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Amplification of a Short Nucleotide Sequence in the Repeat Units of Defective Herpes Simplex Virus Type 1 Angelotti DNA

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It has been shown earlier that the reiterated regions T_{RS} and I_{RS} bracketing the U_S segment of herpes simplex virus type 1 Angelotti DNA are heterogeneous in size by stepwise insertion of one to six copies of a 550-base-pair nucleotide sequence. Considerably higher amplification of this sequence was observed in defective viral DNA: up to 14 copies were detected to be inserted in the repeat units of a major class of defective herpes simplex virus type 1 Angelotti DNA, dDNA1, which originated from noncontiguous sites located in U_L and the inverted repeats of the S component of the parental genome. Physical maps were established for the cleavage sites of *Kpn*I, *Pst*I, *Xho*I, and *Bam*HI restriction endonucleases on the repeats of dDNA1. The map position of the insertion sequence was determined. It was demonstrated that the amplified inserts were not distributed at random among or within the repeats. A given total population of dDNA1 molecules consisted of different homopolymers, each of which contained a constant number of inserts in all of its repeats. Assuming that a rolling-circle mechanism is involved in the generation of full-length defective herpes simplex virus type 1 Angelotti DNA from single repeat units, these data suggest that the 550-base-pair sequence is amplified in the repeats before the replication process.

Virions containing defective viral DNA appear in the virus offspring in the course of serial passages of herpesviruses at high multiplicities of infection (1–7, 9, 11–14, 17, 18, 20). The defective DNA has, approximately, a viral DNA unit length and is made up by repetition of restricted portions of the parental genome. The present study is concerned with one major class of defective herpes simplex virus type 1 Angelotti (HSV-1 ANG) DNA, dDNA1, which has the same buoyant density as the parental viral DNA (17). dDNA1 has a repeat sequence of approximately 7×10^6 daltons and originates from noncontiguous sites of the parental genome located between 0.33 to 0.42 and either 0.82 to 0.857 or 0.963 to 1.0 map units on the prototype isomer (9). As has been reported earlier, the reiterated regions T_{RS} and I_{RS} of the S component of HSV-1 ANG standard DNA contain one to six tandem insertions of a 550-base-pair sequence unit. Consequently, the S-terminal and the L-S joint restriction fragments appear as series of DNA bands in agarose gels, equidistant by about 0.35 $\times 10^6$ daltons. By investigating the sequence arrangement of HSV-1 ANG dDNA1, we found that, in some of the repeats of this class of defective DNA, this sequence is amplified as much as 14-fold. One question arising from this finding was whether the inserts were distributed at random within and among the repeat units of individual dDNA1 molecules or whether the total population of dDNA1 molecules consisted of different homopolymers made up of identical repeats containing constant numbers of inserts. To distinguish between these possibilities, we partially digested dDNA1 with a number of restriction endonucleases and examined the digests for the appearance and size distribution of intermediate fragments. Most of the enzymes we used rendered smears which could not be interpreted. *Pst*I, however, cleaved preferentially on one of its two cleavage sites on the dDNA1 repeats. From the fragment patterns of partial *Pst*I digests we concluded that dDNA1 represents a mixture of different homopolymers. Physical mapping of the cleavage sites of various restriction enzymes on the repeat sequence of dDNA1 further revealed that all of the homopolymers have common left-hand and right-hand termini.

MATERIALS AND METHODS

Virus and cells. HSV-1 ANG (1, 13, 15) was propagated on African green monkey kidney cells (RC-37 Rita, Italdiagnostics, Rome, Italy) as described earlier (17).

dDNA1. The development of virus particles containing dDNA1 during serial virus passages at high multiplicities of infection and the isolation of the *ECO*RI-, HpaI-, and *Hin*dIII-resistant dDNA1 from mature virions has been described in detail previously (9, 17). In a final step, defective DNA was purified as DNA sedimenting at the same apparent rate in neutral sucrose gradients as that of viral standard DNA.

