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Structure of Replicating DNA Molecules of *Bacillus subtilis* Bacteriophage φ29

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We isolated φ29 DNA replicative intermediates from extracts of phage-infected *Bacillus subtilis*, pulsed-labeled with [^{3}H]thymidine, by velocity sedimentation in neutral sucrose followed by CsCl equilibrium density gradient centrifugation. During a chase, the DNA with a higher sedimentation coefficient in neutral sucrose and a lower sedimentation rate in alkaline sucrose than that of viral φ29 DNA was converted into mature DNA. The material with a density higher than that of mature φ29 DNA consisted of replicative intermediates, as analyzed with an electron microscope. We found two major types of molecules. One consisted of unit-length duplex DNA with one single-stranded branch at a random position. The length of the single-stranded branches was similar to that of one of the double-stranded regions. The other type of molecules was unit-length DNA with one double-stranded region and one single-stranded region extending a variable distance from one end. Partial denaturation of the latter molecules showed that replication was initiated with a similar frequency from either DNA end. These findings suggest that φ29 DNA replication occurs by a mechanism of strand displacement and that replication starts non-simultaneously from either DNA end, as in the case of adenovirus.

Bacillus subtilis phage φ29 has a double-stranded DNA of molar mass 11.8×10^6 g mol^{-1} (24) with a protein covalently linked to both 5′ termini (12, 14, 23, 34). The protein attached to the DNA is the product of cistron 3, p3 (23), a virus-coded protein induced early after infection (6, 13), which is essential for DNA replication (5, 11, 30, 33).

Adenovirus DNA also has a protein covalently linked to both 5′ ends (7, 20). Replication of adenovirus starts at either DNA end and proceeds by a strand displacement mechanism (1, 17, 28, 29). Rekosh et al. (20) have proposed a model for the initiation of adenovirus DNA replication in which the terminal protein would act as a primer.

In the case of phage φ29, nothing is known about the origin or mechanism of DNA replication. In this report we show the isolation and an analysis of the structure of φ29 DNA replicative intermediates with an electron microscope. As in adenovirus (17), the two major types of φ29 DNA replicating molecules are unit-length duplex DNA molecules with one single-stranded branch (type I) and unit-length DNA molecules with one double-stranded region and one single-stranded region extending a variable distance from one end (type II). Partial denaturation of type II molecules shows that initiation of replication takes place with a similar frequency from either DNA end. The results presented in this paper suggest that phage φ29 DNA replication occurs by a mechanism of strand displacement initiated non-simultaneously at either end of the DNA.

MATERIALS AND METHODS

Bacteria and phage. Host bacteria were *B. subtilis* 110NA *try*$^-$ *spoA*$^-$ *su*$^-$ and *B. subtilis* 168 MO-99 [*met*$^-$ *thr*$^-$]$^+$ *spoA*$^-$ *su*$^{+3}$ (18). φ29 was the mutant *sus*14(1242) that produces a normal burst and delayed lysis of the bacteria infected under restrictive conditions (5).

Reagents and enzymes. [*methyl*-^{3}H]thymidine and [^{14}C]uracil were obtained from the Radiochemical Centre. Sarkosyl NL97 was a gift from Geigy Chemical Co., and *p*-(hydroxyphenylazo)-uracil was a gift from Imperial Chemical Industries. Nuclease-free pronase, B grade, was from Calbiochem, and fungal proteinase K, chromatographically purified, was from Merck & Co., Inc. Glyoxal was from Aldrich Chemical Co. Proteinase K-treated φ29 DNA, labeled with [^{14}C]uracil, was a gift from R. P. Mellado.

Isolation of replicating φ29 DNA molecules. *B. subtilis* 110NA *su*$^-$ was grown at 30°C in defined medium (6). When the cell concentration was 10^8/ml, the bacteria from 15 ml of culture were concentrated fivefold in defined medium containing 1 mM amino acids, uridine (200 μg/ml), and *p*-(hydroxyphenylazo)-uracil (100 μg/ml) to inhibit host DNA replication (4) and infected with mutant *sus*14(1242) at a multiplicity of 10. At 50 min postinfection, [^{3}H]thymidine (100

μCi/ml) was added, and 1.5 min later the incorporation was terminated by adding 25 mM sodium azide and by placing the cell culture in a Dry Ice bath. After centrifugation, the cell pellet was suspended in 0.5 ml of a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7), 0.2 mM EDTA, and lysozyme (2.5 mg/ml) and incubated for 3 to 4 min to allow the lysis of the infected bacteria. A buffer (0.5 ml) containing 1 mM HEPES (pH 7), 0.2 mM EDTA, and 2% sodium dodecyl sulfate was then added, and the mixture was incubated for 1 h at 37°C to release the DNA from the bacterial membrane (R. P. Mellado and M. Salas, unpublished data). The sample was placed on top of a 39-ml linear 5 to 20% sucrose gradient in 1 mM HEPES (pH 7)–0.2 mM EDTA–0.1% sodium dodecyl sulfate and centrifuged for 17 h at 18,000 rpm and 20°C in an SW27 rotor. Fractions of about 1 ml were taken from the top of the gradient, and the acid-insoluble radioactive material was determined in a sample. The fractions containing both unit-length and replicating DNA molecules, the latter with a sedimentation coefficient higher than that of native DNA, were pooled and treated with glyoxal to prevent base pairing and pronase as described previously (17). After phenol extraction, the sample was concentrated with 2-butanol (26) to a volume of about 3 ml; Sarkosyl was added to a final concentration of 0.1%, and the density was adjusted with solid CsCl to about 1.71 g/cm^3. After centrifugation for 60 h at 40,000 rpm in a Beckman type 65 rotor, fractions were collected from the bottom of the tube and the total radioactivity was determined in a sample of each fraction. Density was determined in selected fractions of the gradient. The fractions indicated below were pooled and dialyzed against a buffer containing 10 mM Tris-hydrochloride (pH 7.5) and 1 mM EDTA.

Electron microscopy. The formamide-cytochrome *c* spreading technique (8, 31) was used, with 80 mM Tris-hydrochloride (pH 8)–7 mM EDTA–30 mM NaCl–50% formamide–0.007% cytochrome *c* in the spreading solution and redistilled water in the hypophase.

For the partial denaturation experiments, the DNA solution containing 35 mM Tris-hydrochloride (pH 8), 15 mM EDTA, 70 mM NaCl, and 2.5% glyoxal was heated for 1 min at 66°C and immediately quenched in ice water. Formamide and cytochrome *c* to final concentrations of 50 and 0.007%, respectively, were then added, and the DNA was spread over a hypophase of redistilled water.

The film was picked up on standard carbon-coated grids, stained with uranyl acetate, and rotary shadowed with platinum-carbon.

Micrographs were taken in a JEOL 100B electron microscope at 80 kV with a magnification of 10,000, determined with a carbon grating replica of 2,160 lines/mm from Balzers Union.

The contour lengths of the DNA molecules were measured with an SAC Digitizer, and the data were processed in a PDP 11/45 minicomputer with a DOS/BATCH operating system.

