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DNA of Epstein-Barr Virus

V. Direct Repeats of the Ends of Epstein-Barr Virus DNA

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Previous data indicated that Epstein-Barr virus DNA is terminated at both ends by direct or inverted repeats of from 1 to 12 copies of a 3×10^5-dalton sequence. Thus, restriction endonuclease fragments which include either terminus vary in size by 3×10^5-dalton increments (D. Given and E. Kieff, J. Virol. **28**:524-542, 1978; S. D. Hayward and E. Kieff, J. Virol. **23**:421-429, 1977). Furthermore, defined fragments containing either terminus hybridize to each other (Given and Kieff, J. Virol. **28**:524-542, 1978). The 5′ ends of the DNA are susceptible to lambda exonuclease digestion (Hayward and Kieff, J. Virol. **23**:421-429, 1977). To determine whether the terminal DNA is a direct or inverted repeat, the structures formed after denaturation and reannealing of the DNA from one terminus and after annealing of lambda exonuclease-treated DNA were examined in the electron microscope. The data were as follows. (i) No inverted repeats were detected within the *Sal*I D or *Eco*RI D terminal fragments of Epstein-Barr virus DNA. The absence of "hairpin- or pan-handle-like" structures in denatured and partially reannealed preparations of the *Sal*I D or *Eco*RI D fragment and the absence of repetitive hairpin- or pan-handle-like structures in the free 5′ tails of DNA treated with lambda exonuclease indicate that there is no inverted repeat within the 3×10^5-dalton terminal reiteration. (ii) Denatured *Sal*I D or *Eco*RI D fragments reanneal to form circles ranging in size from 3×10^5 to 2.5×10^6 daltons, indicating the presence of multiple direct repeats within this terminus. (iii) Lambda exonuclease treatment of the DNA extracted from virus that had accumulated in the extracellular fluid resulted in asynchronous digestion of ends and extensive internal digestion, probably a consequence of nicks and gaps in the DNA. Most full-length molecules, after 5 min of lambda exonuclease digestion, annealed to form circles, indicating that there exists a direct repeat at both ends of the DNA. (iv) The finding of several circularized molecules with small, largely double-strand circles at the juncture of the ends indicates that the direct repeat at both ends is directly repeated within each end. Hybridization between the direct repeats at the termini is likely to be the mechanism by which Epstein-Barr virus DNA circularizes within infected cells (T. Lindahl, A. Adams, G. Bjursell, G. W. Bornkamm, C. Kaschka-Dierich, and U. Jehn, J. Mol. Biol. **102**:511-530, 1976).

Primate B lymphocytes are the only cells that have been infected with Epstein-Barr virus (EBV) in vitro (12, 20). The outcome of infection of normal B lymphocytes with EBV is that as many as 1% of the cells may be transformed into lymphoblasts that can be grown indefinitely as continuous cell lines in vitro (9, 16). Some of these transformants and some lymphocytes infected with EBV in vivo give rise to progeny infected cells that are permissive of virus replication. Both cell type and virus strain determine the frequency with which progeny infected cells permit virus replication (6, 17, 19). Several continuous lymphoblastoid cell lines are sufficiently permissive of virus replication that it has been possible to purify the virus and characterize the viral DNA (7, 8, 11, 21-23, 25, 28; D. Given and E. Kieff, manuscript in preparation).

The B95-8 clone of marmoset lymphocytes transformed by the supernatant virus from a continuous lymphoblastoid cell line established from a patient with infectious mononucleosis contains 5 to 10% permissively infected cells and is unusually productive of virus (18). The DNA of virus produced by B95-8 cultures, EBV (B95-8) DNA, has therefore been the prototype for

studies of EBV DNA (for review see E. Kieff, D. Given, N. Raab-Traub, A. L. T. Powell, W. King, and T. Dambaugh, Biochim. Biophys. Acta, in press.). EBV (B95-8) DNA is a linear, double-stranded DNA with a density in cesium chloride of 1.718 g/cm^3 and a size of 105 × 10^6 daltons (22). Although the DNA of virus that has accumulated in the extracellular fluid of cultures is extensively nicked to an average single-strand size of 10^6 (7), the largest single strands of the DNA of virus labeled for 24 h with [^{3}H]thymidine prior to purification from the extracellular fluid are 50 × 10^6 daltons (22). All molecules of EBV (B95-8) DNA have the same sequence organization (Fig. 1) and consist of a short unique region of 10 × 10^6 daltons, from 1 to 10 copies of a tandem reiteration (each copy of which is 1.9 × 10^6 daltons), and a long unique region of approximately 87 × 10^6 daltons (7, 25; Given and Kieff, manuscript in preparation; Kieff et al., in press).

Both ends of the DNA are heterogeneous in length (7, 8). The heterogeneity in mobility of the terminal fragments is unaffected by treatment of the DNA with DNase, indicating that the variation in mobility in agarose is not due to single-strand DNA (7). The size of the terminal restriction endonuclease fragments from either end of the DNA varies over a range of 3 × 10^6 daltons in 12 discrete increments, each differing by approximately 3 × 10^5 daltons (7). Terminal fragments from either end of the DNA hybridize to each other, and there is no homology between the termini and the *Eco*RI I fragment, which extends to within 10^6 daltons of the terminus of the short unique region, or the *Hsu*I D fragment, which extends to within 3 × 10^6 daltons of the long unique region of the DNA (7). The similarity in size of the incremental units at both ends of the DNA and the high degree of homology between terminal fragments from the opposite ends of the DNA suggest that there is a variable number of copies of a direct or inverted repeat of the same sequence at opposite ends of the DNA (7). The presence of an *Eco*RI cleavage site, 10^6 daltons from the short unique end of the DNA, and a *Hsu*I cleavage site, approximately 3 × 10^6 daltons from the long unique end of the DNA, permits the localization of the reiteration to within 10^6 daltons from the short unique end of the DNA and 3 × 10^6 daltons from the long unique end of the DNA (7).