Restriction endonucleases. *Bam*HI was a product of BRL, Inc., Rockville, Md. All other restriction endonucleases were purchased from New England Biolabs, Boston, Mass. Enzyme digestions were performed by the prescription of the producers.

Gel electrophoresis of dDNA1 restriction fragments. Electrophoresis was carried out in 0.6 or 0.8% vertical agarose (Seakem, Richmond, Calif.) slab gels at 40 V for 18 h at room temperature. The gels were then stained with ethidium bromide and photographed under UV light.

RESULTS

Evidence of multiple insertions of a 550-base pair nucleotide sequence dDNA1. dDNA1 was digested with various restriction enzymes, and the fragments were separated in agarose gels. Completion of the digestions was carefully tested as follows: excess enzyme was multiply added at 2-h intervals after the first 2-h digestion period, and, in parallel assays, digestions were continued for 24 h as controls. The cleavage patterns obtained with *Xho*I, *Pst*I, *Bam*HI, and *Kpn*I (Fig. 1) represented complete digestion in each case.

All of the patterns displayed remarkable "step ladders" of DNA fragment bands, starting with base fragments of different molecular weights. The individual steps differed by 0.35×10^6 daltons in each case. This finding suggests that the repeat sequences of dDNA1 contain multiple insertions of 550-base-pair sequences. The different intensities of the individual step fragments further suggest that the number of dDNA1 molecules present decreases with the increasing number of insertions that they contain.

Sequence or organization of the repeat units of HSV-1 ANG dDNA1. In addition to the major step ladders, all of the fragment pat-