RESULTS

Sedimentation analysis of φ29 DNA replicative intermediates. Figure 1 shows the sedimentation analysis of the DNA synthesized during a 1.5-min pulse with [^{3}H]thymidine by phage φ29-infected *B. subtilis* at 50 min postinfection. At neutral pH, a main peak of radioactivity cosedimented with mature φ29 ^{14}C-labeled DNA used as a marker (Fig. 1A), but there was also some radioactive material with a sedimentation coefficient greater than that of unit-length φ29 DNA. This material disappeared after a chase with an excess of cold thymidine (Fig. 1B). When the pulse-labeled material was centrifuged through an alkaline sucrose gradient, most of the radioactivity sedimented at a rate lower than that of mature φ29 DNA (Fig. 1C). Since no radioactive material sedimented faster than unit-length φ29 DNA, the existence of covalent circles or structures resulting from a hairpin mechanism in the replication of φ29 DNA is unlikely. The radioactive material sedimenting slower than mature φ29 DNA disappeared after a chase and was converted into unit-length DNA (Fig. 1D). Uninfected bacteria, pulse-labeled and chased under the same conditions as the φ29-infected cells, produced no radioactive material under the peak of φ29 DNA (Fig. 1A to D). The above results suggested that the material with a sedimentation rate greater than that of mature φ29 DNA in a neutral sucrose gradient and lower than that of phage DNA in an alkaline gradient consisted of φ29 DNA replicative intermediates.

To determine the density of the [^{3}H]DNA which sedimented in a neutral sucrose gradient in the region corresponding to both mature φ29 DNA and replicative intermediates, the fractions collected from a preparative gradient were pooled, treated with glyoxal and pronase as described above (17), and subjected to equilibrium centrifugation in CsCl. Figure 2A shows that the DNA appeared in a peak with an average density higher than that of mature φ29 DNA. The increased buoyant density of replicating φ29 DNA molecules could have been due to the formation of single-stranded DNA tails in the replication process (see below).

Electron microscopy of φ29 replicative intermediates. The fractions corresponding to mature φ29 DNA and replicative intermediates, labeled in a 1.5-min pulse at 50 min postinfection and separated in a preparative neutral sucrose gradient as described above, were pooled, treated with glyoxal and pronase, and centrifuged to equilibrium in CsCl (Fig. 2B). Fractions 9 to 15, 19 to 24, and 25 to 29 were pooled, dialyzed, and examined with the electron microscope as described above. The DNA in fractions 25 to 29 consisted mainly of mature double-stranded molecules. Fractions 9 to 15 contained

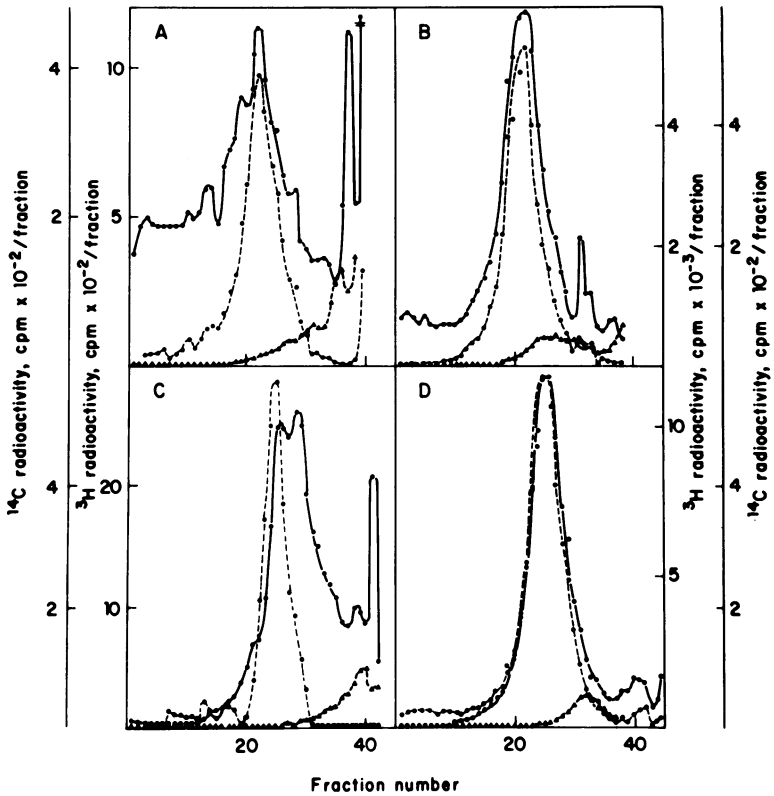

FIG. 1. *Neutral and alkaline sucrose gradients of φ29-infected B. subtilis, pulse-labeled and chased. B. subtilis 110NA su⁻ (1 ml) was infected with mutant sus14(1242) and, after 50 min, labeled with [^{3}H]thymidine (100 μCi/ml, 46 Ci/mmol) as described in the text. At 51.5 min, a sample was removed, a 200-fold excess of nonradioactive thymidine was added to the remaining material, and the incubation was continued for 20 min. After lysis, all of the samples were incubated for 3 h at 37°C in the presence of 1% sodium dodecyl sulfate and proteinase K (200 μg/ml). [^{14}C]labeled φ29 DNA (6,000 cpm) was added to each ^{3}H-labeled sample, and they were sedimented in either neutral or alkaline 5 to 20% sucrose gradients in an AH650 rotor of a Sorvall ultracentrifuge either for 2 h (neutral gradients) or for 2.5 h (alkaline gradients) at 42,000 rpm and 20°C. Fractions were collected from the bottom of the gradient, and the trichloroacetic acid-precipitable radioactivity was determined. A control of uninfected cells was pulse-labeled and chased under the same conditions as the infected B. subtilis. (A) Neutral gradient, pulse; (B) neutral gradient, chase; (C) alkaline gradient, pulse; (D) alkaline gradient, chase. Symbols: ●——●, ^{3}H radioactivity, infected cells; ▲- - -▲, ^{3}H radioactivity, uninfected cells; ○- - -○, ^{14}C radioactivity. Sedimentation is from right to left.*

single-stranded DNA with or without a small double-stranded region at one end; most of these molecules were shorter than unit-length φ29 DNA and were not analyzed further. Fractions 19 to 24 contained replicative intermediates. Figures 3 and 4 show some typical electron micrographs. As a control, more than 99% of *B. subtilis* DNA, labeled with [^{3}H]thymidine before infection, sedimented to the bottom of a neutral sucrose gradient and did not overlap with the main peak of mature φ29 DNA and replicative intermediates.

