

Replication of herpes simplex virus DNA: Localization of replication recognition signals within defective virus genomes

(rolling circle/replicative origin/DNA processing/transfection)

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ABSTRACT Serially passaged herpes simplex virus type 1 (HSV-1) strain Justin was previously shown to contain defective virus genomes consisting of head-to-tail reiterations of sequences derived from the end of the S component of the standard virus DNA. Cotransfection of purified monomeric defective genome repeat units with foster helper virus DNAs onto rabbit skin cells resulted in regeneration and replication of concatemeric defective DNA molecules which were successfully encapsidated. Thus, defective HSV-1 (Justin) genomes contain, within their limited DNA sequences, a sufficient set of recognition sites required for HSV DNA replication and packaging. The arrangement of repeat units within the regenerated defective virus genomes was consistent with their replication by a rolling circle mechanism in which a single repeat unit served as the circularized template. This replication occurred most actively late after infection and could be shown to be inhibited by low concentrations of phosphonoacetate known to inhibit the HSV-specified viral DNA polymerase selectively. The resultant concatemers were shown to be cleaved to $M_r 100 \times 10^6$ DNA molecules which were terminated at one end with the proper *ac* end sequence of the parental standard virus DNA.

The standard genome of herpes simplex virus type 1 (HSV-1) is a linear, double-stranded DNA molecule with $M_r \approx 100 \times 10^6$ (1, 2). Topologically, the standard HSV DNA is terminally reiterated and consists of two covalently linked components, L and S, each of which contains a stretch of unique viral DNA sequences surrounded by inverted repeats: the unique sequences of L (U_L) surrounded by the *ab* and *b'a'* inverted repeat sequences, and the unique sequences of S (U_S) bounded by the *ac* and *c'a'* inverted repeats (3-6). The L and S components have been shown to invert relative to each other, yielding equimolar amounts of four isomeric structures. (3, 5-8).

Whereas recently plaque-purified virus stocks predominantly contain standard HSV genomes, defective virus populations obtained through serial undiluted propagation of such stocks have been shown to contain variable proportions of variant DNA molecules in which greater than 90% of the parental viral DNA sequences have been replaced by multiple head-to-tail reiterations of sequences derived from defined locations of the parental genome (9-15). On the basis of the origin of DNA sequences contained within their repeat units, defective HSV genomes have been divided into two classes. Class I defective genomes contain reiterations of G+C-rich DNA sequences arising predominantly from the end of the S component (10, 12-15). Class II defective genomes contain a small set of DNA sequences derived from the end of S covalently linked to DNA sequences from U_L within map coordinates 0.35-0.45 (11, 13, 15). Both classes of defective genomes can be generated upon parallel propagation of separate virus series from a given HSV strain (13, 15).

Because defective HSV genomes represent subsets of viral

DNA sequences which are successfully amplified and encapsidated in the infected cell, a series of investigations was undertaken to identify the genome functions which allow for this propagation; this, in turn, would imply the activity of these same functions in the replication of the parental HSV genome.

MATERIALS AND METHODS

Cells and Viruses. Human epidermoid 2 (HEp-2) cells and African Green Monkey cells were obtained from Flow Laboratories (Rockville, MD). The plaque purification and serial propagation of HSV-1 (Justin) stocks have been described (10).

Structural Analyses of Viral DNA. Viral DNA was prepared from infected cell lysates by treatment with proteinase K and CsCl equilibrium centrifugation as described (16). Analyses of viral DNA with restriction enzymes (New England BioLabs) and λ exonuclease (New England BioLabs) were as described (14). When redigestion of restriction enzyme fragments was required, electrophoresis was performed in 0.4% low-melting-point agarose gels (Bethesda Research Laboratories, Rockville, MD). After the gel was stained with ethidium bromide (0.5 μ g/ml), agarose slices containing bands of interest were melted at 68°C, cooled to 37°C, and incubated in the second enzyme reaction mixture. Resultant DNA fragments were electrophoresed in 0.5% agarose gels and processed for autoradiography as described (14).

Transfection of Cells with Viral DNA. Transfection of viral DNAs into rabbit skin cells was performed according to the procedure published by Ruyechan *et al.* (17).