EBV (B95-8) DNA has previously been shown to exist in a circular form in cells transformed by the virus (1, 2, 14). If the reiterations at opposite ends of the DNA are direct and not inverted relative to each other, base pairing between opposite strands at the opposing termini could be an intermediate in the formation of circular EBV DNA molecules in vivo, and treatment of EBV DNA with lambda exonuclease should permit the DNA to circularize. A previous comparison of the restriction endonuclease fragments of lambda exonuclease-digested and nondigested EBV DNA indicated that the ends of the DNA are susceptible to lambda exonuclease digestion (8). A further expectation of the presence of reiterations at each end of the DNA is that fragments from either terminus should form circular structures when denatured and reannealed.

MATERIALS AND METHODS

Cell culture, virus purification, and preparation of viral DNA. The conditions used to grow cultures of B95-8 cells for virus production and the procedures used to purify virus from the supernatant media of cultures of B95-8 cells have been described previously (4). The virus pellet from 8 liters of supernatant was suspended in 0.5 ml of a solution consisting of 0.1 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride (pH 7.4). One percent (wt/vol) sodium dodecyl sulfate (BDH Laboratories, Poole, England) and 2% (wt/vol) Sarkosyl NL97 (Geigy Laboratories) were added. The mixture was incubated at 60°C for 2 min and extracted with phenol and chloroform containing 2% (vol/vol) isoamyl alcohol. DNA prepared for lambda exonuclease digestion was further purified by centrifugation in neutral sucrose velocity gradients (13). Fractions containing full-length molecules were combined, dialyzed against 0.001 M EDTA and 0.01 M Tris-hydrochloride (pH 7.4) at 4°C, and concentrated by lyophilization.

Digestion of EBV DNA with lambda exonuclease and self-annealing of ends. Lambda exonuclease and [^{3}H]thymidine-labeled N4 DNA were obtained from Lucia Rothman-Denes, University of Chicago. Exonuclease buffer consists of 0.002 M $MgCl_2$, 0.005 M β-mercaptoethanol, and 0.067 M glycine-KCl (pH 9.6). Exonuclease reactions were terminated by the addition of 2 volumes of a solution consisting of 0.45 M NaCl and 0.045 M sodium citrate (pH 7.4). The extent of solubilization of the ^{3}H-labeled N4 DNA was determined by mixing a sample of the 1% (wt/vol) perchloric acid-soluble component of the reaction with Instagel (Packard Corp.). The amount of enzyme necessary to achieve the maximal rate of digestion of 2 μg of DNA in 0.3 ml of exonuclease buffer was determined by incubation of ^{3}H-labeled N4 DNA with increasing concentrations of enzyme. An amount of enzyme twofold in excess of that required to achieve the maximal rate of digestion of ^{3}H-labeled N4 DNA was used in subsequent reactions with EBV DNA. The rate of digestion of ^{3}H-labeled N4 DNA was determined in parallel with each digestion of EBV DNA. At each interval of digestion of EBV DNA with lambda exonuclease, a sample of the reaction was mixed with 2 volumes of 0.45 M NaCl and 0.045 M sodium citrate (pH 7.4). The DNA was annealed at 68°C for 45 min.

Preparation of terminal fragments of EBV

DNA, denaturation, and reannealing. From 5 to 10 μg of EBV DNA was incubated in 0.200 ml with 25 to 50 units of *SalI* or *Eco*RI restriction endonuclease (both obtained from Bethesda Research Laboratories, Bethesda, Md.), and the resultant fragments were separated on 0.4% agarose gels (1 by 28 cm) as described previously (7, 8). The gels contained 0.5 μg of ethidium bromide per ml. The *SalI* D or *Eco*RI DE DNA fragments were identified under UV illumination and cut from the gel in slices. The agarose slice was dissolved by incubation for 15 min at 45°C in 5 volumes of a solution consisting of 5 M sodium perchlorate and 0.1 M Tris-hydrochloride (pH 7.6). The solution was loaded onto a 0.2-ml column of hydroxyapatite (Biogel HTP, Bio-Rad Laboratories, Richmond, Calif.). The column was washed with 2 ml each of 0.04 M PB (PB consists of an equimolar mixture of monobasic and dibasic sodium phosphate) and 0.18 M PB, and the DNA was eluted with 0.4 ml of 0.5 M PB. The DNA was dialyzed at 25°C against a solution consisting of 3 M NaCl, 0.01 M EDTA, and 0.05 M Tris-hydrochloride (pH 7.4), containing Dowex 50 resin in the H+ form to remove the ethidium bromide from the DNA. The DNA was then dialyzed at 5°C against 0.005 M Tris-hydrochloride (pH 7.4). From 0.1 to 0.5 μg of DNA in 0.005 M Tris-hydrochloride (pH 7.5) was concentrated to 20 μl by lyophilization, reconstituted to 50 μl in a solution consisting of 40% vol/vol formamide, 0.36 M NaCl, 0.001 M EDTA, and 0.05 M Tris-hydrochloride (pH 7.4), sealed in a glass micropipette, denatured at 95°C for 5 min, and incubated for 18 h at 58°C.

Electron microscopy of DNA. EBV DNA was spread in aqueous and formamide spreading solutions. φX174, φX174 RFII (both obtained from N. Cozzarelli, University of Chicago), or PM2 (Boehringer Mannheim Corp., Indianapolis, Ind.) DNAs were used as internal standards. For aqueous spreading, 50 μl of a solution consisting of 0.025 μg of EBV and 0.025 μg of φX174 RFII or PM2 DNAs, 10 μg of cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.), 0.001 M EDTA, and 0.5 M ammonium acetate (pH 7.5) was run down a glass slide onto the surface of a hypophase which consisted of 0.25 M ammonium acetate (pH 7.5). For formamide spreading, 50 μl of a solution consisting of 0.025 μg of EBV DNA, 0.025 μg of PM2 DNA, and 0.025 μg of φX174 DNA, 10 μg of cytochrome *c*, 40% (vol/vol) formamide, 0.001 M EDTA, and 0.01 M Tris-hydrochloride (pH 7.6) was run down a glass slide onto the surface of a hypophase which consisted of 10% (vol/vol) formamide, 0.001 M EDTA, and 0.01 M Tris-hydrochloride (pH 7.6). The DNA cytochrome *c* film was transferred to a Parlodion (T. Pella, Inc., Tustin, Calif.) coated copper grid (T. Pella, Inc.) by surface contact and stained with uranyl acetate in 90% ethanol. The grids were shadowed with 80% platinum/20% palladium wire (T. Pella, Inc.) and examined in a Siemens 102 microscope at 60 kV.