As in the case of adenovirus (17), two major types of structures were found in the peak of φ29 DNA replicative intermediates. We will designate them type I and type II, in accordance with the nomenclature used for adenovirus. Type I molecules consisted of linear double-stranded DNA, mostly with one single-stranded tail (Fig. 3A), although more than one single-stranded tail was also seen at a lower frequency (Fig. 4A and B and Table 1). Type II molecules consisted of linear DNA partially double stranded and partially single stranded (Fig. 3B). A combination of type I and type II molecules, designated type I/II molecules (17), was also observed. They consisted of linear molecules partially double stranded and partially single stranded, with a single-stranded tail coming out from the double-stranded region (Fig. 3C). Type I/II molecules

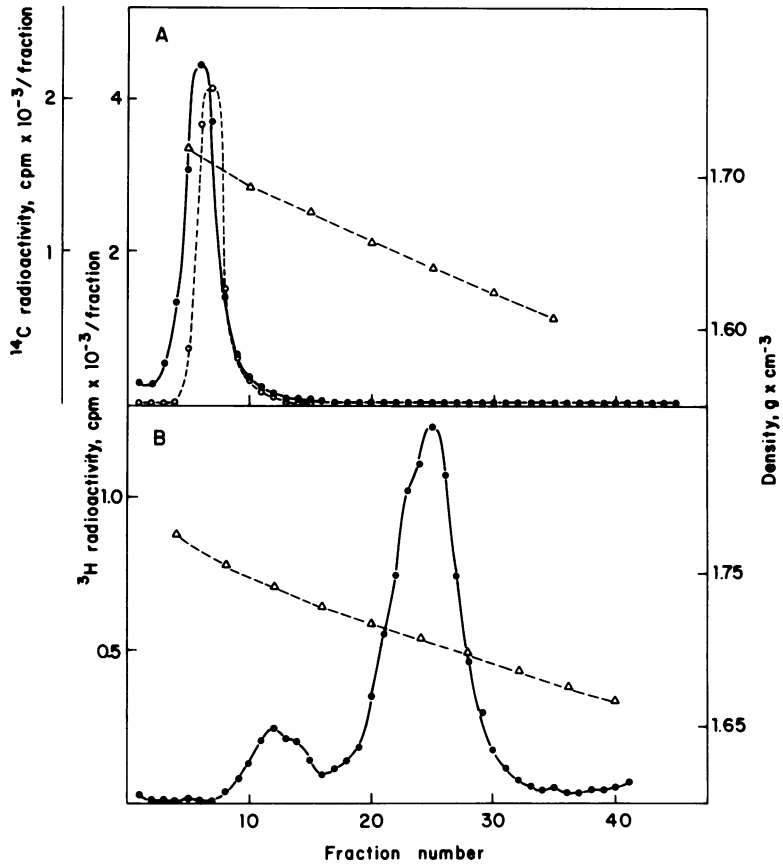

FIG. 2. *Cesium chloride gradient centrifugation of pulse-labeled φ29-infected B. subtilis. B. subtilis 110NA su⁻ (3 ml) was infected with mutant sus14(1242) and, after 50 min, pulsed for 1.5 min with [^{3}H]thymidine (46 Ci/mmol, at a concentration of 40 μCi/ml in [A] or 100 μCi/ml in [B]). At 51.5 min, the cells were lysed, treated with 1% sodium dodecyl sulfate for 1 h at 37°C, and centrifuged in a 5 to 20% neutral sucrose gradient (39 ml of total volume) as described in the text. A marker of φ29 ^{14}C-labeled DNA (50,000 cpm) was added in (A) before centrifugation. The fractions containing unit-length φ29 DNA and replicative intermediates were pooled, treated with glyoxal and pronase, and centrifuged in a CsCl gradient to equilibrium as described in the text. Fractions were collected from the bottom of the tube, and the trichloroacetic acid-precipitable radioactivity was determined. The density of selected fractions of the gradient was previously determined. (A) Analytical gradient; (B) preparative gradient. Symbols: ●——●, ^{3}H radioactivity; ○- - -○, ^{14}C radioactivity; △- - -△, density.*

with two single-stranded tails were also seen, although very rarely (Fig. 4C).

Table 1 shows that type I, type II, and type I/II molecules accounted for about 84% of the total number of molecules scored. Type II molecules were the most abundant (55%), and type I molecules were present in a smaller proportion (20%). If the total number of single-stranded tails in type I molecules is considered, the number (63 molecules) increases to 24% of the total, although it does not reach the expected value of 55%. This is probably because type II molecules are better unfolded than type I molecules and more non-analyzable molecules, which were not scored, were found for type I than for type II molecules. Type I/II molecules accounted for 9% of the total. Another class of molecules observed, which we designate type III molecules (14% of the total), consisted of linear DNA partially double stranded, with single-stranded regions at both ends (Fig. 3D), and linear DNA molecules with double-stranded regions at the two ends and an internal single-stranded portion (type IV molecules; 2% of the total). Although in a small proportion, molecules with branch migration were also found (Fig. 4A).

Length determination of φ29 replicative intermediates. Length measurements were made for types I, II, I/II, and III molecules, since they were present in greater amounts. Figure 5A

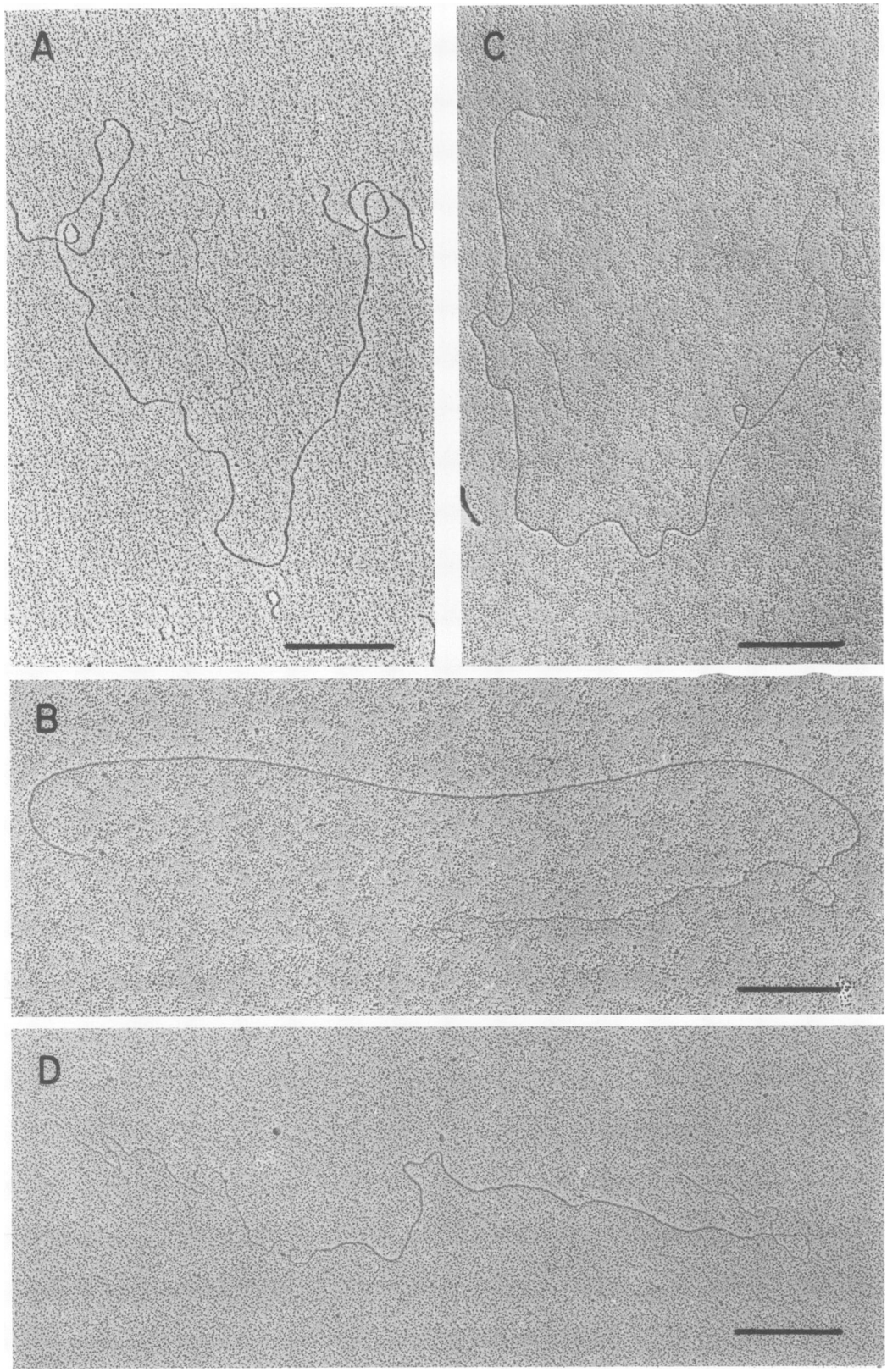

FIG. 3. *Electron micrographs of replicating φ29 DNA molecules. (A) Type I molecule with one single-stranded tail; (B) type II molecule; (C) type I/II molecule with one single-stranded tail; (D) type III molecule. Bar = 0.5 μm.*

