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Anatomy of Herpes Simplex Virus DNA

V. Terminally Repetitive Sequences

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Native DNA from four strains of herpes simplex virus 1 (HSV-1) circularized after digestion with the lambda exonuclease, indicating that the molecules were terminally repetitious. In two strains, the terminal repetition was evident in nearly 50% of the DNA molecules. Maximal circularization was observed when only 0.25 to 0.5% of the DNA was depolymerized by the exonuclease, suggesting that the minimal size of the terminally repetitious regions is in the range of 400 to 800 bases pairs. More extensive exonuclease treatment resulted in a reduction in the frequency of circularization. To determine whether the terminally repetitive regions themselves contained self-annealing sequences that were precluding circularization of more extensively digested DNA, the terminal fragments from HinIII restriction endonuclease digests were isolated, denatured, and tested for their ability to self-anneal. The results of hydroxyapatite column chromatography and electron microscope examination of the terminal regions are consistent with this hypothesis.

Previously, Wadsworth et al. (12) showed that herpes simplex virus 1 (human herpesvirus 1, HSV-1) DNA consists of two regions, designated as *L* and *S*, comprising 82 and 18% of the DNA, respectively. Both *L* and *S* regions are bounded by inverted, terminally repetitive sequences; those of the *L* region, *ab* and *b'a'*, each consist of 6% of the total DNA, whereas those of the *S* region, *ac* and *c'a'*, each consist of 4.3% of the DNA. The arrangement of sequences in the DNA can be represented as *ab-l-b'a'a'c'-s-ca*, in which the junction of the *L* and *S* regions lies between the internal *a'-a'* sequences. Measurements of partially denatured HSV-1 DNA molecules indicate that the maximal size of the *a* sequences, shared by the *L* and *S* regions, is 1% of the DNA. Further analyses of the DNA (8) indicated that the orientation of the *L* and *S* regions relative to each other is random; i.e., HSV DNA populations consist of four kinds of molecules differing only in arrangement of these regions. One consequence of the random orientation of the *L* and *S* regions is that restriction endonucleases, such as HinIII, that do not cleave within the *ab* and *ac* sequences produce a set of four terminal fragments, which are represented in the digests in a 0.5 M concentration relative to the molar concentration of intact DNA.

This paper concerns the *a* sequence, which is reiterated at both ends of the HSV-1 DNA molecule and is shared by the *L* and *S* regions. Estimates of the length of this sequence ranged from 1 to 2% of the DNA (5), based on circularization of the DNA after lambda exonuclease digestion, to a maximum of 4 to 5% of the DNA (11), based on measurements of molecules circularized after exonuclease III treatment. Earlier (12) we showed that the length of the terminally repetitive sequences could not exceed 1% of the DNA on the basis of measurements of partially denatured HSV-1 DNA molecules. In this paper, we report the results of experiments designed to investigate some of the properties of the *a* sequence.

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MATERIALS AND METHODS

Cells and virus. The procedure for maintenance and infection of HEp-2 cells was described elsewhere (3, 10). The HSV-1 virus strains used in this study were F1, MP HFEM-STH2, and Justin. HSV-1 (F1) was isolated from a facial lesion (3) and passaged a maximum of four times at low multiplicity in HEp-2 cells. HSV-1 (Justin) was obtained from Albert Sabin. The virus was plaque-purified (4) and passaged subsequently at low multiplicity. HSV-1 (HFEM-STH2) was obtained from A. Buchan, University of Birmingham, U.K.

Purification of viral DNA. The procedures for purification of viral DNA were the same as reported

elsewhere (12). The DNA preparations used in these studies were found to be free from defective DNA molecules on the basis of analytical centrifugation and restriction enzyme cleavage patterns.

Electron microscopy of DNA. The formamide spreading technique of Davis et al. (2) was used throughout. The procedures used in this laboratory for the measurement of molecules were published elsewhere (12).

Hydroxyapatite column chromatography of DNA. Hydroxyapatite powder was suspended in 0.04 M sodium phosphate buffer (NaPB), and a 0.2-ml column was poured and washed with 0.04 M NaPB. On the basis of extensive calibrations, single- and double-stranded DNAs were eluted with 0.19 and 0.40 M NaPB, respectively.

Preparation of lambda exonuclease and digestion conditions. The lambda exonuclease was purified, by the method of Radding (9), from *Escherichia coli* 1100 (T11) cells induced with an empirically determined dose of mitomycin C (5 μg/mg). Purification was carried through the phosphocellulose step, but the DEAE-cellulose fraction was found to be free from detectable contaminating nucleases and was used for the experiments presented. The standard conditions for digestion of DNA were: 0.067 M glycine-KOH (pH 9.6), 0.003 M $MgCl_2$, 2 μg of DNA per ml, 25 C. Bovine serum albumin at 50 μg/ml and 2-mercaptoethanol at 1 mM were included in the reaction. Reactions were stopped by adding 1/10 volume of 20× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate). To achieve very brief exposure to the nuclease, all components, except the $MgCl_2$, were added to the reaction, mixed, and equilibrated to 25 C. The $MgCl_2$ was then added, and the reaction mixture was agitated, incubated for the indicated times, and then quenched with 1/10 volume of 20× SSC. The conditions for annealing were to heat the DNA at 75 C for 30 min after which the heating bath was turned off and allowed to come to room temperature. The total time for annealing varied from 2 to 5 h.

