

Knotted DNA from bacteriophage capsids

(bacteriophage P2/DNA packaging/DNA topoisomerases)

LEROY F. LIU*, LUKE PERKOCHA†, RICHARD CALENDAR†, AND JAMES C. WANG‡

*Department of Physiological Chemistry, Johns Hopkins Medical School, Baltimore, Maryland 21205; †Department of Molecular Biology, University of California, Berkeley, California 94720; ‡The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Communicated by Robert L. Baldwin, June 9, 1981

ABSTRACT The majority of the DNA prepared from tailless capsids of bacteriophage P2 by the phenol extraction procedure consists of monomeric rings that have their cohesive ends joined. Electron microscopic and ultracentrifugal studies indicate that these molecules have a complex structure that is topologically knotted; they have a more compact appearance and a higher sedimentation coefficient when compared with regular nicked P2 DNA rings. Linearization of these rings by thermal dissociation or repair of the cohesive ends by DNA polymerase I in the presence of all four deoxynucleoside triphosphates gives molecules that are indistinguishable from normal P2 DNA that has been similarly treated. The knotted nature of the majority of P2 head DNA is further supported by analyzing the products when these molecules are treated with ligase and the ligase-treated molecules are subsequently nicked randomly with DNase I. The data are consistent with the notion that, if such a molecule is first converted to a form that contains only one single-chain scission per molecule, strand separation gives a linear strand and a highly knotted single-stranded ring. The results suggest that the DNA packaged in tailless P2 capsids is arranged in a way that leads to the formation of a complex knot when the ends join. In an intact phage particle, the anchoring of one terminus of the DNA to the head-proximal end of the tail [Chattoraj, D. K. & Inman, R. B. (1974) *J. Mol. Biol.* 87, 11–22] presumably diminishes or prevents this kind of joining. The novel knotted DNA can be used to assay type II DNA topoisomerases that break and rejoin DNA in a double-stranded fashion.

Mature P2 phage DNA consists of double-stranded molecules 33 kilobases long (1, 2) with 19-base cohesive ends (3, 4) that allow the formation of circular and concatemeric molecules in solution (1, 2). P2 phage preparations typically contain equal proportions of viable phage particles and tailless DNA-containing capsids (heads) (5). The two cohesive ends of the P2 DNA in the mature phage are not hydrogen bonded and are asymmetrically oriented with the left end anchored on the head-proximal end of the tail (6). When DNA is extracted from a concentrated preparation of mature P2 phage, a viscous solution is obtained, due to the formation of hydrogen-bonded concatemers via pairing of the cohesive ends (1). We observed, however, that DNA extracted from concentrated P2 heads is much less viscous than the corresponding DNA from mature phage particles. In this communication, we present evidence that the DNA from tailless capsids (henceforth referred to as head DNA) represents a novel class of topological isomers of hydrogen-bonded monomeric circular P2 DNA. The apparent low viscosity of head DNA relative to that of DNA from mature phages (henceforth referred to as phage DNA) is due to the presence of complex topological knots.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Bacteria and Bacteriophages. *Escherichia coli* C strains C-1a (7) and C-520 (8), carrying the amber suppressor *supD*, were used as hosts to grow stocks of phage P2. The phage used were the clear plaque mutant P2 *vir1* (9); the temperature-sensitive head-finishing mutant P2 *vir22* Lts37, which carries a 1.5-kilobase deletion (10–12); and the lysis-deficient amber mutant P2 *vir1* am12 (13). Phage stocks were prepared as described by Lengyel *et al.* (14) in LB broth (15)/2 mM MgCl₂/0.5 mM CaCl₂. The phage preparations were precipitated with 10% polyethylene glycol and then suspended in P buffer (14) and incubated at 37°C for 30 min to digest free DNA. Thereafter, debris was removed by centrifugation (13). CsCl was added to a density of 1.42 g cm⁻³, and the solution was centrifuged to sedimentation equilibrium (14). Two bands, the denser one containing the tailless capsids and the lighter one containing the viable phage, were visible due to light scattering. Each band was collected separately by puncturing the side of the centrifuge tube with a syringe. The phage and capsids were then centrifuged separately to obtain higher purity and concentration. CsCl was removed from the purified phage by dialysis against P buffer.

DNA Extraction. DNA was extracted from phage or heads at A₂₆₀ = 5–15, using buffer-saturated phenol (16). The aqueous phase was dialyzed against sterile 10 mM Tris·HCl/50 mM NaCl/100 μM EDTA, pH 8.0.

Enzymatic Reagents. Pronase (Calbiochem) and fungal proteinase K (Merck) were prepared at 20 mg/ml in 10 mM Tris·HCl, pH 8.0, and autolyzed at 37°C for 90 min before use. Ribonuclease A (Calbiochem) was prepared at 10 mg/ml in 10 mM Tris·HCl/0.1 mM EDTA, pH 8.0, and heated at 80°C for 10 min before use to inactivate DNase. DNA polymerase I from *E. coli* (17) was the gift of A. Kornberg. Purified *E. coli* DNA ligase (18) was the gift of P. Modrich. DNase I was purchased from Worthington. Bovine plasma albumin and NAD⁺ were from Calbiochem.