Length measurements. The negatives (40 by 60 mm) were enlarged 10 times with a Besseler enlarger (model 4XZ). The DNA was traced and measured with a map reader (model 620-300, Keuffel and Esser Corp., Chicago, Ill.). The length of PM2 DNA used in these experiments was 1.86 ± 0.08 (mean ± 1 standard deviation) × the length of φX174 RFII DNA when both DNAs were photographed on the same negative. The length of single-strand EBV DNA was measured relative to the length of φX174 DNA in formamide on the same negative. The length of double-strand EBV DNA in formamide or aqueous spreads was measured relative to PM2 DNA on the same negative. The molecular weights of single- and double-strand EBV DNA were determined from the length relative to φX174 DNA (26) and PM2 DNA, assuming no difference in the ratio of mass per length between these DNAs of different guanine-plus-cytosine content (5).

RESULTS

Denaturation and reannealing of terminal fragments of EBV DNA. As indicated in Fig. 1, *SalI* and *Eco*RI restriction endonucleases cleave EBV DNA at 9×10^6 and 7×10^6 daltons, respectively, from the long unique terminus (7). The terminal *Eco*RI D and *SalI* D fragments were separated in 0.4% agarose gels, denatured, reannealed, spread in aqueous or formamide spreading solution, and examined in the electron microscope. The molecular weight of single-strand regions was determined from the length relative to circular φX174 DNA, and the molecular weight of double-strand regions was calculated from the length relative to PM2 DNA. The results were as follows. (i) As expected from previous analyses of the size of EBV DNA strands in alkaline sucrose gradients (7, 22), the length of most single-strand DNA molecules was slightly less than φX174 DNA. However, single-strand DNA molecules as long as 4×10^6 or 5

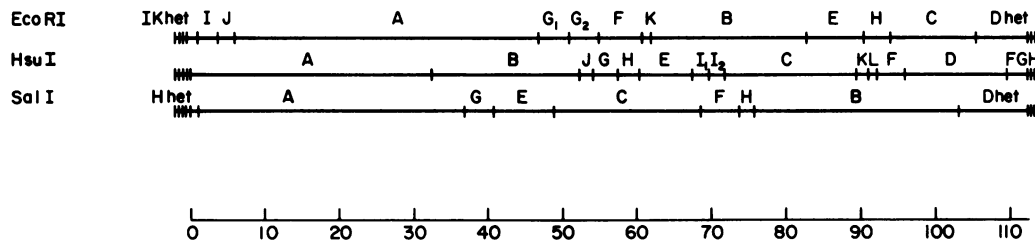

FIG. 1. *Arrangement of EcoRI, HsuI, and SalI restriction endonuclease fragments of EBV (B95-8) DNA (7). Fragments are designated by letter (7) and are drawn in proportion to size (8).*

$\times 10^6$ were occasionally seen in the spreads of *Eco*RI D or *Sal*I D, respectively. No double-strand "hairpin- or pan-handle-like" regions were evident in these molecules or in shorter single strands, indicating that there is no inverted repeat within the terminal segment of the long unique region. (ii) Circular molecules were seen in both aqueous and formamide spreads, indicating the presence of direct repeats within the *Eco*RI D and *Sal*I D fragments (Fig. 2A and B). The mechanism by which direct repeats lead to the formation of circles is shown in Fig. 3A. The circles appeared to consist predominantly of double-strand DNA, although short single-strand regions were apparent in some formamide preparations, and bushes were frequently seen in aqueous spreads. In formamide, almost all circular molecules had two attached single strands emanating from separated points of the circle (Fig. 2A). Most circles in aqueous spreads had at least two discernible bushes (Fig. 2B). In the absence of inverted repeats in these DNA fragments, the frequent finding of circular structures with two attached and separated single strands indicates the presence of a directly repeated sequence. Approximately 10% of the circles had one or two attached double strands (Fig. 2D). The finding of a few circles with one or two double-strand tails presumably arises by hybridization of a third or a fourth DNA strand to the nonrepeated DNA component of the single-strand tail(s) of DNA, which is annealed into circles through its repeated DNA component. (iii) The length of 36 circular molecules with single-strand tails was determined in formamide spreads (Fig. 2C). The size of the smallest circles in formamide was 2.5×10^5 to 3×10^5 daltons, suggesting that the size of the repeated unit was 2.5×10^5 to 3×10^5 daltons. The size of the largest circles was 2.5×10^6 daltons, suggesting that some molecules contain 8 to 10 repeating units. (iv) The largest double-strand DNA attached to a circle in the aqueous spreads was 10×10^6 daltons (Fig. 2D). The largest single strand of DNA attached to a circle in formamide spreads of the *Sal*I D fragment was 4.5×10^6 daltons (Fig. 2E). These data indicate that the direct repeats are at least 9×10^6 daltons from the terminal *Sal*I cleavage site.

Lambda exonuclease digestion and annealing of EBV DNA. To demonstrate that native EBV DNA does not contain complementary single-strand regions that permit circularization, the DNA was incubated under annealing conditions and examined in the electron microscope after spreading in formamide. Three hundred untangled molecules appearing much longer than the internal PM2 form II DNA were surveyed. No circular molecules were seen. Single-strand regions were not discernible, either at the ends of the DNA or internally. The length of 20 DNA molecules clearly larger than the admixed PM2 DNA was 16.4 ± 0.7 PM 2 units (Fig. 4B).