Restriction endonuclease digestion and isolation of DNA fragments. Native HSV-1 DNA was cleaved with the HinIII restriction endonuclease. The reaction mixture consisted of 3 μg of DNA/ml, 50 mM NaCl, 10 mM Tris-hydrochloride (pH 7.6), and 5 mM $MgCl_2$. The reaction was stopped after 2.5 h at 37 C by the addition of EDTA. The DNA was subjected to electrophoresis on 0.35% agarose columns for approximately 18 h as described by Hayward et al. (7). Ethidium bromide-stained bands were located by UV light illumination and sliced out for extraction of the DNA. The agarose was dissolved by shaking the slices with 8 volumes of saturated KI at 37 C for 10 min. Hydroxyapatite was added to adsorb the DNA and was spun out by low-speed centrifugation. The DNA was eluted in a small volume of 0.4 M NaPB and phenol-extracted to remove traces of agarose. The DNA was then dialyzed against 0.01 M Tris-hydrochloride (pH 8.4)-0.001 M EDTA buffer at 4 C.

RESULTS

Circularization of HSV-1 DNA after exonuclease digestion. DNA prepared from four strains of HSV-1 was digested for varying lengths of time with the lambda exonuclease, annealed, and spread for electron microscopy. The result of these experiments, shown in Fig. 1 as the frequency of circularization plotted as a function of the amount of the DNA depolymerized by the exonuclease, indicated that the DNAs from all of the strains tested circularized after digestion with the exonuclease. In two DNA preparations, 46% of the molecules could be circularized. The reasons for the variation in the maximal frequency of circularization of the DNA from different strains of viruses are unknown but are not due to contaminating nucleases since the same preparation of lambda exonuclease was used throughout.

In experiments designed to estimate the length of the terminally repetitive sequences (solid lines in Fig. 1), it was found that depolymerization of 0.25 to 0.5% of the DNA, corresponding to 400 to 800 base pairs, resulted in maximal circularization. The frequency of circularization dropped precipitously upon continued digestion. Attempts to utilize electron microscopy to visualize and measure the size of the *a* sequence were unsuccessful. DNA preparations digested 0.25 to 0.5% contained circles on which the point of closure could not be found (Fig. 2 and 3). Circles arising from more extensively digested preparations could not be used for a direct measurement of the terminally repetitive sequences for two reasons. First, the

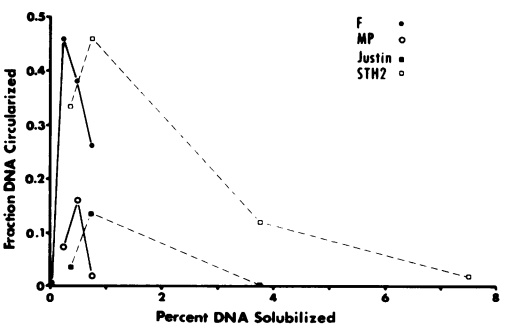

FIG. 1. *Circularization of HSV-1 DNA as a function of lambda exonuclease digestion. The amount of digestion was determined by measurements of the amount of radioactivity solubilized or extrapolated from a plot of amount of digestion as a function of time. The frequency of circularization was determined by direct counting of circular and linear molecules by electron microscope examination. Only those linear molecules judged by inspection to be full length were counted. Approximately 80% of the ends were digested by the nuclease.*

lambda exonuclease can digest internal regions of HSV DNA (S. Wadsworth and B. Roizman, manuscript in preparation) and, therefore, not all single-stranded gaps on the circularized molecules would be adjacent to the point of closure. This would not pose a serious difficulty if clearly identifiable duplex regions bounded by single-stranded gaps could be routinely observed on circularized molecules. Such regions were not observed; largely knotted structures were found. Examples are shown in Fig. 4 and 5, with the presumptive closure magnified. The precipitious decline in the frequency of circularization cannot be explained by contaminating nucleases because the enzyme preparation used in this study had no activity against single-stranded ϕX174 DNA.

Analysis of the terminally reiterated sequences. Of the various hypotheses that might explain the precipitious decline in frequency of circularization upon continued digestion of DNA, we have investigated one: that the terminally reiterated sequences contain sequences capable of self-annealing. The hypothetical self-annealing sequences could be either true palindromes, represented as X-X', in which X' is an inverted, complementary sequence to X, or interrupted palindromes represented as $XYZX'$. This hypothesis predicts that digestion of the DNA with the lambda exonuclease beyond the internal X' sequence would result in self-annealing and in a decline in the frequency of circularization.