RESULTS

Temporal and Enzymatic Relationship Between Defective and Standard Genome Replication. The purpose of this series of experiments was to determine the temporal and enzymatic relationships between the replication of defective genomes and the replication of standard genomes in the infected cell. To follow the accumulation of defective and standard genomes, cells were infected with passage 15 (P15) of the Justin series at 1 plaque-forming unit per cell and labeled with [3 H]thymidine from 2 hr after infection until time of harvest (Fig. 1). In estimating the amount of defective and standard DNAs we made use of the previous observation (10) that the class I (Justin) defective genomes displayed a buoyant density higher than that of standard virus DNA. These analyses revealed that, whereas the initial rate of variant DNA synthesis paralleled that of the helper virus DNA, defective virus genomes accumulated more rapidly

Abbreviations: HSV, herpes simplex virus; U_L , unique sequence of L; U_S , unique sequence of S; P15, passage 15; PAA, phosphonoacetate.

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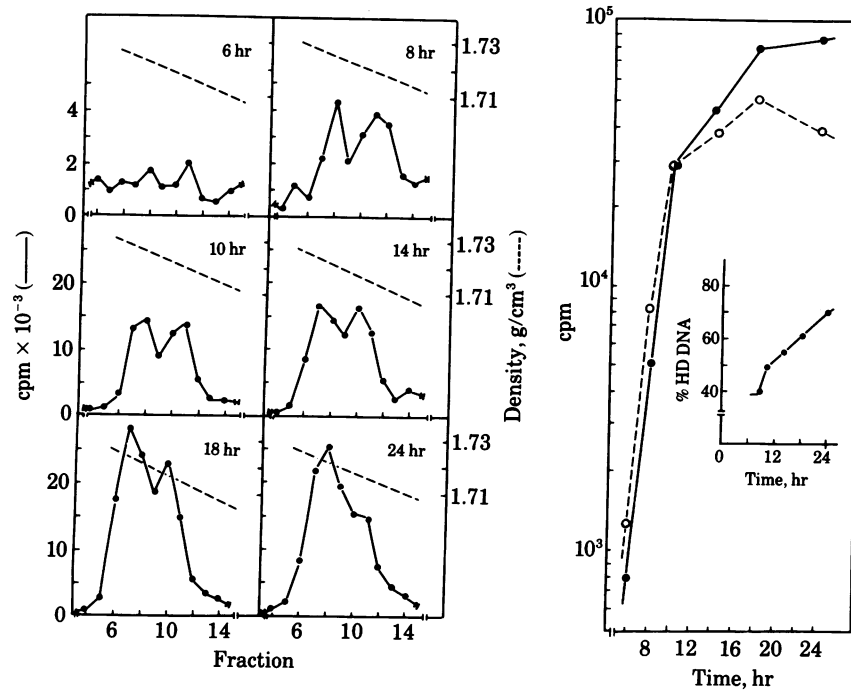


FIG. 1. Time course of standard and defective HSV-1 (Justin) genome replication. Replicate cultures infected with Justin P15 were labeled, beginning at 2 hr after infection, with [³H]thymidine (1 μCi/ml) and harvested at the times shown. After lysis and proteinase K digestion, the cell lysates were centrifuged to equilibrium in CsCl. (Left) Regions of the gradients containing HSV DNA. The buoyant densities were estimated from the refractive indices and are in slight error due to the presence of NaDodSO₄ in the gradients. The heavy and light peaks in each gradient represent defective- and helper-virus DNA, respectively. (Right) Distribution of counts in heavy (●) and light (○) viral DNA as determined from the corresponding gradients shown in Left. (Inset) Percentage of viral DNA present as defective-virus genomes at indicated times after infection. HD, high density.

at later time intervals. Thus, the defective genomes constituted 40% of the total viral DNA at 8 hr and 70% of the total viral DNA at 24 hr after infection.

In order to determine whether the synthesis of HSV defective genomes involved the viral DNA polymerase, cells infected with defective Justin populations were treated with phosphonoacetate (PAA) at concentrations ranging from 20 to 300 μg/ml. These concentrations of the drug were previously shown to inhibit the HSV-specified DNA polymerase selectively (18). After isolation of viral DNA the relative proportions of defective- and helper-virus DNA were determined from analyses of *Eco*RI digests. The ratio of defective- to helper-virus DNA was unaffected by increasing concentrations of PAA added either immediately prior to infection (data not shown) or during the infection (Fig. 2). Thus, defective-virus DNA replication was as sensitive to PAA as helper-virus DNA replication.