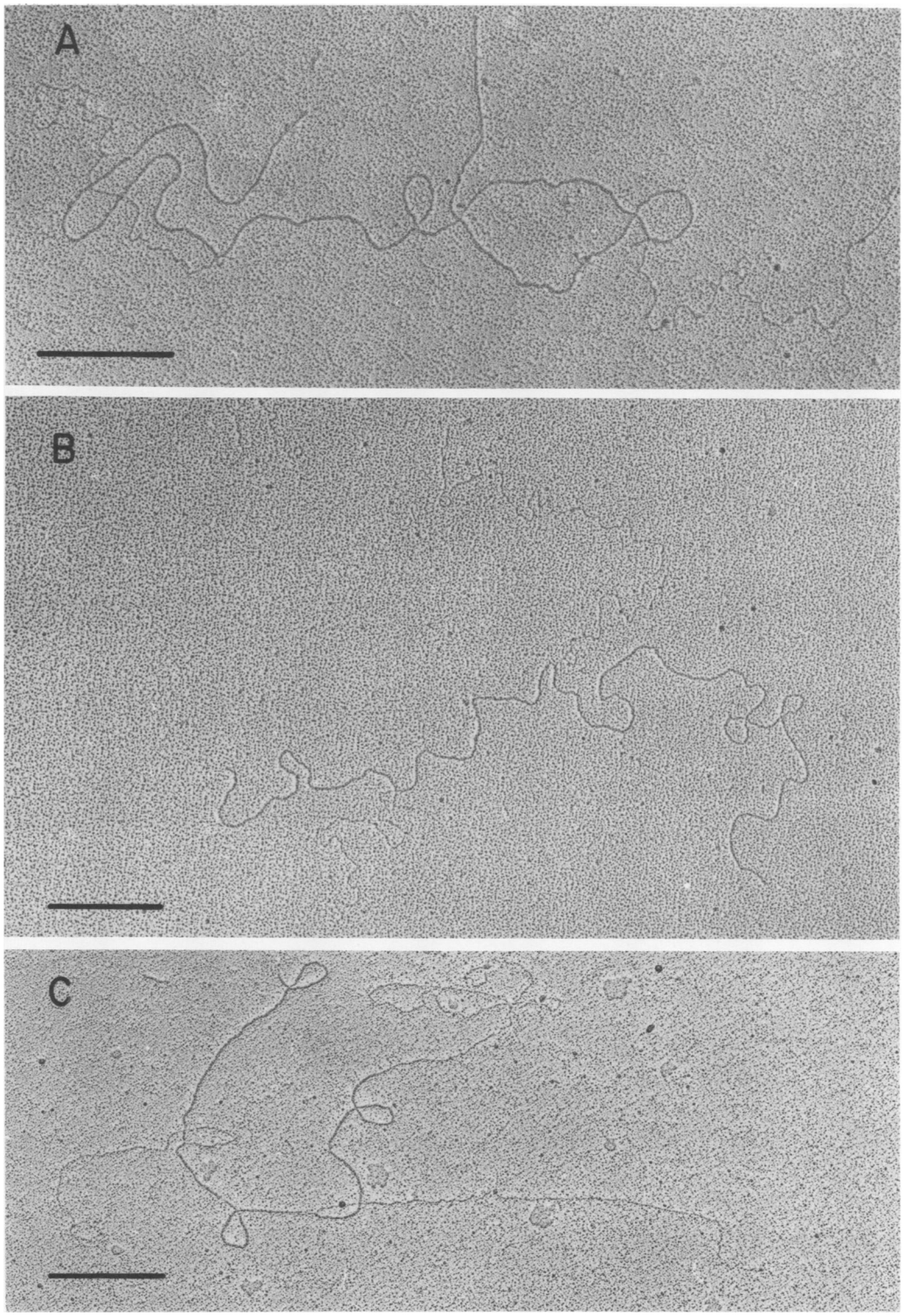

FIG. 4. *Electron micrographs of type I or type I/II replicating ϕ29 DNA molecules with two single-stranded tails. (A) Type I molecule with two single-stranded tails moving in opposite directions; (B) type I molecule with two single-stranded tails moving in the same direction; (C) type I/II molecule with two single-stranded tails moving in the same direction. Bar = 0.5 μm.*

shows a histogram of the length of the double-stranded region in type I molecules, with an average value of 7.02 ± 0.47 μm. This length was greater than that (6.26 ± 0.18 μm) reported previously for φ29 DNA (24), which was determined using ethidium bromide as a spreading agent (16). This difference was probably due to the different technique used here to spread the DNA. Figure 5B shows the ratio between the length of the double-stranded region with the value more similar to that of the single-stranded tail and the total double-stranded DNA length. As expected, there was a random distribution of the intersections of the single-stranded tails with the duplex region in type I molecules.

TABLE 1. *Approximate frequencies of the different types of replicating φ29 DNA molecules*

DNA molecule	No.	% of analyzable molecules[a]
Type I		
One single-stranded branch	43	20
Two single-stranded branches	10	
Type II	145	55
Type I/II		
One single-stranded branch	22	9
Two single-stranded branches	1	
Type III	36	14
Type IV	6	2

[a] About 28% of the total number of molecules were non-analyzable.

The length of the single-stranded tails in type I and type I/II molecules and the corresponding double-stranded regions was also determined. Figure 5C shows that when the ratio of single- to double-stranded DNA length was plotted, there was a maximum at a value of 1. These data suggest that initiation of replication occurs at or near the end(s) of φ29 DNA. The distribution obtained, mainly toward the positions with a ratio smaller than 1, was probably due to the difficulty in visualizing clearly the ends of single-stranded DNA regions and to a suscepti-