The second series of experiments was designed to test this hypothesis in two ways: by hydroxyapatite chromatography of denatured DNA and by direct visualization of self-annealed structures. First, the terminal DNA fragments C1, C2, and D (8) electrophoretically purified from HinIII digests of HSV-1 DNA were denatured and chromatographed on hydroxyapatite to test for self-annealing of terminal sequences. As a control, fragment G, an internal fragment, and whole, sheared DNA were also denatured and chromatographed on hydroxyapatite. The prediction is that the terminal fragment presumed to contain self-annealing sequences will chromatograph as duplex DNA after denaturation, whereas fragments such as G, not containing *a* sequences, will chromatograph as single-stranded DNA after denaturation.

The results of these experiments (Fig. 6 and Table 1) indicate that denatured DNA from terminal regions possesses some structural feature causing it to chromatograph as duplex DNA. Moreover, sequences from each and of the molecule seem to have this feature since fragments C1 and C2 are from opposite ends of the molecule (8). Neither denatured DNA that had been sheared to 600 to 800 bases nor denatured DNA from the internal fragment G behaved in this fashion on hydroxyapatite. Electron microscope examination of the C1 DNA eluted from hydroxyapatite with 0.4 M NaPB revealed that the DNA is single stranded even though it chromatographed as duplex DNA.

Additional electron microscopic studies were undertaken following a report of R. Hyman (personal communication) that single-stranded ends of DNA molecules form terminal loops upon self-annealing. In our studies, intact, native DNA was digested to approximately 4 to 5% with the lambda exonuclease, annealed in 66% formamide, and spread for electron microscopy. These experiments readily showed DNA molecules with single-stranded tails ending in tiny loops. Representative molecules are shown in Fig. 7. The single-stranded loops presumably arise from self-annealing of complementary sequences separated by non-complementary sequences. The measured lengths of the loops ranged from 600 to 1,600 bases relative to ϕX174 DNA, with the average being approximately 1,000 bases. Approximately 22% (7/33) of the lambda exonuclease-digested DNA molecules exhibited these terminal loops, whereas only 4% of the broken ϕX174 DNA molecules in the same fields had folded into a form that could have been interpreted as the terminal loops.

DISCUSSION

Terminally repetitive sequences are generally identified from analyses of DNA molecules allowed to self-anneal after digestion with a 3' or 5' exonuclease. Two preceding studies, i.e., those of Grafstron et al. (5) and of Sheldrick and Berthelot (11), demonstrated the circularization of HSV-1 DNA digested with lambda exonuclease and *E. coli* exonuclease III, respectively. The salient features of the results presented in this paper are as follows. (i) Terminally repetitive sequences were present in the DNA of four strains of HSV-1 differing in the history of their propagation outside the human host. We could not detect defective DNA molecules by us (4) or by others (1) of the kind reported in these preparations. Because the frequency of circularization of the DNA is considerably higher than the detection limit for defective molecules, we conclude that the presence of an identical noninverted sequence at both termini is a general property of HSV-DNA. (ii) A remarkable and totally unanticipated finding