Replication Recognition Sites Within the Defective Genome Repeat Units. Three sets of experiments were done in order to test whether defective genome repeat units could be replicated and encapsidated in cells coinfecting with defective- and helper-virus DNA. The design of these experiments was similar to that reported (19) for simian virus 40 defective genomes and involved the cotransfection of rabbit skin cells with "foster" helper-virus DNA and monomeric defective Justin repeat units. These monomeric repeat units were prepared by *Eco*RI digestion of purified high-density defective virus DNA. Virus stocks obtained from the transfected cells were propagated for three to five passages and were subsequently analyzed for the presence of regenerated defective genomes. The foster helper used in the Co-1 and Co-2 experiments was standard HSV-1 (F) DNA, whereas the helper in experiment Co-3 was standard HSV-1 (Justin) DNA. In all cases, virus stock from parallel control transfections using helper-virus DNA alone contained no detectable defective virus genomes at the passage levels used (P3

to P5) for the restriction enzyme analyses. In contrast, virus stocks derived from the Co-1 through Co-3 transfections contained $M_r 10^8$ high-density DNA molecules that were entirely resistant to both *Hind*III and *Bgl* II—a characteristic feature of the original defective Justin genome (10, 14).

Single and double enzyme cleavage patterns of purified defective virus DNA (*Bgl* II/*Hind*III-resistant) are shown in Figs. 3 and 4. As summarized in Fig. 5, these analyses revealed that the *Eco*RI-cleaved repeat unit seeds, as well as shortened repeat units trimmed at both *Eco*RI-cleaved ends, were capable of autonomous replication, generating progeny defective genomes that were either indistinguishable from the defective-virus genomes in the original Justin series [including retention of the *Eco*RI site (Fig. 5*b*)] or contained small discrete deletions in the region of the repeat units flanking the *Eco*RI site (Fig. 5*c*). Progeny virus stocks also contained smaller quantities (mostly in Co-1 progeny) of more highly deleted repeat units which were tentatively mapped within the limits shown in Fig. 5*d* on the basis of their retention or loss of various restriction enzyme sites.

Defective Genomes Are Replicated by Rolling Circle Mechanism. The different types of repeat units present in early passages of the Co-1 through Co-3 transfections were clearly segregated in separate $M_r 100 \times 10^6$ DNA molecules. This conclusion was readily apparent from the observation that *Eco*RI cleavage of the *Bgl* II/*Hind*III-resistant DNA ($M_r 100 \times 10^6$) yielded predominantly only two types of bands. The first migrated as $M_r 100 \times 10^6$ DNA and therefore corresponded to virus genomes which consisted entirely of multiple reiterations of *Eco*RI-resistant repeats; the second band corresponded in size to monomeric repeat units ($M_r 5.5 \times 10^6$) and therefore must have arisen from cleavage of defective genomes consisting entirely of *Eco*RI-sensitive repeats. Similarly, only a minimal degree of intermixing between *Eco*RI-sensitive and -resistant