EBV DNA or ^{3}H-labeled N4 DNA in a parallel control was incubated with lambda exonuclease for varying intervals of up to 20 min. After an initial lag of approximately 1 min, the rate of degradation of ^{3}H-labeled N4 DNA was 225,000 daltons per min from each end (Fig. 4C). The exonuclease-digested, annealed EBV DNA was examined in aqueous and formamide spreads. The data obtained on examination of the DNA after 1, 3, 5, 10, and 20 min of lambda exonuclease digestion were as follows. (i) After 1 min of lambda exonuclease digestion, some molecules had a single-strand end as long as 0.3×10^6 to 0.4×10^6 daltons. Occasional molecules (less than 5%) had short, 0.8×10^5- to 2×10^5-dalton, double-strand, hairpin-like structures within the single-strand region (Fig. 5A). Almost all molecules had discernible single-strand tails at only one end. Three hundred DNA molecules clearly larger than the PM2 standard were surveyed. One circular full-length EBV DNA molecule was seen (Fig. 4A). (ii) After 3 min of lambda exonuclease digestion, the longest single-strand region at the end of the DNA molecule was 10^6 daltons. Although almost every molecule had one single-strand end, only 20% of the molecules had clearly discernible single-strand tails at both ends. Approximately 10% of the single-strand ends had a short double-strand region (Fig. 5A). Internal single-strand regions were clearly discernible in some molecules (Fig. 5B). Two full-length circular EBV DNA molecules were seen per 300 DNA molecules clearly larger than PM2 DNA (Fig. 4A). (iii) By 5 min of lambda exonuclease digestion, the longest single-strand ends were 1.6×10^6 daltons and approximately 30% of the molecules had a short double-strand region within one single-strand tail (Fig. 5A). No molecules were seen that had such structures within both single-strand tails. The longest single-strand region observed distal to the double-strand segment was 0.4×10^6, and the longest single strand proximal to the double-strand region was 10^6. Thus, despite the frequent presence of long single-strand regions on both sides of the double strand, hairpin-like structures, multiple double-strand regions separated by single-strand DNA were never observed, indicating that the palindrome could not be within a repeated single-strand DNA unit shorter than 0.4×10^6 daltons. Six circular molecules were seen per 300 molecules that were clearly longer than PM2

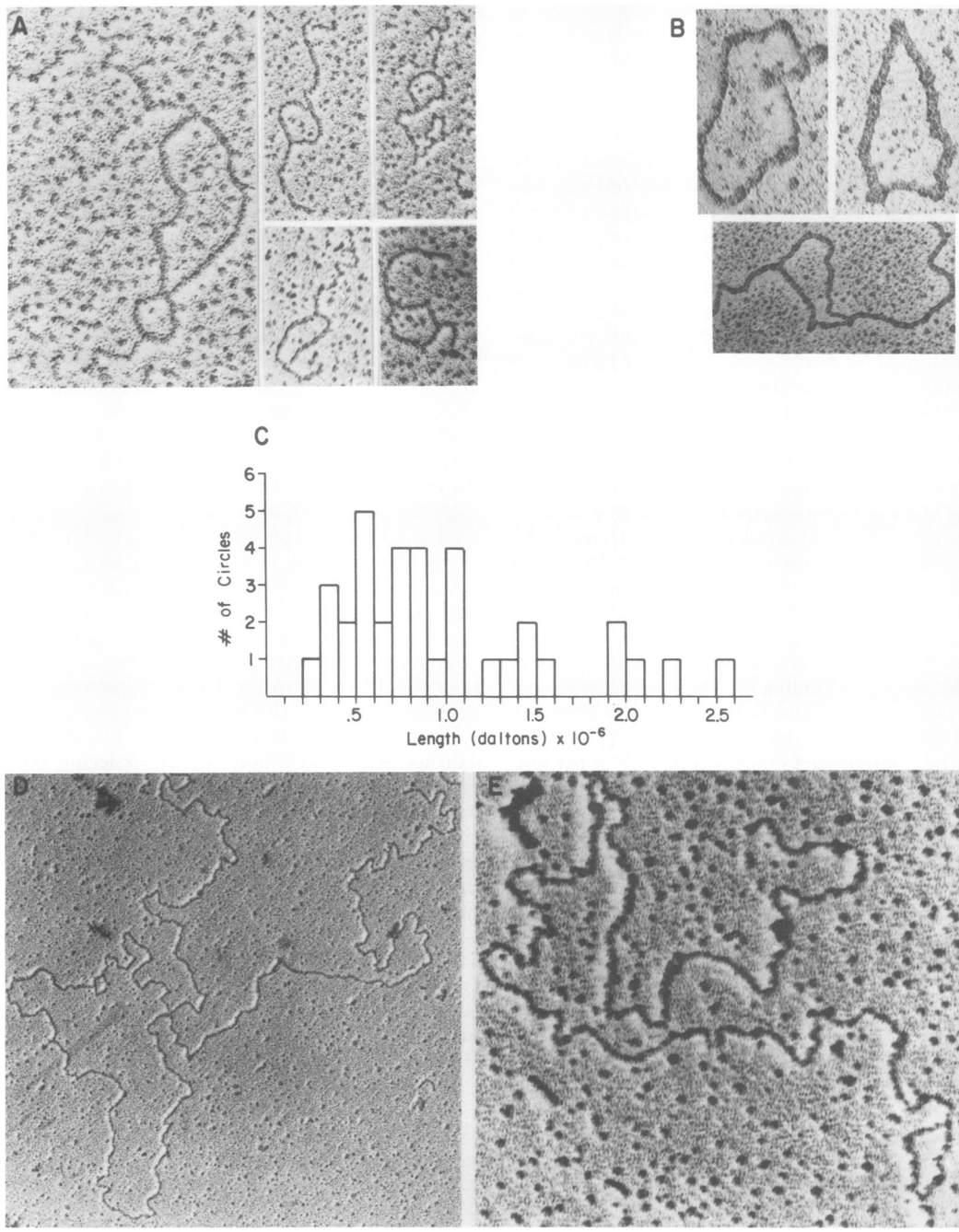

FIG. 2. *Electron photomicrographs and histogram of size of circles formed by the denatured and reannealed SalI D fragment. SalI D is the heterogeneous terminal fragment of the long unique region of EBV (B95-8) DNA. (A) Circular structures with two single-strand tails in formamide spreads. (B) Circular structures with single-strand bushes (above) or two double-strand tails (below) in aqueous spread. (C) Histogram of the size of the circles formed by annealing of the denatured SalI D fragment measured in formamide relative to PM2 form 2 DNA and φX174 DNA as internal markers. (D) Circular structure in aqueous spread with attached double-strand tails of 8×10^6 and 10×10^6 daltons. (E) Circular structure in formamide spread with 4.5×10^6-dalton tail.*