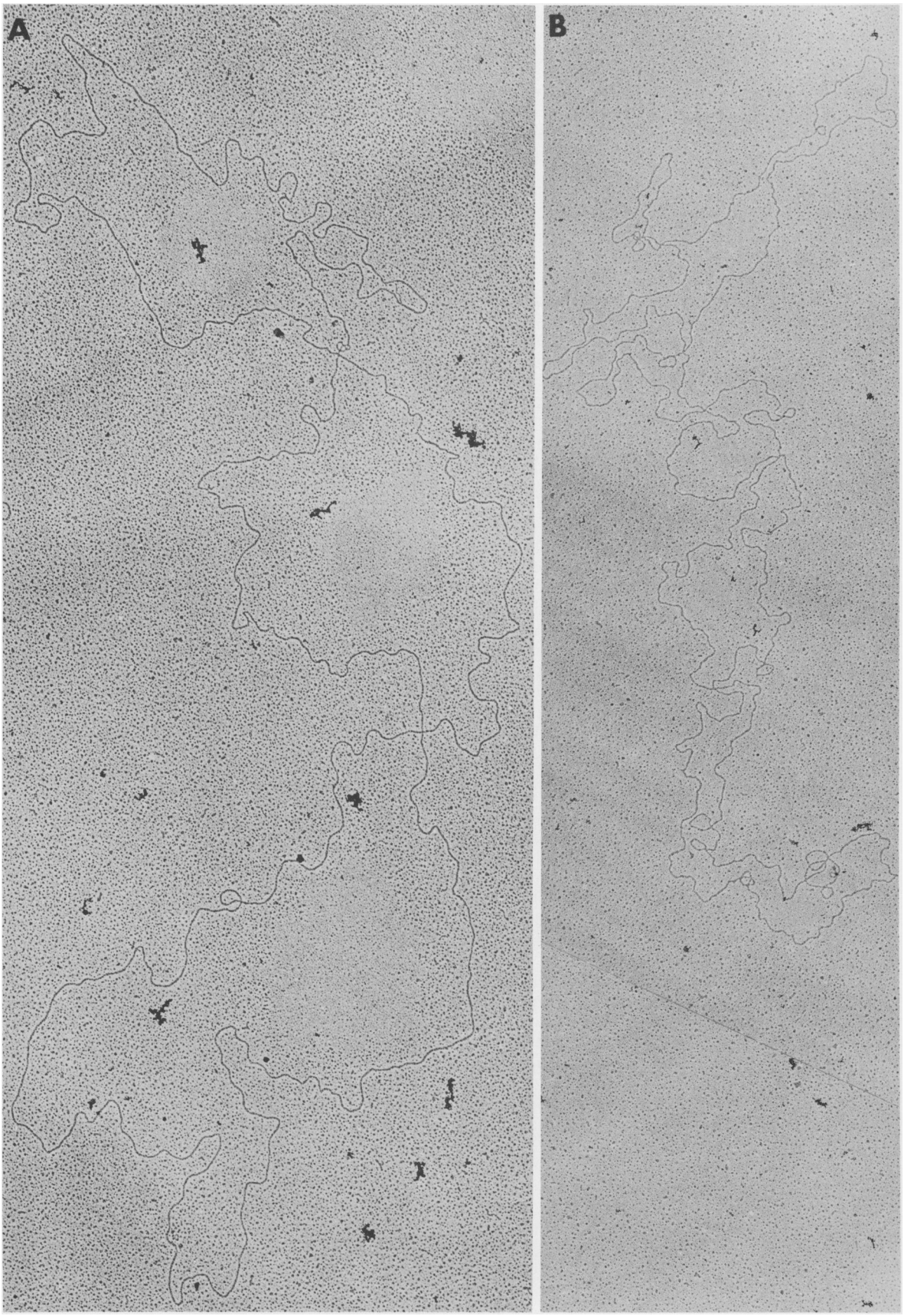

FIG. 2

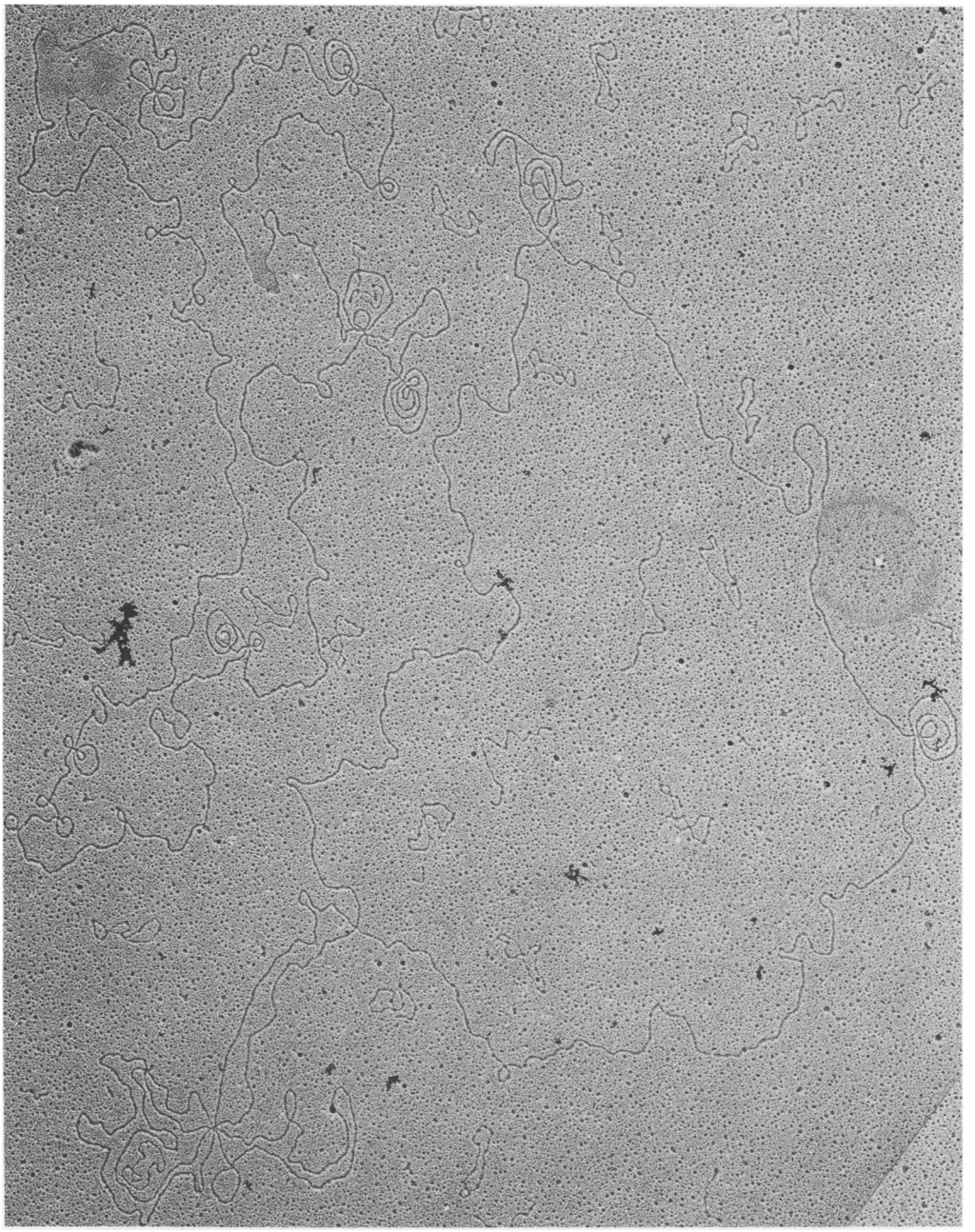

FIG. 2 and 3. *Electron micrographs of three representative DNA molecules circularized after digestion with the lambda exonuclease to approximately 0.5%. In this and other micrographs φX174 and PM2 DNAs are present. These are included as standards for measurements (12) of single- and double-stranded molecules, respectively.*