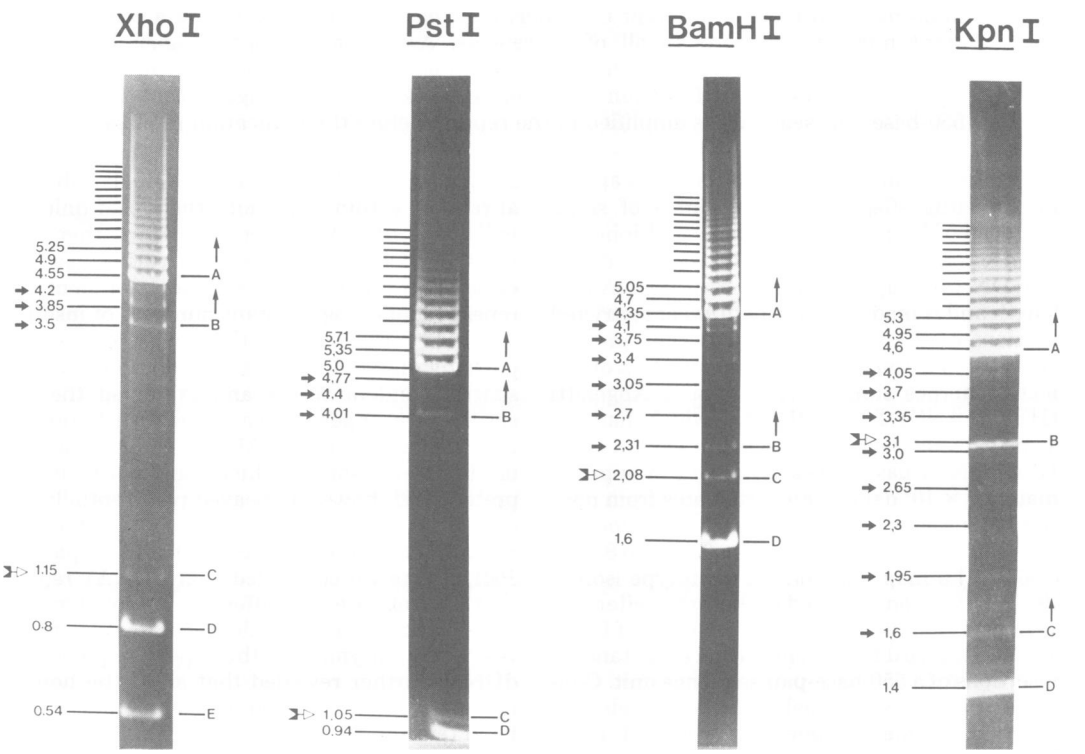

FIG. 1. *Digestion of HSV 1 ANG dDNA1 with the restriction endonucleases XhoI, PstI, BamHI, and KpnI. Ethidium bromide-stained restriction fragments electrophoretically separated on different agarose gels (0.5 to 1%). The numbers represent the molecular weights $\times 10^6$ of the fragments which were estimated by calibrating the gels using HindIII restriction fragments of λ DNA (19) and simian virus 40 DNA (15) and HaeIII restriction fragments of φX174RF DNA (10) as markers. Open arrows, left-hand terminal fragments; Filled arrows, right-hand terminal fragments.*

terns shown in Fig. 1 (and a number of patterns resulting from other restriction enzymes which are not shown) contained a second minor step ladder of fragments (*Xho*I-B, *Pst*I-B, *Bam*HIB, and *Kpn*I-C) which also are equidistant by 0.35 $\times 10^6$ daltons. Furthermore, each of the patterns contained one minor fragment (*Xho*I-C, *Pst*I-C, *Bam*HI-C, and *Kpn*I-B) which when added together with the base step of the corresponding minor step ladder, equals the molecular weight of the base step fragment of the major step ladder. The molarities of the DNA fragment bands were estimated from their molecular weights and by optical scanning of the stained gels. By taking the molarity of the discrete minor fragment (indicated by the open arrow in Fig. 1) for the individual pattern as one (i.e., only one fragment is present per dDNA1 molecule), we found the sum of the molarities of the minor step ladder fragments to be approximately one. It was assumed that the fragments of the minor step ladders and the 1 M fragments in each of the individual patterns shown in Fig. 1 represent left-hand and right-hand terminal fragments of the complete dDNA1 molecules. This assumption implies that the left-hand terminal fragments created by *Xho*I, *Pst*I, *Bam*HI, and *Kpn*I do not contain multiple 550-base-pair inserts, whereas all of the corresponding right-hand terminal fragments do, and, hence, each of the latter is displayed as a step ladder of bands in agarose gels.

The fragments *Xho*I-D, *Xho*I-E, *Pst*I-D, *Bam*HI-D, and *Kpn*I-D, all of which were more than 10 M as judged by optical scanning, were considered to represent internal segments of the dDNA1 repeat sequence units. The same high molarity was determined for the sum of the major step ladder fragments (*Xho*I-A, *Pst*I-A, *Bam*HI-A, and *Kpn*I-A), which apparently also represent internal DNA fragments.

From the above data, the sequence arrangement of the dDNA1 repeats and the map positions of the *Kpn*I, *Xho*I, *Pst*I, and *Bam*HI cleavage sites on the repeat units were derived. The resulting physical maps (Fig. 2) were confirmed by a series of double digestions of dDNA1 with different restriction enzymes. Four representative cleavage patterns obtained are shown in Fig. 3. Each of them agreed with the corresponding double cleavage pattern predicted from the physical maps (Fig. 2).