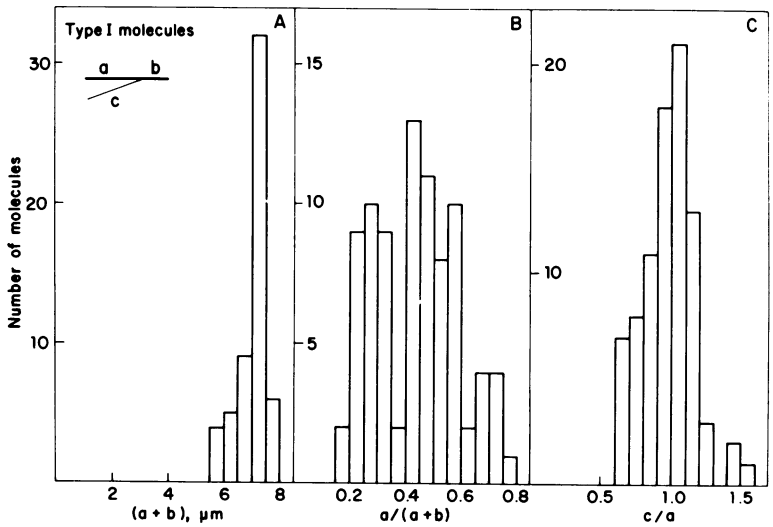

FIG. 5. *Length distribution, position of the growing points, and length of single-stranded branches in type I replicating φ29 DNA molecules. Replicating φ29 DNA molecules were isolated and prepared for electron microscopy as described in the text. In the drawings of the different types of molecules in this and the following figures, the heavy line represents double-stranded DNA and the light line represents single-stranded regions. (A) Length of the double-stranded DNA in 56 type I molecules. (B) Position of the growing points in type I molecules. The histogram represents the ratio between the length of the double-stranded region with a length similar to that of the single-stranded tail (a) and that of the total length of duplex DNA (a + b). Fifty-six type I molecules with a total of 67 growing points were measured. The determination of the growing points of 18 type I/II molecules was also included in the histogram. In this case, the ratio between the length of the double-stranded region with a length similar to that of the single-stranded tail and that of the total length of the DNA (double and single stranded) was determined. (C) Length of single-stranded branches in type I molecules. The histogram represents the ratio between the length of the single-stranded tail (c) and that of the double-stranded region with a similar length (a). Fifty-six type I molecules with a total of 67 tails were measured. The length of the single-stranded branches of 18 type I/II molecules was also included in the histogram.*

bility to degradation greater for single-stranded than for double-stranded DNA.

Figure 6A shows the length distribution of type II and type I/II molecules. The lengths of double- plus single-stranded regions (excluding the single-stranded tails in type I/II molecules) gave an average value of 7.15 ± 0.70 μm, similar to that obtained for type I molecules. The point of transition between double- and single-stranded regions in type II and type I/II molecules was randomly located (Fig. 6B), although molecules with a longer duplex DNA region were more abundant than those with a longer single-stranded region. This result was probably due to the fact that molecules with long single-stranded DNA regions were mainly present in the peak corresponding to fractions 9 to 15 of Fig. 2B.

The length of the double- and single-stranded regions in type III molecules was 7.13 ± 0.45 μm (Fig. 7A), similar to the values obtained for type I and type II molecules. The length of each of the single-stranded regions in type III molecules is shown in Fig. 7B. Since no defined maximum was detected, there seemed to be a random distribution in the transition points between single- and double-stranded DNA regions.

Partial denaturation map of type II replicating DNA molecules. The small number of type I molecules with two single-stranded tails, each growing from a different end (6 out of a total of 56), does not permit the conclusion that replication can be initiated at either DNA end. To test this possibility, partial denaturation of replicating DNA molecules was carried out, and the map of type II molecules was obtained. Figure 8A shows the partial denaturation map of φ29 DNA taken from the work of Sogo et al. (25). It can be seen that the right end was always open, whereas the left end was closed in most cases. Figure 8B and C shows the partial denaturation maps of type II molecules in which the partially denatured double-stranded region, cor-

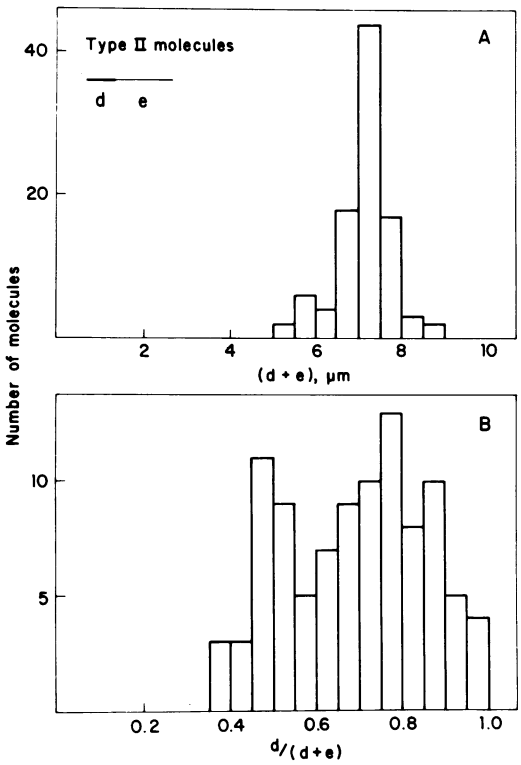

FIG. 6. *Length distribution and position of the growing points in type II replicating φ29 DNA molecules. Replicating φ29 DNA molecules were isolated and prepared for electron microscopy as described in the text. (A) Length of the double- and single-stranded unbranched regions in 74 type II and 23 type I/II molecules. (B) Position of the transition between double- and single-stranded regions in type II molecules. The histogram shows the ratio between the double-stranded region and that of the total DNA length in 74 type II and 23 type I/II molecules.*

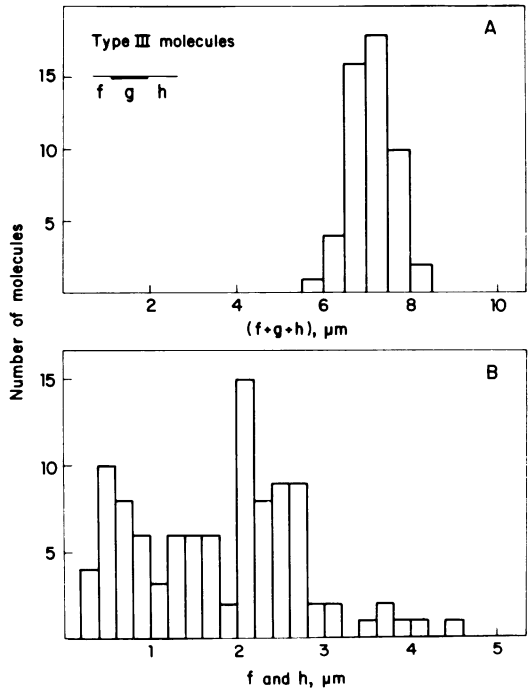

FIG. 7. *Length of the total DNA and of the single-stranded regions in type III replicating DNA molecules. Replicating φ29 DNA molecules were isolated and prepared for electron microscopy as described in the text. (A) Total DNA length (f + g + h) in 51 molecules. (B) The length of each of the two single-stranded regions (f and h separately) in 51 molecules was plotted in the histogram.*

responding to the initiation of replication, could be clearly assigned to the left (Fig. 8B) or right (Fig. 8C) DNA ends. A total of 32 and 23 type II molecules were found to initiate at the left and right DNA ends, respectively. Figure 9 shows electron micrographs of partially denatured type II molecules with one end closed (Fig. 9A) and one end open (Fig. 9B), corresponding to the left and right ends of φ29 DNA, respectively.