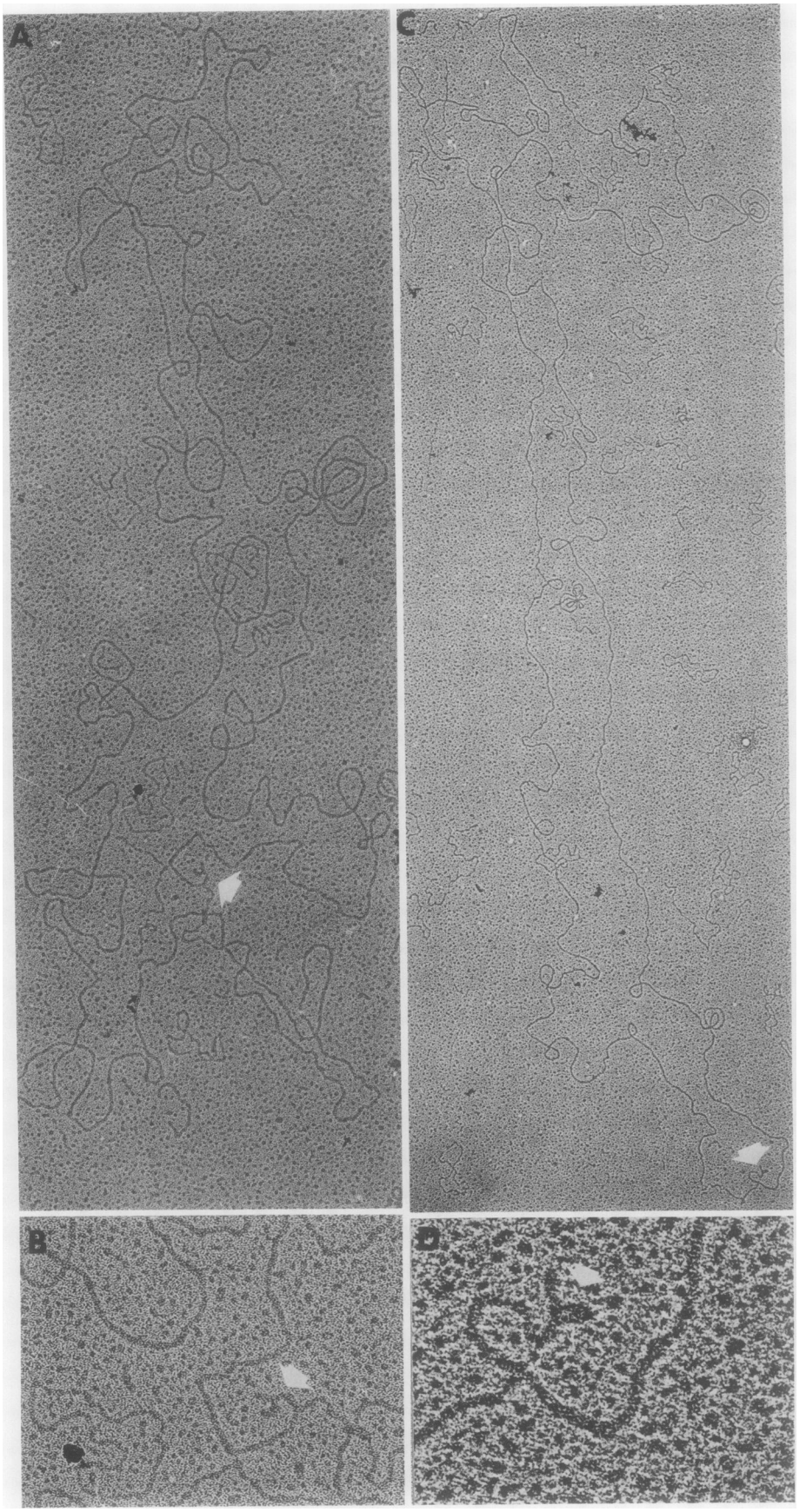

FIG. 4

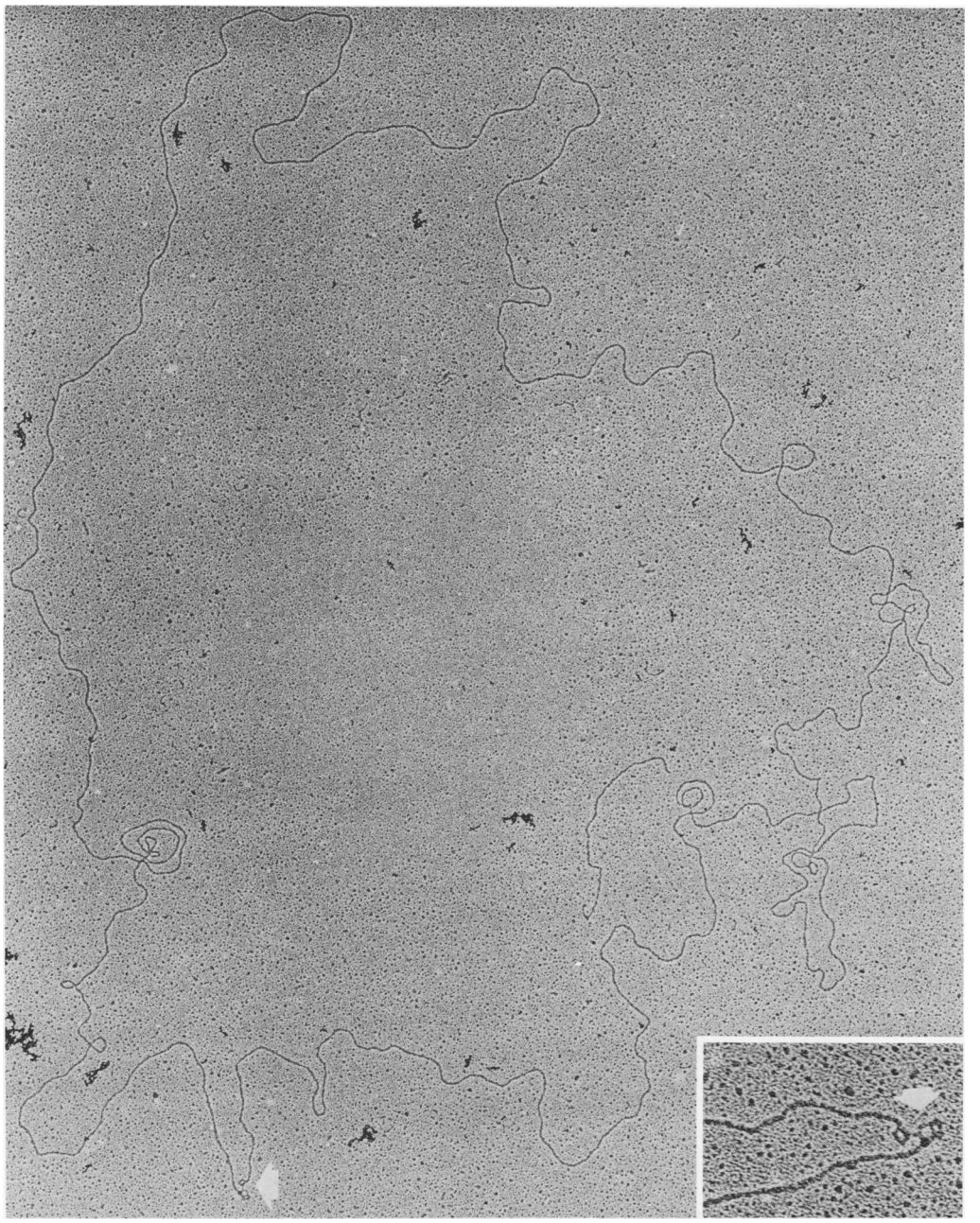

FIG. 4 and 5. *Electron micrographs of three representative DNA molecules circularized after digestion with the lambda exonuclease to 5%. The presumptive closures of the circles in Fig. 4A and C are shown in Fig. 4B and D, respectively. The presumptive closure of the circle in Fig. 5 is shown in the insert.*