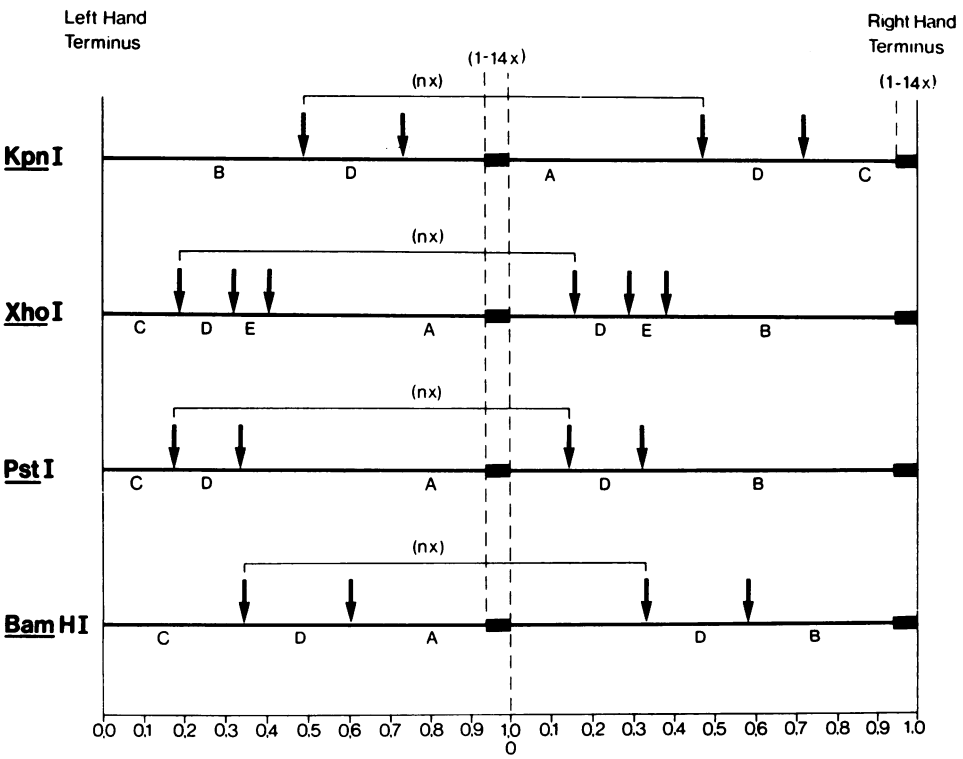

FIG. 2. *Scale maps of KpnI, XhoI, PstI, and BamHI cleavage sites on the repeat sequence of HSV-1 ANG dDNA1. The black bars represent the 550-base-pair inserts. n equals the number of repeats per unit-length molecule of dDNA1 and varies from 9 to 17 depending on the copy number of inserts per repeat.*