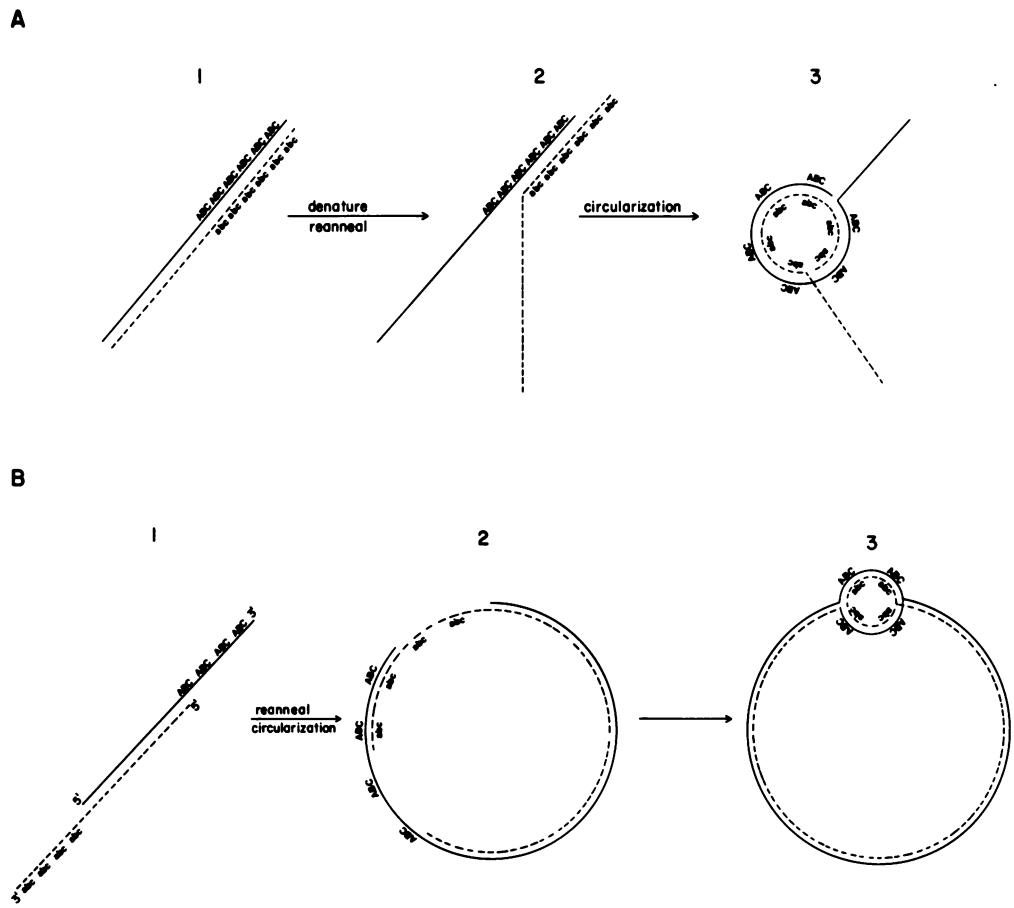

FIG. 3. *(A) Suggested model for circularization of terminal DNA fragments after denaturation and reannealing. The SalI D fragment consists of 9 × 10⁶ daltons of unique DNA and from 1 to 12 copies of a 3 × 10⁵-dalton reiterated segment, designated ABC on one strand and abc on the complementary strand (A1). After denaturation and adjustment to conditions which permit annealing, nucleation may occur in the reiterated segment either out of register or between strands with different numbers of repeats, resulting in an intermediate structure (A2). Further annealing results in partially or fully double-strand circles with two single-strand tails (A3). Double-strand tails may occur if additional DNA strands anneal to the unique DNA. (B) Suggested model for formation of an at least partially double-strand circle at the joint region following annealing of lambda exonuclease-digested EBV DNA. Lambda exonuclease digestion of EBV DNA results in formation of 3′ single-strand tails at opposite ends (B1). The presence of direct repeats at both ends allows annealing between the homologous sequences in the repeated DNA at each 3′ end (B2). If the nucleation site is out of register relative to the unique DNA regions at each end, further annealing may generate a partly double-strand circle at the joint (B3).*

DNA (Fig. 4A, 6, and 7). The length of another 100 molecules clearly longer than PM2 DNA was measured. Ninety-five molecules ranged in size from 7 to 15 PM2 units. Only one linear EBV DNA molecule had a length greater than 15 PM2 units. Three additional full-length circular molecules were seen and are included in the histogram of size of circular molecules (Fig. 4B). Thus, the frequency of circularization of full-length EBV DNA molecules after 5 min of lambda exonuclease treatment was very high. The ends of four circular molecules were joined through circles of 0.7 × 10^6 daltons (Fig. 7). The mechanism by which the reiterated sequences at the ends of EBV lead to the formation of small circles at the juncture between the ends is shown in Fig. 3B. (iv) After 10 or 20 min of lambda exonuclease digestion, single-strand tails as long as 2 × 10^6 daltons were seen at the end of some molecules. Two full-length circular molecules were seen in formamide spreads of DNA annealed after 10 or 20 min of lambda exonuclease digestion. However, most DNA molecules were extensively degraded by 10 min. Thus, by