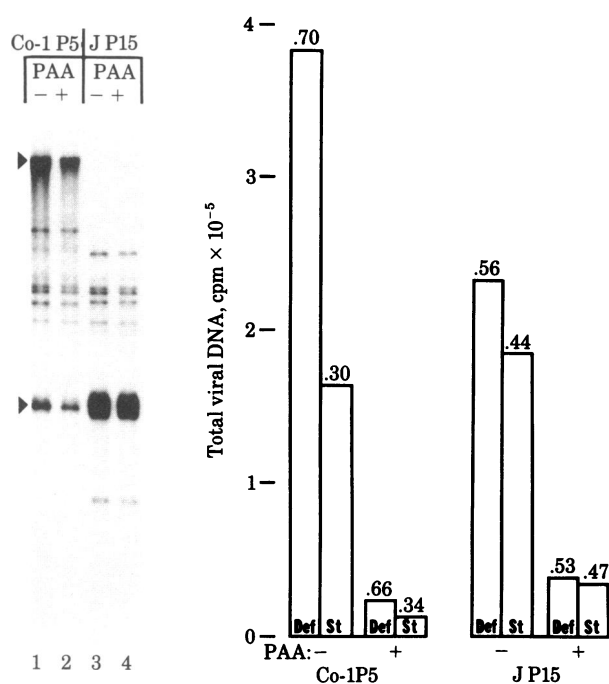


FIG. 2. Inhibition of defective and standard HSV genome replication by PAA. Two pairs of replicate cell cultures were infected with either Justin P15 or P5 of the Co-1 viral pool (described later in the text) at 1 plaque-forming unit per cell. At 7 hr later, one of each pair of infections was treated with PAA at 100 μ g/ml. [³²P]Orthophosphate labeling was continuous from 8 to 23 hr after infection and then viral DNA was harvested. (Left) *Eco*RI digestion profiles; the triangles point to the major defective genome-containing bands. (Right) Total incorporation of ³²P into defective- and helper-virus bands as estimated from the total amount of RNase-resistant, trichloroacetic acid-precipitable ³²P in the CsCl-purified viral DNA extracts and the relative distribution of radioactivity in the various *Eco*RI bands as estimated from densitometric scans of the autoradiograms.

repeats was observed after 15 serial undiluted propagations of Co-1 and Co-2 (Fig. 3).

These results exclude the possibility that the defective genome repeat unit seeds gave rise to concatemers by intermolecular recombinations between separate repeats inasmuch as early passages did not contain appreciable amounts of DNA molecules consisting of mixtures of different repeat unit types. Furthermore, the data make it unlikely that the regenerated defective genomes were replicated from full-length concatemeric templates because no significant accumulation of concatemers with more than one repeat unit type was observed even after 15 serial passages. The results concerning the regenerated defective genomes as well as previous data demonstrating the uniform head-to-tail reiterated organization of the original defective genomes strongly support the hypothesis that defective genomes are replicated by a rolling circle mechanism (20) using monomeric repeat units as the replicative templates.

Correct Termination of Regenerated Defective Genomes.

It had been shown (14) that defective DNA molecules in the original Justin series terminated at one end with the original *ac* sequence of the parental standard virus DNA, whereas at the other end they terminated at random locations within their last repeat units. λ exonuclease digestion of defective virus DNA in the Co-1 and Co-2 stocks revealed that the regenerated defective genomes displayed identical structural features. The exonucleolytic digestion affected only the band that comigrated with the S terminal fragment (*Kpn* K) of standard Justin DNA (Fig. 6), which is also the terminal fragment of the Justin HD

DNA (21). The fact that the unique terminal fragment of the regenerated defective genomes comigrated with that of the original Justin P15 high-density DNA is all the more significant in light of the fact that the foster helper HSV-1 (F) genome (used in the Co-1 and Co-2 transfections) contains a terminal fragment (*Kpn* K) that is larger by M_r 0.3×10^6 than the corresponding S end fragment of Justin DNA (21) (Fig. 6). The data therefore show that the major regenerated defective genome species were not products of recombination with the HSV-1 (F) foster helper during either initial defective genome regeneration or subsequent cycles of replication.

DISCUSSION

Previous studies have firmly established that the replication of the complex HSV genome occurs in the nucleus of the infected cell by a virally encoded PAA-sensitive DNA polymerase (18). Furthermore, several studies have revealed that at least one route of HSV DNA replication involves replicative intermediates consisting of long head-to-tail concatemers of viral DNA (22, 23). Less-well established are the nature of replicative structures, the number and location of replication origins within the HSV DNA, and the significance of a great variety of other different putative replicative intermediates which have been observed in preparations of replicating HSV DNA (reviewed in ref. 6). According to one model proposed by Roizman and co-workers (6, 23), the synthesis of mature HSV DNA occurs by rolling of circles formed after exonucleolytic digestion of the terminally redundant DNA ends. The resultant concatemers are subsequently matured by specific cleavages and recombination repair synthesis into the four isomeric forms of viral DNA.

In comparison with the complex structural features of standard HSV DNA and its replicative intermediates, defective-virus genomes offer a simpler model system for the analyses of certain aspects of HSV DNA replication. Several conclusions can be drawn from our studies with respect to the replication of defective HSV genomes. The first is that defective-virus DNA molecules are capable of being autonomously replicated by helper-virus *trans* replication functions without being covalently associated with helper-virus DNA. This conclusion is supported by the reproducible conservation in the cotransfection experiments of unaltered (*Eco*RI-sensitive) defective genomes as well as by the retention of the HSV-1 Justin rather than the HSV-1 F-type terminal fragment.