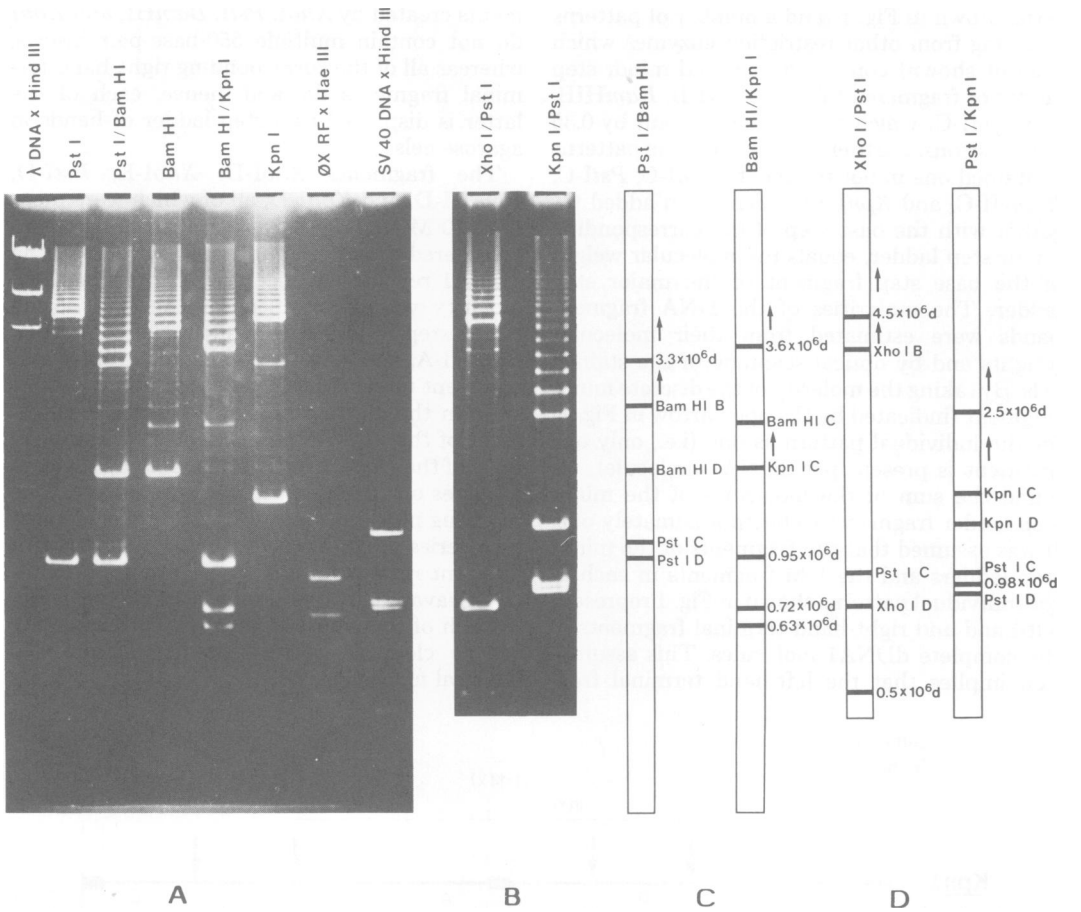

FIG. 3. *PstI-BamHI, BamHI-KpnI, XhoI-Pst, and PstI-Kpn I double digestions of HSV-1 ANG dDNA1. Ethidium bromide-stained restriction fragments electrophoretically separated on agarose gels. The nomenclature of the fragments is as described for Fig. 1 and 2. The molecular weight calibration was performed in each of the two gels (A and B) by using HindIII restriction fragments of λ DNA (19), simian virus 40 DNA (15), and HaeIII restriction fragments of φX174RF DNA (10) as markers and is shown in gel A. For better illustration the fragment patterns of the PstI-BamHI and the BamHI-KpnI double digests were compared with the fragment patterns of single digests with PstI, BamHI, and KpnI in gel A. The fragment patterns of the double digests in A and B are shown schematically in C and D, respectively.*

For example, the double digestion of dDNA1 with *Pst*I and *Bam*HI created the left-hand terminal fragment *Pst*I-C, the fragments *Pst*I-D and *Bam*HI-D, and the series of right-hand terminal fragments *Bam*HI-B. The major step ladder of fragments in this digest had a base fragment of 3.3×10^6 daltons, as expected, and mapped between the *Bam*HI cleavage site D-A and the *Pst*I cleavage site A-D. *Bam*HI-*Kpn*I digestion created the left-hand terminal fragment *Bam*HI-C and the series of right-hand terminal fragments *Kpn*I-C, together with the other fragments predicted from the maps shown in Fig. 2. Similar conclusions can be drawn from the *Xho*I-*Pst*I and the *Pst*I-*Kpn*I cleavage patterns.

As shown in Fig. 2, it is conceivable that the 550-base-pair sequence inserts (1 to 14 copies [at least]) cluster between the map positions of about 0.74 and 1.0; these map coordinates follow from the size of the left-hand terminal fragment *Kpn*I-B (3.1×10^6 daltons) and the base fragment of the right-hand terminal fragments *Kpn*I-C (1.6×10^6 daltons), assuming that the latter contains at last one of the inserts. In the maps shown in Fig. 2, the clusters of inserts are tentatively placed at the right-hand end of the dDNA1 molecules, speculating that part of the insert sequence might function as a signal for cutting the repeat concatemers to viral DNA unit length. According to the sequence arrangement shown in Fig. 2, all dDNA1 molecules had

identical left-hand and right-hand ends. The total sequence complexity of the dDNA1 repeat unit was calculated from the physical maps to be about 6×10^6 daltons (*Kpn*I pattern, 6.0×10^6; *Bam*HI pattern, 5.95×10^6; *Pst*I pattern, 5.94×10^6; *Xho*I pattern, 5.9×10^6 daltons).

