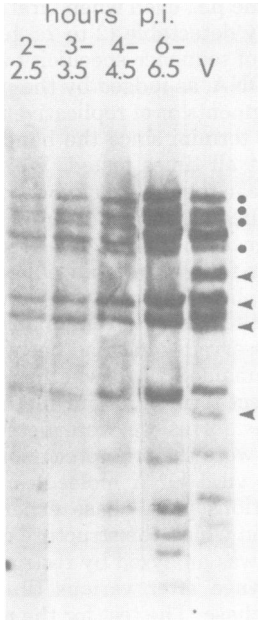

FIG. 1. *Restriction analysis of viral DNA pulse-labeled early and late p.i. Vero cells were infected at a multiplicity of infection of 20 with HSV-1 (MP). Timing of infections was from the end of a 1-h adsorption period. At the times indicated, cells were pulse-labeled for 30 min with 100 μCi of [^{3}H]thymidine (≃40 Ci/mmol) per ml. Cells were washed in ice-cold TE buffer (10 mM tris(hydroxymethyl)aminomethane, pH 7.4–10 mM EDTA) at a concentration of ≃10^6 cells per ml. Cell lysates were prepared by addition of N-lauroyl sarcosine to 1% and heating to 65°C for 3 to 5 min. Lysates were deproteinized by digestion for 4 h at 37°C with 0.05 mg of predigested pronase per ml and diluted with TE buffer to 10 ml. CsCl was dissolved into the lysate by gentle mixing to give a refractive index of 1.4010. Samples were spun for 3 days at 35,000 rpm in a 60 Ti rotor. Fractions were collected, and samples were taken to determine trichloroacetic acid-precipitable counts per minute profiles. Fractions corresponding to viral DNA density were diluted fivefold with TE buffer, pooled, and precipitated along with yeast tRNA with 2 volumes of ethanol. Pelleted and dried precipitates were suspended in 50 μl of water, adjusted to 10 mM tris(hydroxymethyl)aminomethane, pH 7.6–10 mM $MgCl_2$–10 mM NaCl, and the DNA was digested with 2 U of endodeoxyribonuclease BglII for 4 h at 31°C. After addition of EDTA and a sucrose solution containing bromphenol blue as an indicator dye, samples were loaded into a 5-mm, 0.35% agarose gel in 40 mM tris(hydroxymethyl)aminomethane, pH 7.8–5 mM sodium acetate–1 mM EDTA–0.5 μg of ethidium bromide per ml and electrophoresed at 3 V/cm for 18 h. The gel was prepared for fluorography by a modification of the method of Bonner and Laskey (5). It was dehydrated by soaking in three changes of methanol for 1 h each and stained for 3 h with 4% 2,5-diphenyloxazole in methanol. The gel was soaked in water to precipitate 2,5-diphenyloxazole and dried under vacuum onto a sheet of Whatman 3MM paper. Du Pont Cronex 2DC X-ray film was used, and exposures were at −70°C. Mature, viral DNA was extracted from virions. The terminal (0.5 M) fragments are indicated by arrows, and the L/S joint (0.25 M) fragments are indicated by circles.*

conclusions: (i) the rapidly sedimenting replicative intermediates seen at the peak of DNA synthesis (1, 12, 16) are generated from forms of the viral genome whose termini are linked head-to-tail; (ii) replication per se is not restricted to a single-sequence isomer; (iii) terminal fragments are never labeled during replication, even at times before the appearance of rapidly sedimenting forms (1, 12, 16); (iv) endless replicative intermediates labeled at the peak of viral DNA synthesis can be processed into end-containing (presumably unit-length linear) DNA molecules, but replication and maturation do not appear to be tightly linked.