DISCUSSION

Pulse-chase experiments suggest that the pulse-labeled radioactive DNA with a sedimentation coefficient greater than that of mature

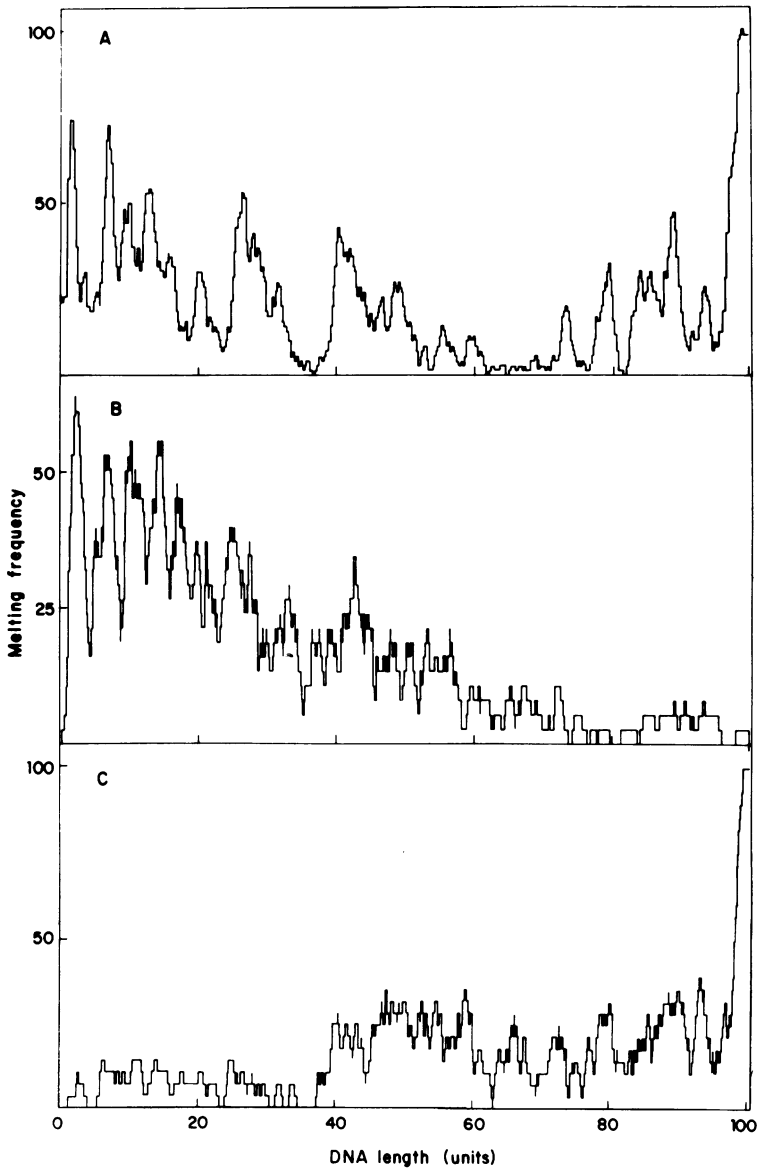

FIG. 8. *Partial denaturation maps of type II replicating DNA molecules. (A) Partial denaturation map of φ29 DNA taken from the work of Sogo et al. (25). (B) Partial denaturation map of 32 type II replicating DNA molecules with a denaturation degree of 25%. The double-stranded region, corresponding to the initiation of the replication, can be assigned to the left end of the DNA. (C) Partial denaturation map of 23 type II replicating DNA molecules with a denaturation degree of 22%. In this case, it can be seen that the double-stranded region corresponds to the right end of the whole genome. Five mature φ29 DNA molecules, partially denatured, were included in (B) and (C) to determine clearly the ends of the histograms.*

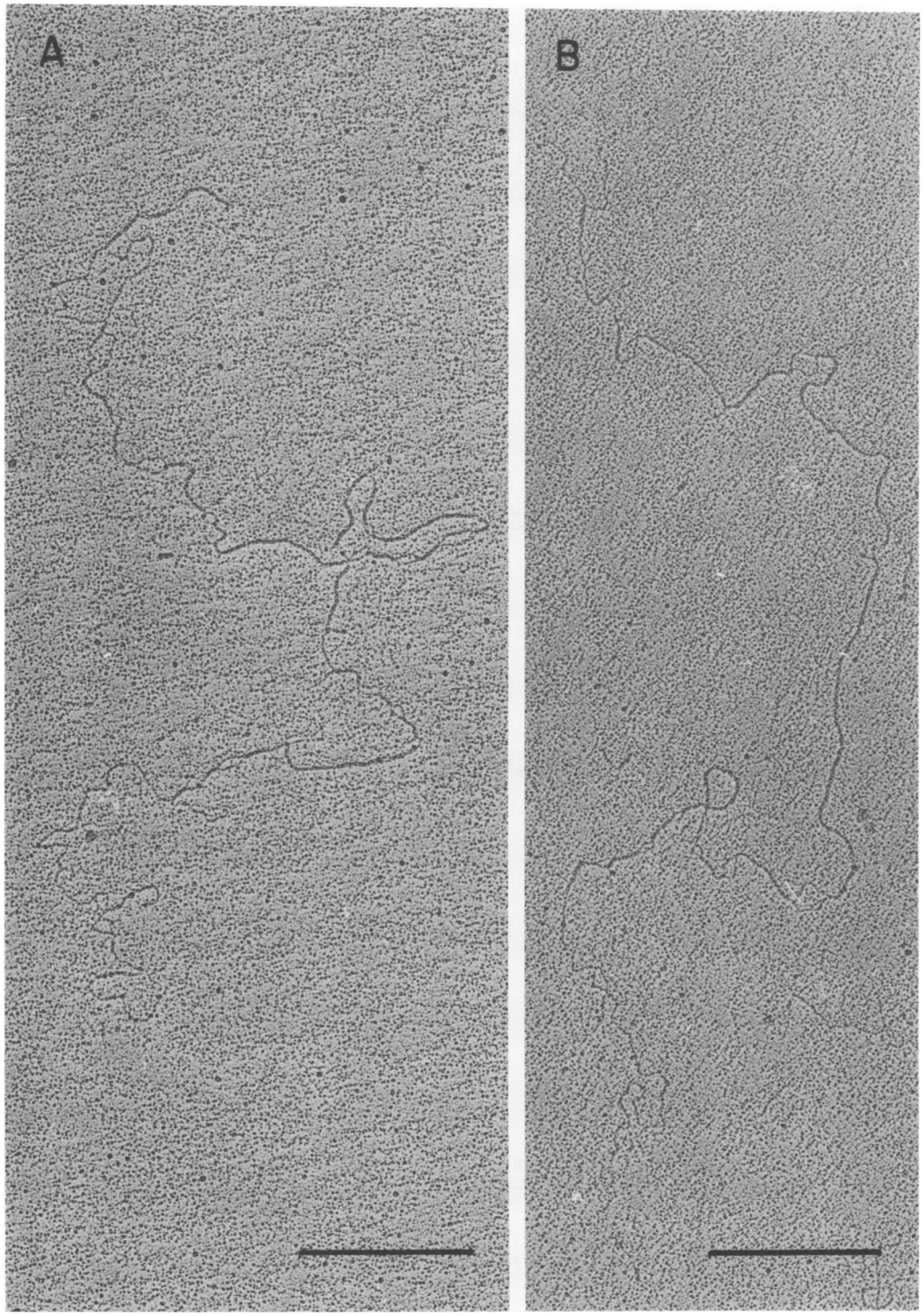

FIG. 9. *Electron micrographs of partially denatured type II molecules. (A) Partially denatured molecule in which the double-stranded end is closed, corresponding to initiation at the left end of the genome. (B) Partially denatured molecule in which the double-stranded end is open, corresponding to initiation at the right end of the genome. Bar = 0.5 μm.*

DNA consists of replicative intermediates.