The second conclusion is that defective genome repeat units and, by extension, region 0.94–1.00 of the standard virus DNA contain two separate recognition sequences involved in the propagation of defective genomes. The first sequence directs the specific cleavage of HSV DNA concatemers prior to or coincidental with encapsidation; it is located at or near the normal S terminus of both the standard virus and the original defective genomes and is also recognized for cleavage even in "permuted" *Eco*RI-generated repeat unit monomers. The second sequence serves as an initiation site for DNA replication by viral-specific DNA replication machinery. The exact location of this second sequence is currently unknown, but preliminary evidence obtained in cotransfection experiments using submonomer defective genome fragments suggests that it is located outside the *ac* region (0.99–1.00) shared by the class I and class II defective HSV genomes (ref. 15; unpublished data). Thus, it is possible that the U_L sequences of class II defective genomes contain a separate origin for viral DNA replication. On the basis of the location of replication loops in replicating linear HSV DNA molecules, Friedmann *et al.* (24) suggested the presence of multiple replication origins along the standard HSV genome. If one ascribes certain isomeric configurations to the molecules they

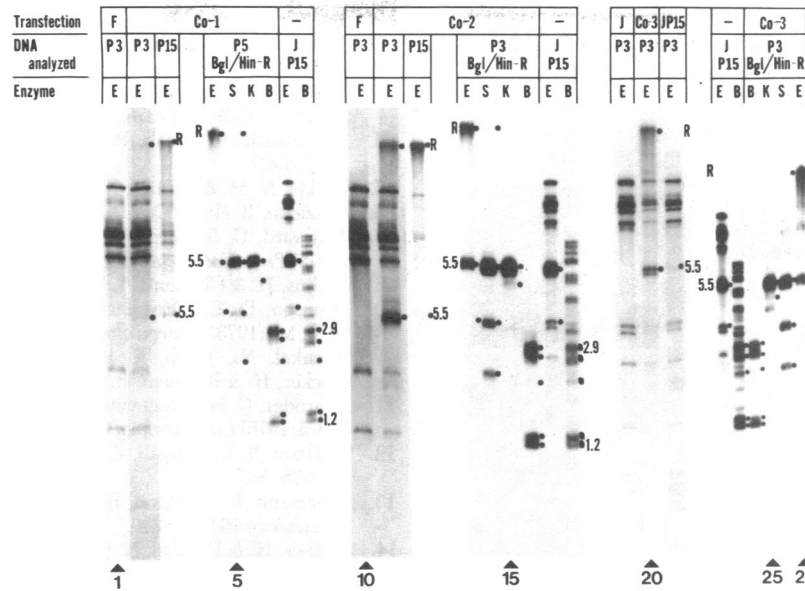


FIG. 3. Structural analyses of viral DNA progeny of the cotransfection experiments. [³²P]Orthophosphate-labeled viral DNAs from the cotransfection experiments (Co-1, Co-2, and Co-3), DNA from the parallel transfections with foster helper-virus DNA alone (F and J), and the original Justin P15 DNA were digested with *Eco*RI (E), *Sal* I (S), *Kpn* I (K), or *Bam*HI (B). Lanes 4–8, 13–16, and 24–27 show the digestion patterns of defective-virus DNA that was first purified in low-melting-point agarose gels on the basis of its resistance to *Bgl* II and *Hind*III (*Bgl*/Hin-R), resistance to analysis endonuclease (*Eco*RI or *Sal* I).

measured, the locations of these sites could correspond to the S and U_L sequences present in class I and class II defective genomes. The existence of two separate origins for viral DNA replication has also been shown in the case of pseudorabies virus DNA (25).

Finally, our results strongly support the hypothesis that defective HSV genomes are replicated by a rolling circle mechanism and therefore that the HSV infected cells must contain the enzymatic and structural functions necessary to support this type of replication. In this light it is noteworthy that Becker *et al.* (26) previously proposed, on the basis of electron microscopic studies, that defective HSV-1 (HF) genomes are replicated by a rolling circle mechanism. In addition, Ben-Porat and Rixon (27)

have shown that standard pseudorabies virus DNA replication proceeds in two temporal phases, only the later of which proceeds via large replicative intermediates consisting of head-to-tail concatemers of viral DNA. It is therefore reasonable to suggest that the delayed replication of defective HSV genomes represents the relatively late emergence of viral DNA replication machinery that can direct rolling circle viral genome replication. Furthermore, it would seem from the data that defective virus genomes are more efficient than their helper virus counterparts in utilizing this late synthetic DNA replication machinery.