These observations are very significant to our understanding of the mechanism of HSV DNA replication. First, to account for the lack of labeling of free termini, we have to assume that, if the replicative intermediates are truly concatemers, they either are highly polymerized or

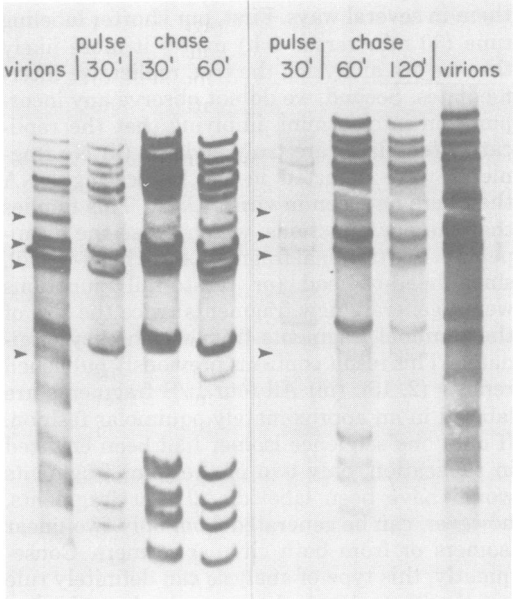

FIG. 2. *Appearance of terminal fragments during a cold thymidine chase. Infected Vero cells were pulse-labeled with [^{3}H]thymidine from 5.5 to 6 h p.i., and label was chased for various times with a 100-fold excess of cold thymidine. The restriction analysis was performed as described in the legend to Fig. 1. Arrows indicate the positions of the terminal fragments.*

contain unidirectional replicative forks which always move away from termini. Although the latter explanation would be consistent with a rolling circle model, the simpler interpretation of the templates being circles at all times during infection seems more appealing and will be expanded upon below.

Second, our data exclude the possibility of only one sequence isomer being involved in replication. Under all of our labeling conditions, the four L/S joint fragments are labeled to a similar extent, even during the first round of replication after a drug-induced block (unpublished data). These results do not imply that all four sequence isomers are capable of initiating a replicative or infectious cycle without complementing functions but merely that the viral replicative machinery does not discriminate between sequence isomers. In contrast, there is still a very strong possibility that intermolecular recombination mechanisms do select for a particular isomer. Specifically, the recent data of Honess et al. (14) support this idea, since these investigators can generate a consistent linkage map between markers in the L and S regions of the genome. The latter observation also lends support to the notion that genetic recombination cannot be the cause of isomer formation.

Third, as indicated above, replicative forms lacking ends can be chased into end-containing, presumably mature, viral DNA molecules; again, there seems to be no sequence specificity to the process. Data obtained in the pseudorabies system (19) indicate that there is an obligatory linkage between maturation of virions and the appearance of unit-length DNA molecules. Coupled with our results, these observations suggest that linear unit-length molecules are end products which are unlikely to reenter the replicating DNA pool.

Although our results would argue against unit-length linear molecules serving as templates for DNA replication, the absence of detectable end fragments and the presence of all four junction fragments in replicating DNA at all stages of infection do not offer conclusive evidence for replicative intermediates being circular. However, evidence has recently been presented for the circular structure of template DNA at least during the first round of pseudorabies virus DNA replication (3). As previously pointed out by Sheldrick and Berthelot (23), the hypothesis of circular templates reduces the problem of the relationship between four linear isomers to that of the interconversion of only two circular isomers and the subsequent choice of an L/S junction for excision. In other words, a simple intramolecular recombination event between L/S junctions can account for all of the isomerization events. If the templates, at least early in the replicative cycle, are actually circles, then our results show that both isomers can replicate. It would not seem surprising, under such a set of assumptions, that viral DNA populations isomerize very rapidly. Although our results do not offer any evidence bearing directly on the actual mechanism of isomerization, they do show that it is not a postreplicative event. Whether isomerization is dependent upon replication is still an open question.

Having argued that the templates for HSV DNA replication may be circular, we are left to explain their aberrant sedimentation behavior as noted by others (12, 16). We feel that two mechanisms, or most probably a combination of the two, could account for the known properties of replicating HSV DNA. First, it is possible that daughter molecules would remain topologically linked at the end of a round of replication, thus generating progressively larger catenated structures, similar to trypanosome kinetoplast DNA (6), as the number of rounds of replication progresses. It has recently been shown that circular simian virus 40 replicative intermediates remain transiently linked in such a structure (25). Second, as pointed out before (24), intermolecular recombination between L/S joints can generate circular concatemers, which would explain the molecules that are larger than unit length detected with electron microscopy (7, 11, 16). Alternatively, these could be cellular contaminants banding at the density of viral DNA.

Although there is at present no conclusive evidence about the mechanism of HSV DNA replication, the framework within which models can be constructed is narrowing. Experiments are under way to further define the anatomy of the replicative intermediates.

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