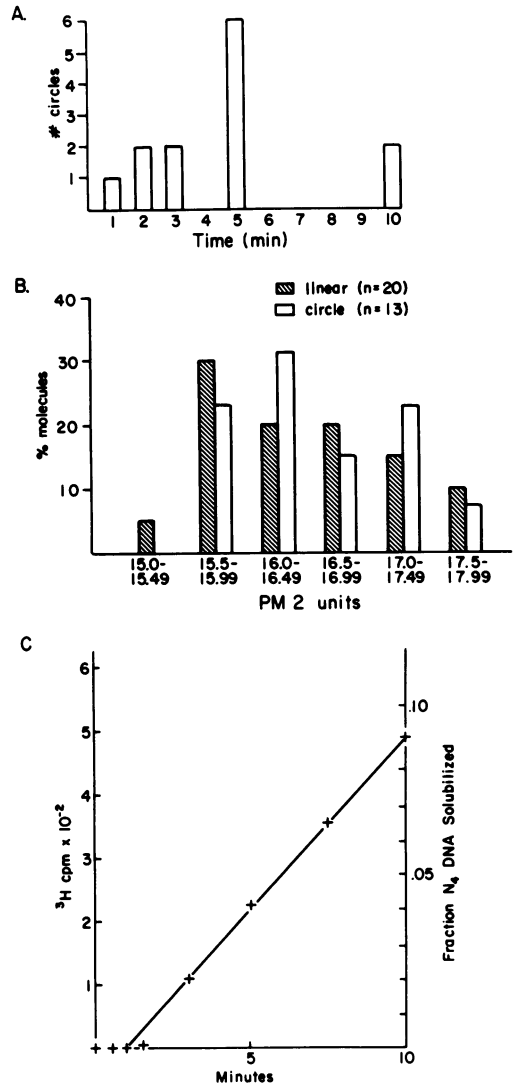

FIG. 4. *(A) Number of circular EBV DNA molecules seen per 300 untangled molecules clearly larger than PM2 DNA after intervals of lambda exonuclease digestion. (B) Histogram of the length of EBV DNA prior to exonuclease digestion and of the length of circular EBV DNA molecules obtained after lambda exonuclease digestion. Measurements are relative to the length of PM2 DNA as an internal standard. (C) Digestion of ^{3}H-labeled N4 DNAs in parallel with the digestion of EBV DNA with lambda 5'-exonuclease. The extent of digestion was assayed by determining the acid-soluble radioactivity.*

10 min of lambda exonuclease digestion, the size of most EBV DNA molecules was between 3 and 5 PM2 units and the length of the single-strand tails of most DNA molecules was 0.5×10^6 to 1×10^6 daltons, probably as a consequence of digestion beyond a nick on the opposite strand, resulting in a loss of the terminal part of the 3' strand. Furthermore, some molecules contained internal single-strand regions of 1×10^6 to 2×10^6 daltons. (v) The size of 13 circular molecules identified in the annealed DNA preparations after various times of lambda exonuclease digestion was 16.5 ± 0.7 PM2 units (Fig. 4B).

DISCUSSION

An unusual finding in this study was the extent to which EBV DNA was digested internally by the lambda exonuclease. Thus, by 3 min of lambda exonuclease digestion, internal single-strand regions were evident in formamide spreads of EBV DNA, and by 5 min the length of native EBV DNA was reduced as a consequence of digestion beyond a nick on the opposite strand. Furthermore, although the concentration of lambda exonuclease used in these preparations was twofold in excess of the amount required to achieve maximal digestion of the ends of N4 DNA, the ends of EBV DNA were frequently not digested after 1 or even 3 min of lambda exonuclease digestion. The lambda exonuclease preparation was free of contaminating endonuclease, and treatment of N4 DNA in parallel with these experiments resulted repeatedly in 25 to 50% frequency of circularization (L. Rothman-Denes, unpublished data). However, EBV DNA differs from N4 DNA in that the DNA obtained from virus which accumulates in the extracellular fluid of B95-8 cultures has single-strand nicks at each 1×10^3 to 3×10^3 bases (7). Although lambda exonuclease has little activity on a nicked DNA template, the activity increases substantially with small gaps (3, 15, 27). It is not known whether there exist small gaps in EBV DNA or whether random nicking of EBV DNA in virions in the extracellular fluid results in the generation of small gaps, as has been demonstrated with herpes simplex DNA (10). Alternatively, the lambda exonuclease preparation could contain a small amount of another exonuclease which could create small gaps at the site of nicks.

Previous data summarized in the Introduction indicated that the ends of EBV DNA consist of a variable number of inverted or direct repeats of a 3×10^5-dalton double-strand DNA segment. The experiments reported here confirm these earlier observations and extend them. Thus, the findings that there is no inverted repeat within 10×10^6 daltons of the terminus of the long unique DNA region and that denatured and reannealed *SalI* D or *Eco*RI D terminal fragments of the long unique region form circles varying from 2.5×10^5 to 25×10^5 daltons in size