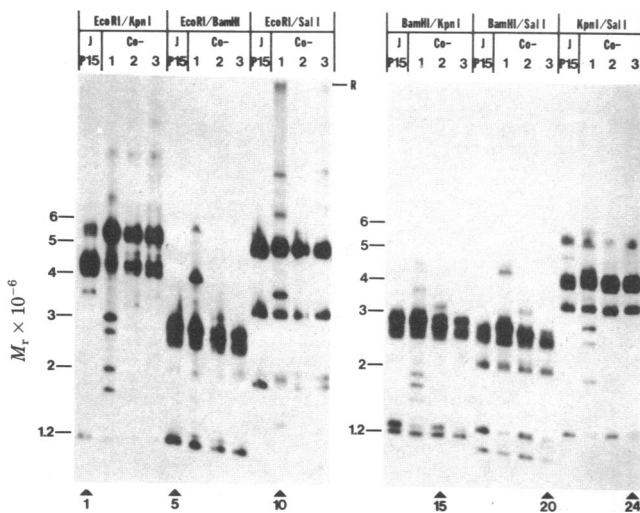


FIG. 4. Structural analyses of purified defective virus DNA progeny from the cotransfection experiments. ³²P-Labeled *Bgl* II/*Hind*III-resistant defective-virus DNAs from Justin P15 (JP15) and Co-1, -2, and -3 virus stocks were analyzed by the various pairs of enzymes indicated in the figure. R, resistance to *Eco*RI and *Sal* I.

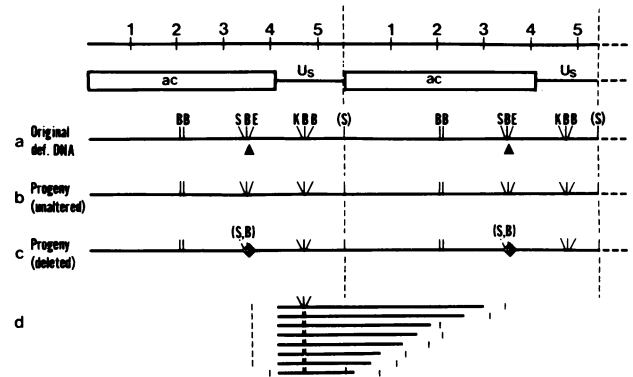


FIG. 5. Schematic representation of repeat units contained in regenerated defective-virus DNA. (a) Physical map of Justin P15 defective genomes. Top line, distances in $M_r \times 10^{-6}$ and the arrangement of U_S and ac sequences (14); ▲, location of the *Eco*RI sites that were cleaved during generation of monomeric repeat unit seed. (b) Representation of regenerated defective-virus DNA progeny which are indistinguishable from the original defective genomes. (c) Some progeny have lost the *Eco*RI site and are therefore resistant to cleavage by that enzyme. Subsets of this DNA have also lost the neighboring *Bam*HI and *Sal*I sites but are otherwise similar to the Justin P15 defective genome. (d) More highly deleted progeny are represented by their repeat unit lengths and are located within the limits indicated on the basis of their retention or loss of restriction endonuclease sites. Enzyme abbreviations are as in Fig. 3.