When the DNA with a buoyant density in CsCl greater than that of phage φ29 DNA was analyzed with the electron microscope, type I, type II, and type I/II molecules were found. Length measurements of these molecules suggest for φ29 replication a displacement mechanism similar to the one postulated for adenovirus type 2 with initiation at or near the ends of the DNA (17). Evidence that initiation of φ29 DNA replication may take place at either end of the DNA has been obtained by partial denaturation of type II replicating DNA molecules. Since type I DNA molecules with two single-stranded branches corresponding to the displaced parental strands initiating simultaneously at the two DNA ends are found very rarely, it may be suggested that initiation of φ29 replication takes place at or near either DNA end, but non-simultaneously.

For the replication of the displaced parental strand, two possible nonexclusive mechanisms were postulated for adenovirus type 2 (17): (i) before the parental strand has been completely displaced, initiation would take place at the opposite end of the DNA molecule; (ii) the parental strand would be completely displaced, giving rise to a double-stranded DNA molecule and a single-stranded DNA molecule (Fig. 10A). Since the 3′ ends of the two DNA strands of adenovirus types 2 and 5 have the same sequence of 102 and 103 nucleotides, respectively (2, 27), the initiation of the replication at the 3′ end of the displaced strand could take place through circle formation by hybridization of the complementary sequence at the 5′ and 3′ termini of the DNA (17). In any case, a newly synthesized molecule of the terminal protein of adenovirus DNA could recognize the 3′ ends and prime replication by the covalent attachment of the initiating nucleotide (20).

In phage φ29 DNA, there is no evidence for the existence of a large region of inverted terminal repetition, as in adenovirus DNA. Denaturation of protein-free φ29 DNA and renaturation at a low DNA concentration did not give rise to the formation of single-stranded circles (A. Talavera, personal communication) under conditions in which circles were produced with adenovirus DNA (9, 32). The partial denaturation map of φ29 DNA indicates that under conditions in which the right end is open, the left end remains closed (25). Nevertheless, the existence of a small region of inverted terminal repetition cannot be ruled out. A nucleotide sequence determination of both φ29 DNA ends is being carried out to answer this question.

An alternative mechanism to initiate φ29 DNA replication would be that a newly synthesized molecule of the terminal protein of φ29

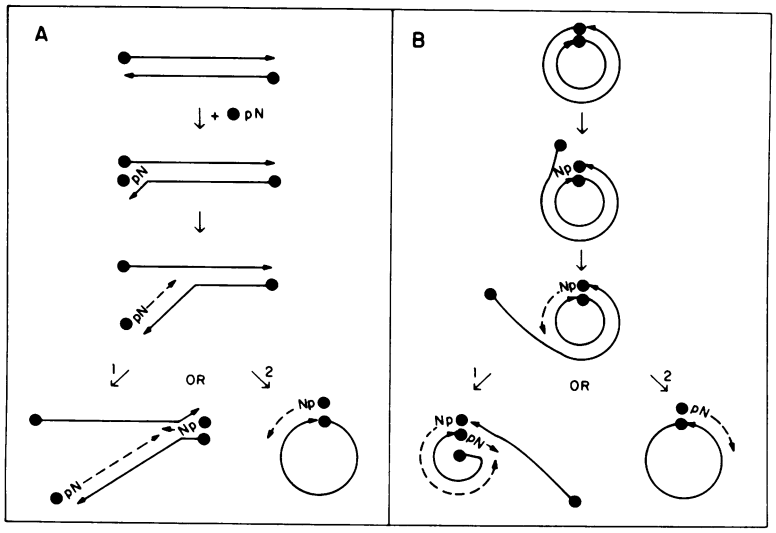

FIG. 10. *Possible models for φ29 DNA replication and for the role of the terminal protein in initiation of replication. The model is an adaptation of those proposed by Rekosh et al. (20) and Lechner and Kelly (17), considering replication on linear DNA (A) or circular DNA (B). The dots at the 5′ ends of the DNA represent the terminal protein. The dots followed by pN represent the protein linked to the initiating nucleotide (or deoxynucleotide) that is providing the free 3′-OH group to elongate the chain. Continuous lines represent parental DNA, and discontinuous lines represent newly synthesized DNA. (1) and (2) are alternative models to initiate the replication of the displaced strand. Only the initiation of replication of one of the DNA strands has been drawn for simplicity.*

DNA, p3, which we have shown is needed for the initiation of replication (R. P. Mellado, M. A. Peñalva, M. R. Inciarte, and M. Salas, submitted for publication), primes replication not by interaction with the 3′ end of the DNA as proposed in the case of adenovirus (20), but rather by interaction with the parental protein (Fig. 10). This is consistent with previous results showing that a functional parental protein is needed to carry out ϕ29 DNA replication (23). In this case, it must be assumed that before the parental DNA strand has been completely displaced, initiation would take place at the opposite end of the DNA molecules (Fig. 10, possibility 1A or B). However, if the replication is initiated on the completely displaced parental strand, some kind of protein-DNA interaction must be postulated. If there were a small region of identical sequence at the ends of ϕ29 DNA, a circle could be formed by an interaction of the protein bound at the 5′ end of the displaced strand with the 3′ end (Fig. 10, possibility 2A or B). Having a circle, a newly synthesized molecule of protein p3 could interact with the parental protein subunit and initiate replication from the 3′ end. If this were the case, single-stranded circles should be found as replicative intermediates if no protease treatment were included in the isolation procedure.

Besides type I, II, and I/II replicative intermediates, another class of molecules is found both in ϕ29- and in adenovirus type 2-infected cells, which consists of linear DNA partially double stranded with single-stranded regions at both ends. In the case of phage ϕ29, the single-stranded regions of these molecules (type III) have a random length. Although these molecules are 14% of the total in ϕ29 and 9% of the total in adenovirus (17), their presence must be explained. A possibility to account for this type of molecule is that if a circle is formed to initiate the replication of the displaced strand, the interaction between the protein and the 3′ end of the DNA could take place occasionally at random with internal regions of the molecule around the 3′ end. Therefore, type III molecules would be abortive type II DNA. Another possibility for explaining type III molecules is that they arise by recombination between two type II molecules in which each of the displaced parental single strands is being replicated in the opposite direction. This recombination event would also produce abortive DNA molecules.

It is well established that in vitro the protein linked to ϕ29 DNA is able to interact with itself, giving rise to the formation of a variety of circular structures and concatemers (19, 23). The same result is obtained in the case of the adenovirus DNA-protein complex (3, 15, 21, 22). Girard et al. (10) have analyzed with an electron microscope the replicative intermediates isolated by treatment of adenovirus-infected HeLa cell nuclei with Sarkosyl and have shown the existence of a majority of unit-length circular DNA molecules. It is, however, difficult to establish whether the circles are true intermediates in DNA replication or whether they are formed in vitro during the isolation and processing of the samples.