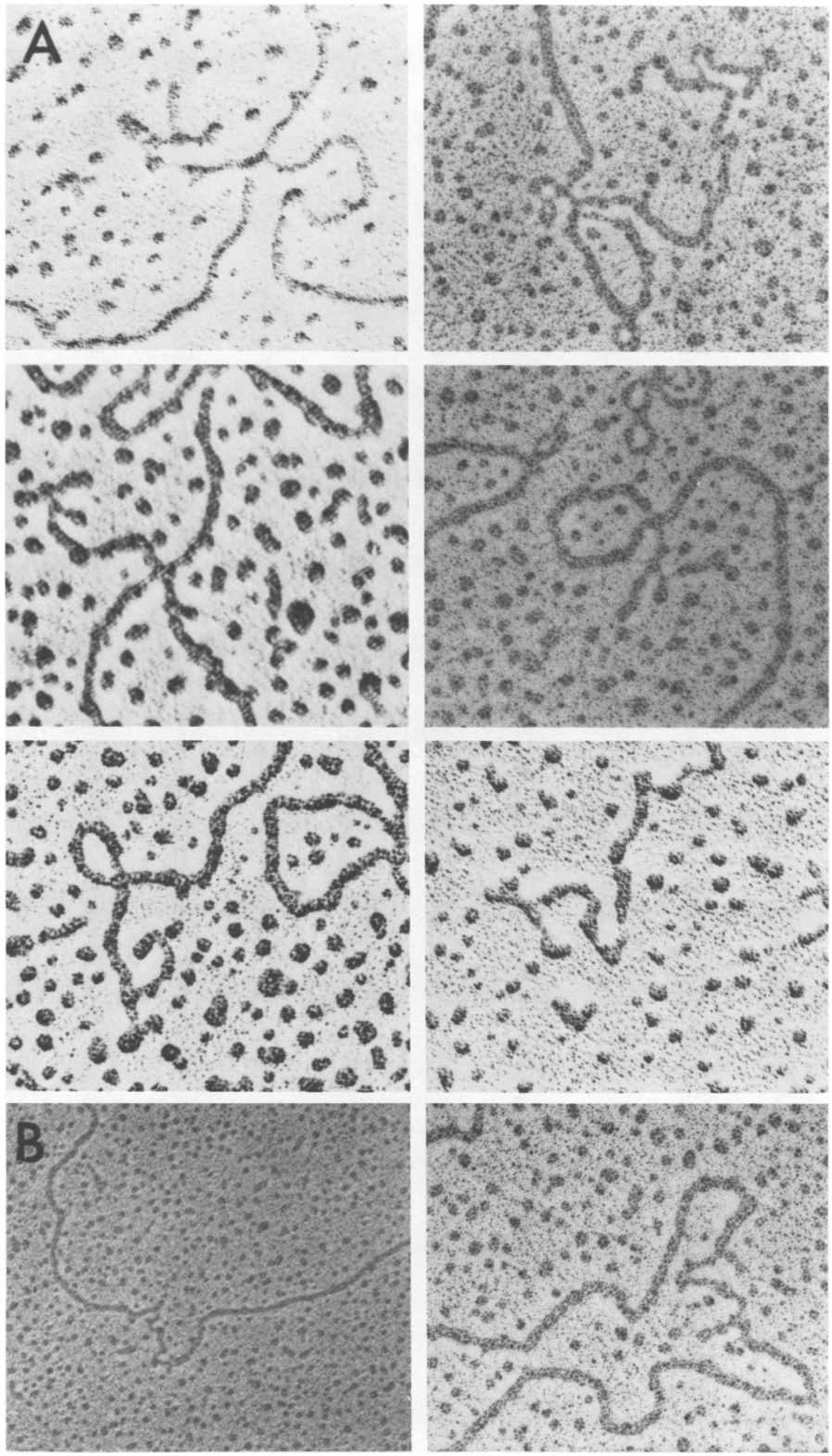

FIG. 5. *(A) Hairpin-like structures found in some single-strand ends generated by lambda exonuclease treatment. (B) Internal single-strand regions visible in EBV DNA after 3 min of incubation with lambda exonuclease.*

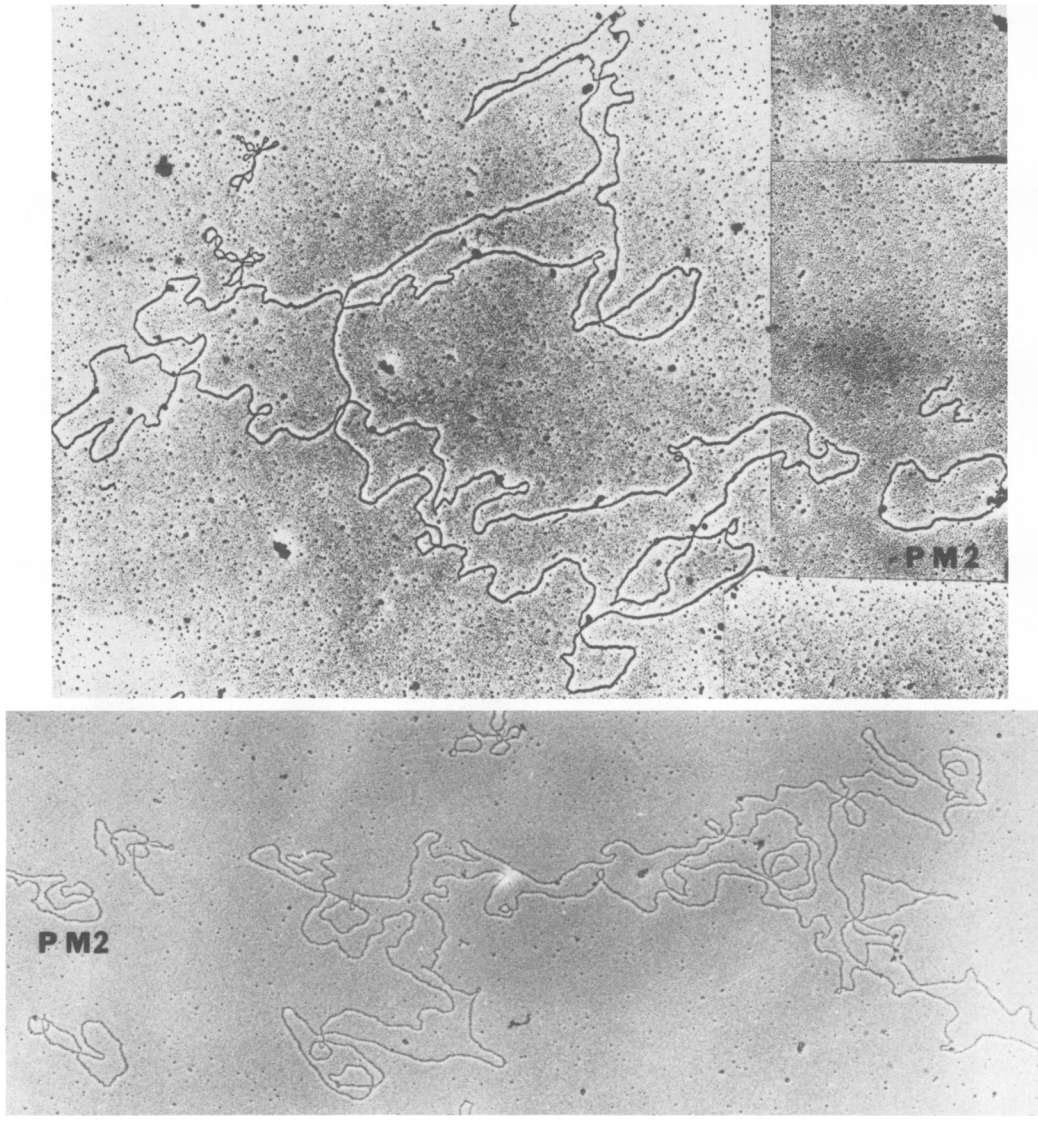

FIG. 6. *Electron micrograph of two circularized EBV DNA molecules spread in formamide after digestion for 5 min with lambda in exonuclease and annealing. The length of these molecules is 16.5 and 17.0 times that of adjacent PM2 DNA.*

indicate that there are direct and not inverted repeats at this terminus. Furthermore, the finding that the ends of EBV DNA treated with lambda exonuclease anneal to form circles at the juncture site indicates that the terminus of the short unique region must also contain direct repeats of the sequence repeated at the terminus of the long unique region.