Nonrandom distribution of the 550-base-pair insertions in the dDNA1 repeat sequence units. The model of the sequence arrangement in dDNA1 (Fig. 2) raises the question of whether individual dDNA1 molecules are made up at random of repeats with different copy numbers of the insertion sequence or whether each of the complete dDNA1 molecules consists of a certain number of identical repeats, each of which has the same number of inserts. As an experimental approach to answer this question, dDNA1 was partially digested with a series of restriction endonucleases, and the obtained fragment patterns were analyzed on agarose gels. Most of the enzymes assayed for this purpose rendered smears of fragment bands. However, *Pst*I endonuclease created partial digestion fragment patterns which allowed a conclusive interpretation. Figure 4 shows a sequential series of "snap shots" representing initial states of the *Pst*I digestion. Beside the creation of the final major step ladder (0.35×10^6 daltons [Fig. 1]), two intermediate fragment step ladders with steps of 0.7×10^6 and of 1×10^6 to 1.1×10^6 daltons can be detected. The corresponding base step fragments have molecular weights of about 11×10^6 and 17×10^6, respectively. A further group of bands which are not clearly resolved on the gel start with fragments of about 23×10^6 daltons. The interpretation of these results is shown in Fig. 5; the different intermediate fragment step ladders apparently represent the origination of dimers, trimers, and of multimers of a higher order of the repeats. In each of the multimers, the terminal 0.94×10^6-dalton fragment *Pst*I-D was cut off. The fact that the step ladders of dimers and trimers, which are clearly resolved in the gels shown in Fig. 4, have step sizes of 0.7×10^6 ($2 \times 0.35 \times 10^6$) daltons and 1×10^6 ($3 \times 0.35 \times 10^6$) daltons, respectively, strongly suggests that the 550-base-pair inserts are not interspersed at random in the repeats of individual dDNA1 molecules. It is conceivable that, in the case of random distribution, all intermediate step ladders would display a common step size of 0.35×10^6 daltons. Thus, one must conclude that each of the steps of an individual step ladder indicates the existence of a certain class of dDNA1 molecules, made up by identical repeats, each of which contains a constant number of inserts. This implicates that different dDNA1 molecules are made up by different numbers of repeats which are heterogeneous in size. Assuming an average size of the complete molecules of about 100×10^6 daltons, the repeat numbers must vary from 17 (1 insert per repeat, 6×10^6 daltons) to 9 (14 inserts per repeat, 10.6×10^6 daltons).