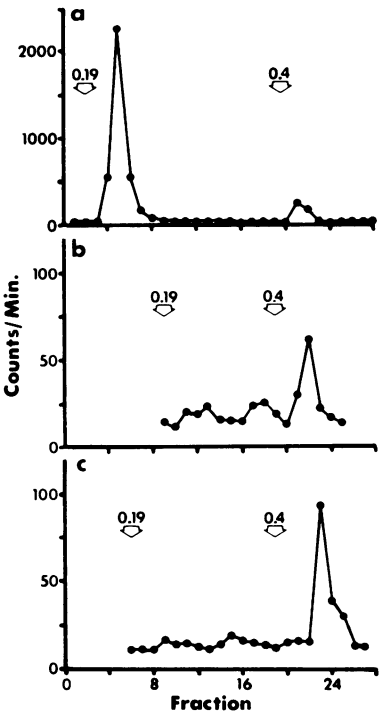

FIG. 6. *Hydroxyapatite column chromatography of HSV-1 DNA. HSV DNA was denatured with 0.1 M NaOH, diluted 10-fold with 0.04 M NaPB, and applied to columns containing 0.2 ml of hydroxyapatite. (a) Sonically treated DNA; (b) HinIII restriction enzyme fragment C1; (c) HinIII restriction enzyme fragment C2.*