The significance of the occurrence of a variable number of direct repeats at both ends of EBV DNA is not fully known. At least three mechanisms can be envisaged by which the occurrence of direct repeats at opposite ends of the DNA could lead to the formation of covalently closed circular DNA such as has been found in cells infected with EBV. The direct repeats at opposite ends could be common binding sites for proteins which blunt-end ligate or covalently join the ends through peptide nucleic acid linkages. Alternatively, the infected cell could contain an exonuclease similar to lambda exonuclease or a recombination function similar to P22 which could permit circularization by base pairing between homologous DNA sequences on

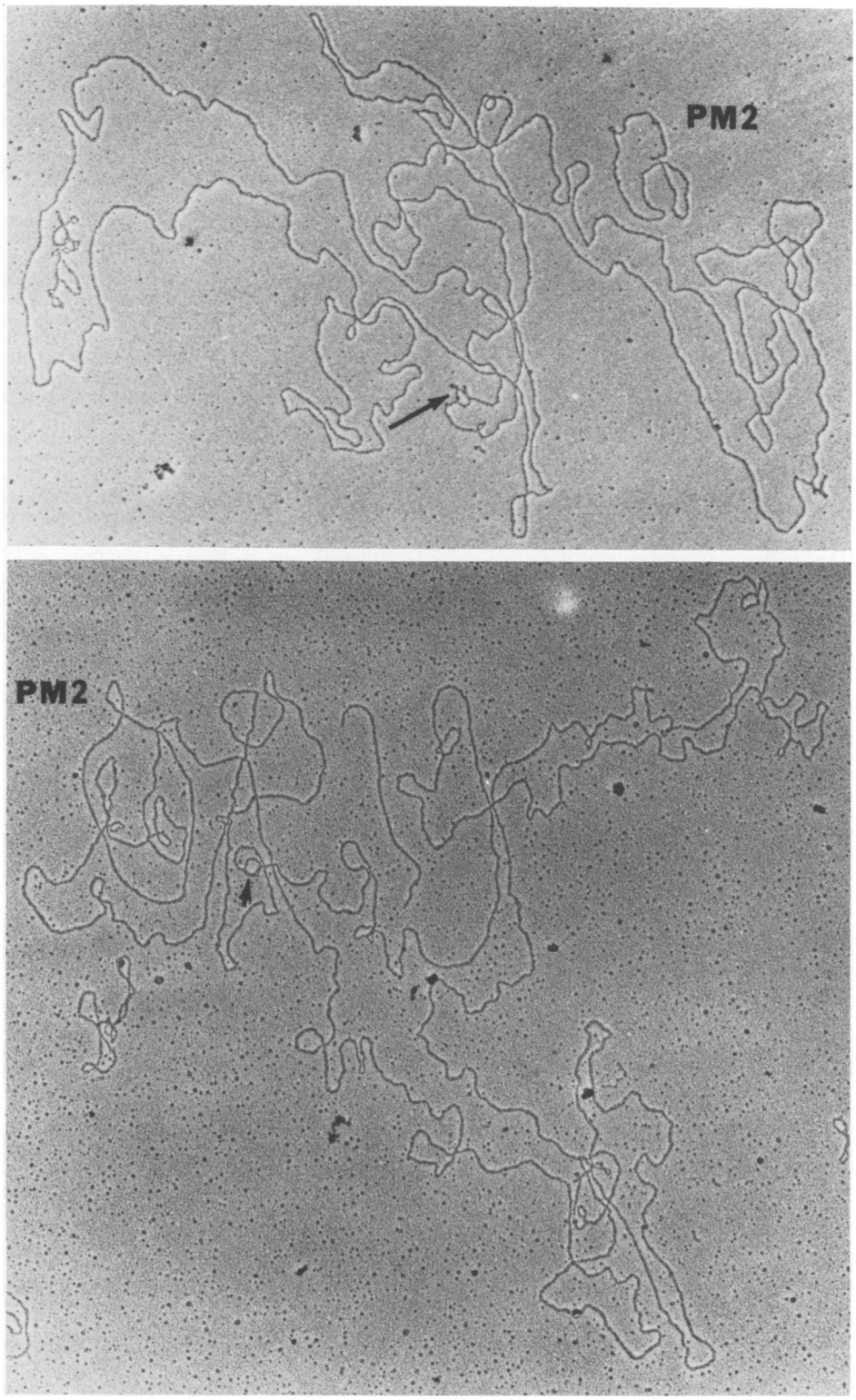

FIG. 7. *Electron micrograph of two circularized EBV DNA molecules spread in formamide after digestion for 5 min with lambda in exonuclease and annealing. The arrow indicates a circle at the juncture between the ends. The length of these molecules was measured relative to adjacent PM2 DNA.*

opposite strands at both ends. Although adenovirus DNA has been shown to have protease-resistant peptide-blocked termini (24), it is unlikely that closed circular EBV DNA contains polypeptide linkages, since treatment of the DNA with proteases does not prevent the isolation of circular molecules (1, 2, 14). Furthermore, the ends of linear EBV DNA are not blocked to lambda exonuclease digestion (8).

Several alternative mechanisms could generate the variation observed between ends of different molecules in the number of copies of the terminal direct repeat. Possibly, the simplest mechanism might be the cleavage of precursor DNA concatemers by limiting concentrations of a nuclease which recognizes a specific site in the reiterated DNA.

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