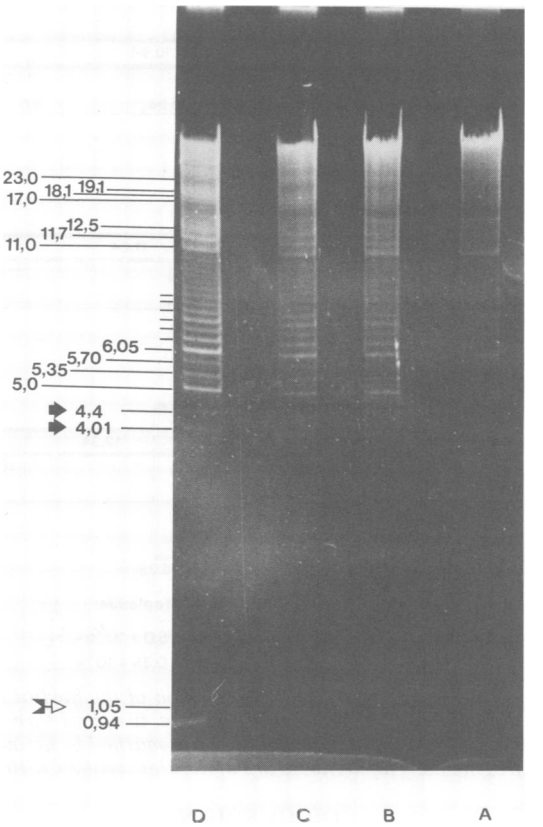

FIG. 4. *Partial digestion of dDNA1 with PstI restriction endonuclease. Amounts (8 μg) of dDNA1 were digested with 0.4 U of PstI (the definition of one enzyme unit was as given by the suppliers [BRL]). Portions of the digests containing 1.5 μg of DNA were taken from the reaction mixture at 4 (D), 6 (C), 8 (B), and 12 min (A) after the start of the reaction and were analyzed on an agarose gel. The numbers represent molecular weights $\times 10^6$ of the fragments, which were determined by using HindIII fragments of λ DNA, simian virus 40 DNA, and HSV-1 ANG viral standard DNA as markers. Filled arrows, right-hand terminal fragments (PstI-B, Fig. 1); open arrow: left-hand terminal fragment (PstI-C, Fig. 1).*

Similar data on the composition of repetitive defective HSV-1 (Justin) DNA has been provided by Frenkel et al (5). By partial denaturation studies, these authors could demonstrate two types of DNA molecules built up either by repeat units with one adenine-plus thymine-rich region or by repeat units with two adenine-plus thymine-rich regions.

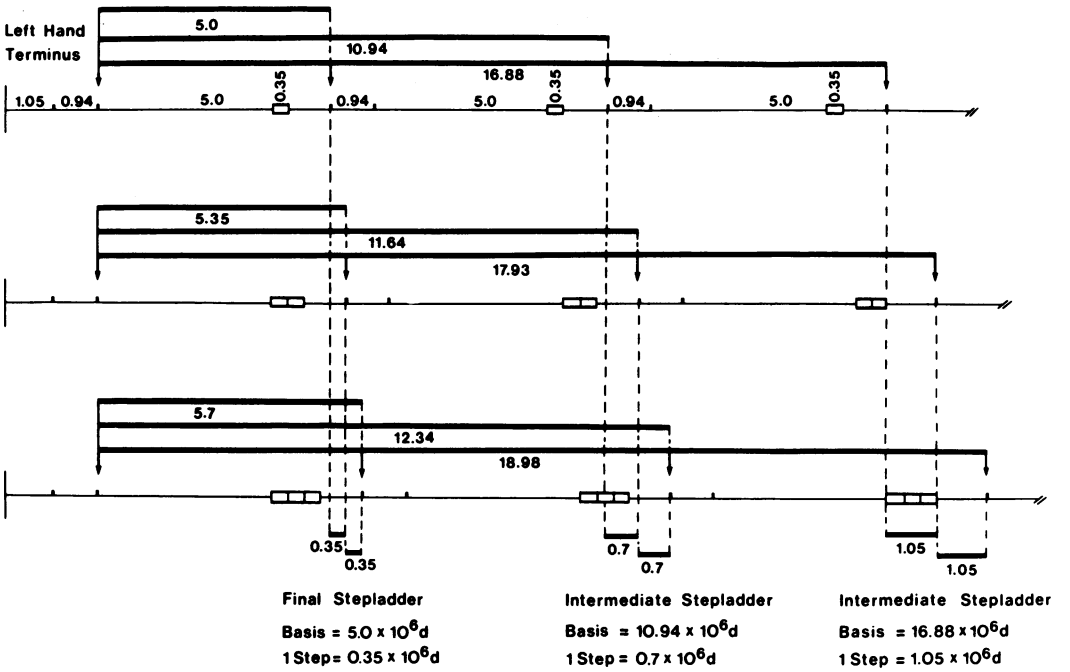

FIG. 5. *Schematical interpretation of the partial digestion of dDNA1 with PstI. The scheme shows three different dDNA1 molecules with one, two, and three inserts per repeat. The inserts are represented by open bars. Preferential cleavage sites are marked by arrows. The black bars indicate the preferentially originating fragments. The numbers are molecular weights* $\times 10^6$