TABLE 1. *Hydroxyapatite chromatography of denatured HSV-1 HinIII Restriction Endonuclease Fragments*[a]

DNA fragment	% Eluting as single strands	% Eluting as double strands
D	0	100
G	78	22

[a] DNA was denatured and applied to hydroxypatite columns in 0.04 M NaPB.

was that the frequency of circularization of HSV DNA decreased upon continued digestion with the lambda exonuclease. The observation that maximum circularization (nearly 50% of the molecules) was observed after only 0.25% of the DNA was depolymerized might explain the relatively low frequencies of circulation observed by Sheldrick and Berthelot (7%) (11) and Grafstrom et al. (16%) (5).

In an attempt to explain the decrease in the frequency of circularization, we explored the simplest in a family of hypotheses: that the terminally repetitive sequences each consist of a terminal sequence, X, situated next to, or some distance away from, its inverted complement, X' (Fig. 8). This hypothesis, as do several other versions on this theme, predicts that digestion beyond the inverted complement X' would result in the annealing of X to its complement X' on the same strand. Circularization of the DNA would then depend on the dimension of X, X' and the putative intervening YZ sequences, which may or may not be present. Several comments should, however, be made concerning this hypothesis and the experiments designed to test its relevance. (i) In as much as HSV DNA circularizes after lambda exonuclease digestion, the data are sufficient to conclude that the a sequence, defined as the terminally repetitive sequence, has the same polarity at both the right and left termini; therefore, the graphic illustration of this hypothesis is constructed accordingly. (ii) The hypothesis predicts that digestion with lambda exonuclease to the inverted complementary sequence X' would produce circularized molecules with single-stranded tails. Although none was seen, we cannot exclude the possibility that the digestions were nearly complete or that the X and X' regions are less than 50 to 100 base pairs long and could not be seen in our microscope. It is noteworthy that the diagram defines X as the double-stranded sequence adjacent to the single-stranded loop. This sequence would have to be rather small since it was not seen in all electron micrographs. (iii) Although the data presented in this paper are consistent with this hypothesis, they do not prove it and other experimental designs may have to be brought to bear on this problem. Irrespective of the actual arrangement of the terminal sequences, our data indicate that the minimum size of the terminal reiterated sequences is 0.25 to 0.5% of the DNA, corresponding to 400 to 800 base pairs. The upper limit of 1% (1,600 base pairs) was deduced from partial denaturation studies reported earlier (12). Our estimates of the size of the terminal reiteration are thus in agreement with those of Grafstrom et al. (6) reported after the completion of our work.