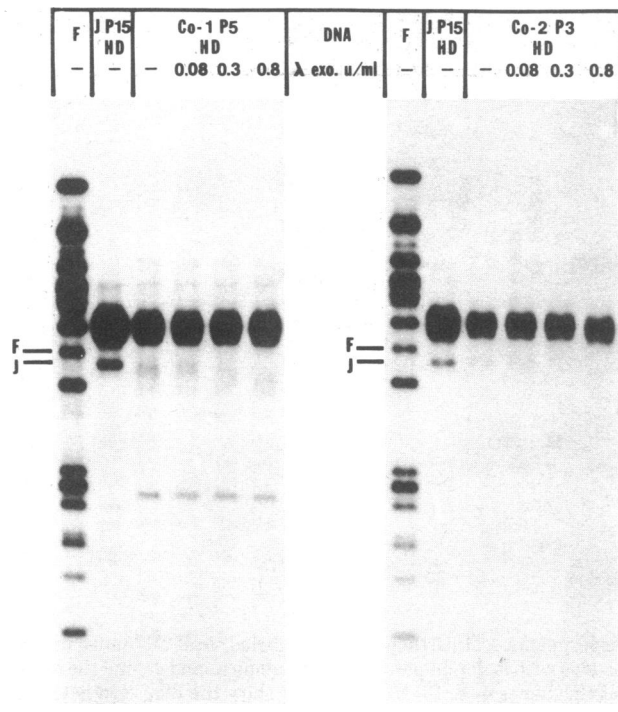


FIG. 6. Identification of the terminal fragments of Co-1 and Co-2 major defective progeny species. High density (HD) DNAs from Co-1 P5 and Co-2 P3 were treated with three concentrations of λ exonuclease and subsequently digested with *Kpn* I. The *Kpn* I K fragments which represent the right-hand end of the S segment of HSV-1 (F) and HSV-1 (Justin) are visible in the F and Justin P15 HD lanes and are indicated at the sides of the autoradiograms by the letters F and J, respectively.

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1. Becker, Y., Dym, H. & Sarov, I. (1968) *Virology* **36**, 184-192.
2. Kieff, E. D., Bachenheimer, S. L. & Roizman, B. (1971) *J. Virol.* **8**, 125-132.
3. Sheldrick, P. & Berthelot, N. (1974) *Cold Spring Harbor Symp. Quant. Biol.* **39**, 667-678.
4. Wadsworth, S., Jacob, R. J. & Roizman, B. (1975) *J. Virol.* **15**, 1487-1497.
5. Wilkie, N. M. & Cortini, R. (1976) *J. Virol.* **20**, 211-221.
6. Roizman, B. (1979) *Cell* **16**, 481-494.
7. Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4243-4347.
8. Delius, H. & Clements, J. B. (1976) *J. Gen. Virol.* **33**, 125-133.
9. Bronson, D. L., Dreesman, G. R., Biswal, N. & Benyesh-Melnick, M. (1973) *Intervirology* **1**, 141-153.
10. Frenkel, N., Jacob, R. J., Honess, R. W., Hayward, G. S., Locker, H. & Roizman, B. (1975) *J. Virol.* **16**, 153-167.
11. Schröder, C. H., Stegmann, B., Lauppe, H. F. & Kaerner, H. C. (1975/1976) *Intervirology* **6**, 270-284.
12. Graham, B. J., Bengali, Z. & Vande Woude, G. F. (1978) *J. Virol.* **25**, 878-887.
13. Stegmann, B., Zentgraf, H., Ott, A. & Schröder, C. H. (1978) *Intervirology* **10**, 228-240.
14. Locker, H. & Frenkel, N. (1979) *J. Virol.* **29**, 1065-1077.
15. Frenkel, N., Locker, H. & Vlazny, D. (1981) in *Herpesvirus DNA: Recent Studies on the Internal Organization and Replication of the Viral Genomes*, ed. Becker, Y. (Nijhoff, The Hague, The Netherlands), in press.
16. Leiden, J. M., Frenkel, N. & Rapp, F. (1980) *J. Virol.* **33**, 272-285.
17. Ruyechan, W. T., Morse, L. S., Knipe, D. M. & Roizman, B. (1979) *J. Virol.* **29**, 677-697.
18. Mao, J. C.-H., Robishaw, E. E. & Overby, L. R. (1975) *J. Virol.* **15**, 1281-1283.
19. Ganem, D., Nussbaum, A. L., Davoli, D. & Fareed, G. L. (1976) *Cell* **7**, 349-359.
20. Gilbert, W. & Dressler, D. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 473-484.
21. Locker, H. & Frenkel, N. (1979) *J. Virol.* **32**, 429-441.
22. Jacob, R. J., Morse, L. S. & Roizman, B. (1979) *J. Virol.* **29**, 448-457.
23. Roizman, B., Jacob, R. J., Knipe, D., Morse, L. S. & Ruyechan, W. T. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 809-826.
24. Friedmann, A., Shlomal, J. & Becker, Y. (1977) *J. Gen. Virol.* **34**, 507-522.
25. Jean, J.-H., Blankenship, M. L. & Ben-Porat, T. (1977) *Virology* **79**, 281-291.
26. Becker, Y., Asher, Y., Weinberg-Zahlering, E., Rabkin, S., Friedmann, A. & Kessler, E. (1978) *J. Gen. Virol.* **40**, 319-335.
27. Ben-Porat, T. & Rixon, F. J. (1979) *Virology* **94**, 61-70.