DISCUSSION

A 550-base-pair sequence of the HSV-1 ANG genome was found earlier to be amplified to six copies in regions T_{RS} and I_{RS} of standard viral DNA (9). Locker and Frenkel (11) and Wagner and Summers (21) have detected variable, strain-specific size heterogeneities of the L ends and the L-S joints of the DNA of HSV-1 strains KOS, F, and Justin and have proposed that this is due to the amplification of a short nucleotide sequence. These authors, however, have analyzed viral DNA from infected cells, whereas in the study presented here, viral DNA extracted from mature virions was investigated. It should be mentioned that we obtained the same results by using HSV ANG dDNA1 isolated from infected cells.

The defective derivative HSV-1 ANG dDNA1 is of special interest as it originates from noncontiguous sites of the parental DNA. dDNA1, besides being part of T_{RS} or I_{RS}, including the 550-base-pair sequence, consists of sequences from U_L. Surprisingly, the 550-base-pair sequence was amplified to much higher copy numbers in the dDNA1 repeats than in the parental DNA. This fact is important in connection with another result of the present study which suggests that the total population of dDNA1 molecules consists of different homopolymers, each of which is made up of identical repeats with a constant copy number of the 550-base-pair insertion sequence. Considering results of Locker and Frenkel (12) which suggest that individual repeat units of defective HSV-1 DNA are elongated to full-size defective viral DNA by a rolling-circle mechanism, it is conceivable that the amplification of the insertion sequence in the dDNA1 repeat occurs before the onset of replication.

Roizman (16) and Jacob et al. (8) have presented a hypothesis of the replication of HSV DNA via a rolling-circle model and the generation of all four isomers of HSV DNA from one isomer. One central feature of this hypothesis is the regeneration of the "a" sequence, which is present at both ends of the molecule and is reiterated in the L-S joint in opposite orientation. Circularization of the DNA molecules by ligation of cohesive ends would lead to the loss of one copy of the "a" sequence which has to be regenerated to achieve complete viral DNA molecules either before circularization or after their excision from concatemeric DNA. The above authors proposed in their model that the regeneration of the "a" sequence would occur after replication via a rolling-circle mechanism and that in the course of the regeneration of the "a"

sequence, the other isomers could be formed.

One conceivable speculation from the present data is that the 550-base-pair sequence in HSV-1 ANG standard DNA and in dDNA1 could be (or could contain) the "a" sequence. The observed amplification of this sequence in standard and defective HSV-1 ANG DNA could then reflect one step of the viral DNA replication in terms of the above-mentioned hypothesis, which, for some reason, does not stop after the regeneration of one copy of the "a" sequence. The fact that the 550-base-pair sequence is amplified to a higher degree in the repeats of dDNA1 than in the parental DNA suggests that aplification can occur in standard DNA as well as in the dDNA1 repeats themselves, i.e., after their formation from U_L and the repeats from the S component. On the other hand, higher copy numbers of the amplified sequence are evidently of no advantage for the replication of the repeats, as can be concluded from the increasing number of insertions which correlate with a decreasing number of dDNA1 molecules (Fig. 1).

Molecular cloning of dDNA1 restriction enzyme fragments and DNA sequencing are now in progress to elucidate the sequence arrangement of dDNA1. It should be mentioned finally that another type of defective HSV-1 ANG DNA, dDNA2 (9) which closely resembles the HD DNA derived from HSV-1 Justin by Frenkel and co-workers (5, 6, 11), also shows multiple insertions of the 550-base-pair unit described in this paper (Kaerner, unpublished data).

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