ACKNOWLEDGMENTS

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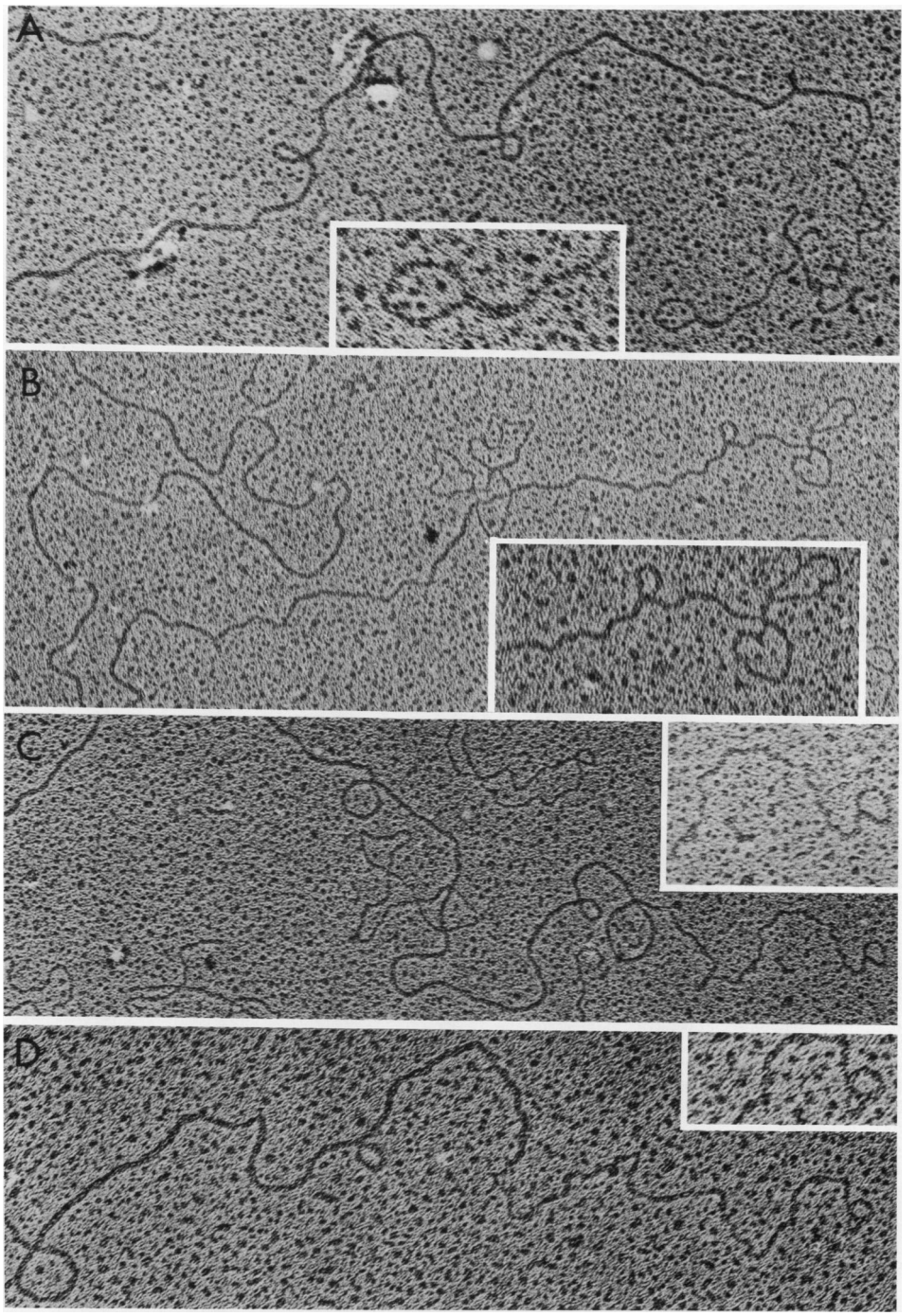

FIG. 7. *Electron micrographs of four lambda exonuclease-digested HSV-1 (F1) DNA molecules self-annealed in 66% formamide buffered with 0.2 M Tris-hydrochloride, (pH 8.0)-0.001 M EDTA. The DNAs were spread by the formamide technique. The insets show the terminal loops.*

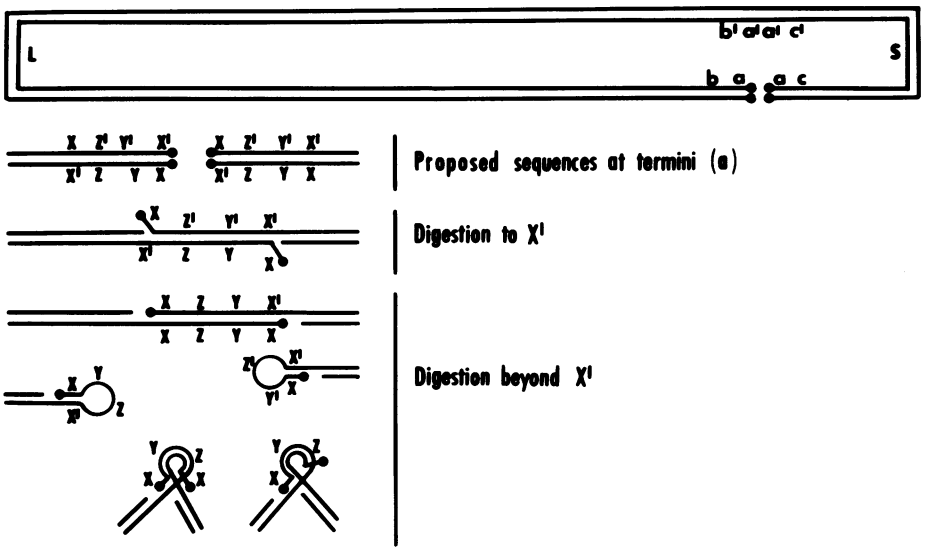

FIG. 8. *Diagrammatic representation of one of several possible sequence arrangements in terminally reiterated regions of HSV-DNA. (Top) Diagramatic representation of HSV-1 DNA molecules showing the L and S regions and the sequences ab, b'a', a'c', and ca. (Bottom) Proposed model for the a sequence and some of the structures that would result from annealing of termini of DNA digested with lambda exonuclease. In this particular model, the left and right reiterated terminal sequences have the same structure and polarity. Slightly different predictions, but basically the same results, are obtained from models constructed so that only portions of reiterated sequences in the two termini have the same polarity. A prediction of this hypothesis is that the single-stranded loops from one terminus are capable of annealing with complementary sequences from the other terminus but with reduced efficiency. We do not wish to imply that the diagrammatic representation of the structures that could result from this renaturation account for the knotted closures seen in circularized molecules digested to 0.5% or more and shown in Fig. 4 and 5.*

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