The following argument, however, favors the idea that both ϕ29 DNA and adenovirus replication might take place on circular DNA (Fig. 10B): if replication occurred on linear DNA and the chances to initiate replication at both ends are similar, type I DNA molecules with two single-stranded branches being displaced in opposite directions would probably be more frequent than has been found both in ϕ29 and in adenovirus type 2 (17) replication since in linear DNA both ends would be free to initiate replication (Fig. 10A). However, if replication occurred on DNA kept circular through protein-protein interaction, there could be some constraint for the simultaneous initiation at the two 3′ ends (Fig. 10B), and, therefore, the above molecules would be rare.

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LITERATURE CITED

1. **Ariga, H., and H. Shimojo.** 1977. Initiation and termination sites of adenovirus 12 DNA replication. Virology **78**:415–424.
2. **Arrand, J. R., and R. J. Roberts.** 1979. The nucleotide sequences at the termini of adenovirus-2 DNA. J. Mol. Biol. **128**:577–594.
3. **Brown, D. T., M. Westphal, B. T. Burlingham, U. Winterhoff, and W. Doerfler.** 1975. Structure and composition of the adenovirus type 2 core. J. Virol. **16**: 366–387.
4. **Brown, N. C.** 1970. 6-(Hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in phage-infected *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **67**:1454–1461.
5. **Carrascosa, J. L., A. Camacho, F. Moreno, F. Jimenez, R. P. Mellado, E. Viñuela, and M. Salas.** 1976. *Bacillus subtilis* phage ϕ29: characterization of gene products and functions. Eur. J. Biochem. **66**:229–241.
6. **Carrascosa, J. L., E. Viñuela, and M. Salas.** 1973. Proteins induced in *Bacillus subtilis* infected with bacteriophage ϕ29. Virology **56**:291–299.
7. **Carusi, E. A.** 1977. Evidence for blocked 5′-termini in human adenovirus DNA. Virology **76**:390–394.
8. **Davis, R., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions

of base sequence homology in nucleic acids. Methods Enzymol. **21**:413-428.

9. **Garon, C. F., K. N. Berry, and J. A. Rose.** 1972. A unique form of terminal redundancy in adenovirus DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **69**:2391-2395.
10. **Girard, M., J. Bouché, L. Marty, B. Revet, and N. Berthelot.** 1977. Circular adenovirus DNA-protein complexes from infected HeLa cell nuclei. Virology **83**: 34-55.
11. **Hagen, E. W., B. E. Reilly, M. E. Tosi, and D. L. Anderson.** 1976. Analysis of gene function of bacteriophage φ29 of *Bacillus subtilis:* identification of cistrons essential for viral assembly. J. Virol. **19**:501-517.
12. **Harding, N., J. Ito, and G. S. David.** 1978. Identification of the protein firmly bound to the ends of bacteriophage φ29 DNA. Virology **84**:279-292.
13. **Hawley, L. A., B. E. Reilly, E. W. Hagen, and D. L. Anderson.** 1973. Viral protein synthesis in bacteriophage φ29-infected *Bacillus subtilis.* J. Virol. **12**:1149-1159.
14. **Ito, J.** 1978. Bacteriophage φ29 terminal protein: its association with the 5′ termini of the φ29 genome. J. Virol. **28**:895-904.
15. **Keegstra, W., P. S. Van Wielink, and J. S. Sussenbach.** 1977. The visualization of a circular DNA-protein complex from adenovirions. Virology **76**:444-447.
16. **Koller, Th., J. M. Sogo, and H. Bujard.** 1974. A method for studying nucleic acid-protein interactions with the electron microscope. Biopolymers **13**:995-1009.
17. **Lechner, R. L., and T. J. Kelly, Jr.** 1977. The structure of replicating adenovirus 2 DNA molecules. Cell **12**: 1007-1020.
18. **Moreno, F., A. Camacho, E. Viñuela, and M. Salas.** 1974. Suppressor-sensitive mutants and genetic map of *Bacillus subtilis* bacteriophage φ29. Virology **62**:1-16.
19. **Ortin, J., E. Viñuela, M. Salas, and C. Vasquez.** 1971. DNA-protein complex in circular DNA from phage φ29. Nature (London) New Biol. **234**:275-277.
20. **Rekosh, D. M. K., W. C. Russell, and A. J. D. Bellet.** 1977. Identification of a protein linked to the ends of adenovirus DNA. Cell **11**:283-295.
21. **Robinson, A. J., and A. J. D. Bellett.** 1974. A circular DNA-protein complex from adenoviruses and its possible role in DNA replication. Cold Spring Harbor Symp. Quant. Biol. **39**:523-531.
22. **Robinson, A. J., H. B. Younghusband, and A. J. D. Bellett.** 1973. A circular DNA-protein complex from adenoviruses. Virology **56**:54-69.
23. **Salas, M., R. P. Mellado, E. Viñuela, and J. M. Sogo.** 1978. Characterization of a protein covalently linked to the 5′ termini of the DNA of *Bacillus subtilis* phage φ29. J. Mol. Biol. **119**:269-291.
24. **Sogo, J. M., M. R. Inciarte, J. Corral, E. Viñuela, and M. Salas.** 1979. RNA polymerase binding sites and transcription map of the DNA of *Bacillus subtilis* phage φ29. J. Mol. Biol. **127**:411-436.
25. **Sogo, J. M., P. Rodeño, Th. Koller, E. Viñuela, and M. Salas.** 1979. Comparison of the A-T rich regions and the *Bacillus subtilis* RNA polymerase binding sites in phage φ29 DNA. Nucleic Acids Res. **7**:107-120.
26. **Stafford, D. W., and D. Bieber.** 1975. Concentration of DNA solutions by extraction with 2-butanol. Biochim. Biophys. Acta **378**:18-21.
27. **Steenbergh, P. H., J. Maat, H. Van Ormondt, and J. S. Sussenbach.** 1977. The nucleotide sequence at the termini of adenovirus type 5 DNA. Nucleic Acid Res. **4**:4371-4389.
28. **Sussenbach, J. S., and M. G. Kuijk.** 1977. Studies on the mechanism of replication of adenovirus DNA. V. The location of termini of replication. Virology **77**:140-157.
29. **Sussenbach, J. S., and M. G. Kuijk.** 1978. The mechanism of replication of adenovirus DNA. VI. Localization of the origin of the displacement synthesis. Virology **84**: 509-517.
30. **Talavera, A., M. Salas, and E. Viñuela.** 1972. Temperature-sensitive mutants affected in DNA synthesis in phage φ29 of *Bacillus subtilis.* Eur. J. Biochem. **31**: 367-371.
31. **Westmoreland, B. C., W. Szybalski, and H. Ris.** 1969. Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science **163**:1343-1348.
32. **Wolfson, J., and D. Dressler.** 1972. Adenovirus-2 DNA contains an inverted terminal repetition. Proc. Natl. Acad. Sci. U.S.A. **69**:3054-3057.
33. **Yanofsky, S., F. Kawamura, and J. Ito.** 1976. Termolabile transfecting DNA from temperature-sensitive mutant of phage φ29. Nature (London) **259**:60-63.
34. **Yehle, C. D.** 1978. Genome-linked protein associated with the 5′ termini of bacteriophage φ29 DNA. J. Virol. **27**: 776-783.