Analytical Ultracentrifugation. Band sedimentation was performed with 30–40 μl of sample and reference solutions in type III 30-mm double-sector centerpieces (19). The bulk sedimentation medium was 3 M CsCl/10 mM Na₃EDTA (neutral 3 M CsCl) or 3 M CsCl/100 mM KOH/10 mM Na₃EDTA (alkaline 3 M CsCl). Centrifugation was at 20,000 rpm and 20°C in a model E (Spinco) analytical ultracentrifuge equipped with a photoelectric scanner. The sedimentation coefficients measured were not corrected for buoyancy or viscosity effects. CsCl density-gradient equilibrium centrifugation was performed in 12-mm double-sector cells at 39,000 rpm and 20°C for a minimum of 60 hr. *Micrococcus luteus* DNA was used as a density marker ($\rho = 1.733$ g cm⁻³).

Electron Microscopy. DNA molecules were visualized in a

Philips 201 electron microscope at 20,000-fold magnification. The aqueous spreading procedure of Davis *et al.* (20) was used unless specified otherwise.

DNA Ligase Reaction. Twenty micrograms of phage or head DNA and 0.8 unit of DNA ligase were incubated for 20 min at 30°C in 150 μ l of 7 μ M NAD⁺/1 mM MgCl₂/5 mM Tris·HCl, pH 8.0/50 μ M Na₃EDTA containing bovine plasma albumin at 0.1 mg/ml. The reaction was stopped by heating at 65°C for 5 min.

DNase I Treatment of Ligated DNA. Four microliters of DNase I (4 μ g/ml) was added to the above mixture and incubated at 22°C. Fifteen-microliter samples were withdrawn at the times indicated in Fig. 5, and the reaction was stopped by addition of 15 μ l of 4 M NaCl/20 mM Na₃EDTA.

DNA Polymerase I Reaction. Two micrograms of P2 DNA was treated with 0.002 unit of DNA polymerase I in 30 μ l of 5 mM MgCl₂/30 mM NaCl/3 mM Tris·HCl, pH 8.0/100 μ M Na₃EDTA containing each of the four deoxyribonucleoside triphosphates at 25 μ M. Incubation was for 15 min at 37°C; this was followed by addition of 30 μ l of 4 M NaCl/20 mM Na₃EDTA to stop the reaction.

Treatment of Condensed DNA with Proteases and RNase A. DNA from tailless capsids (0.5 μ g) was treated with 120 μ g of Pronase or 120 μ g of proteinase K or 60 μ g of RNase A for 30 min at 37°C in 60 μ l of 50 mM NaCl/10 mM Tris·HCl, pH 8.0/10 mM Na₃EDTA. The reactions were terminated by chilling to 0°C and by adding 60 μ l of 4 M NaCl/20 mM Na₃EDTA.

RESULTS

The appearance of P2 Head DNA as a Condensed Form. DNA was extracted from P2 *vir22 Lts37* heads prepared from

a phage stock grown at permissive temperature. This DNA was spread in cytochrome *c* by the aqueous method (Fig. 1) (20), and molecules of about P2 monomer length were observed. Sixty-nine out of 100 molecules were highly condensed, with no visible ends and three or more loops protruding from a central region (Fig. 1). The central region usually contained a dark spot. The remaining DNA consisted of 20 noncondensed circular molecules and 11 linear monomers. The condensed DNA forms are 22% shorter than the linear molecules, suggesting that the dark spot may contain a considerable amount of highly knotted DNA. The dark spots are not artifacts of spreading in cytochrome *c*, as they are also seen after spreading by the protein-free ethidium bromide procedure (21). The condensed structure does not depend on the *vir22* or *Lts37* mutations; the same result was obtained using heads of the clear-plaque lysis-deficient mutant P2 *vir1 am12*. Moreover, the condensed structure was not found in DNA extracted from the complete phage of either genotype: instead, linear monomer, circular monomer, and linear concatemers were observed (data not shown). The microscopy results are consistent with the earlier observation (unpublished) that P2 head DNA is much less viscous than P2 phage DNA at the same concentration.

Treatment of the Condensed Form with Proteases and RNase. P2 head DNA was analyzed by band sedimentation in neutral 3 M CsCl (Fig. 2B). Twenty-eight percent of the material sedimented at 21 S, as did linear monomeric P2 DNA from mature phage (Fig. 2A). Seventy-two percent of the head DNA sedimented as a heterogeneous population with a mean sedimentation coefficient of 45 S. As noncondensed circular P2 DNA sediments only 10% faster than linear molecules (22), the rapidly sedimenting peak (\approx 45 S) most likely represents the

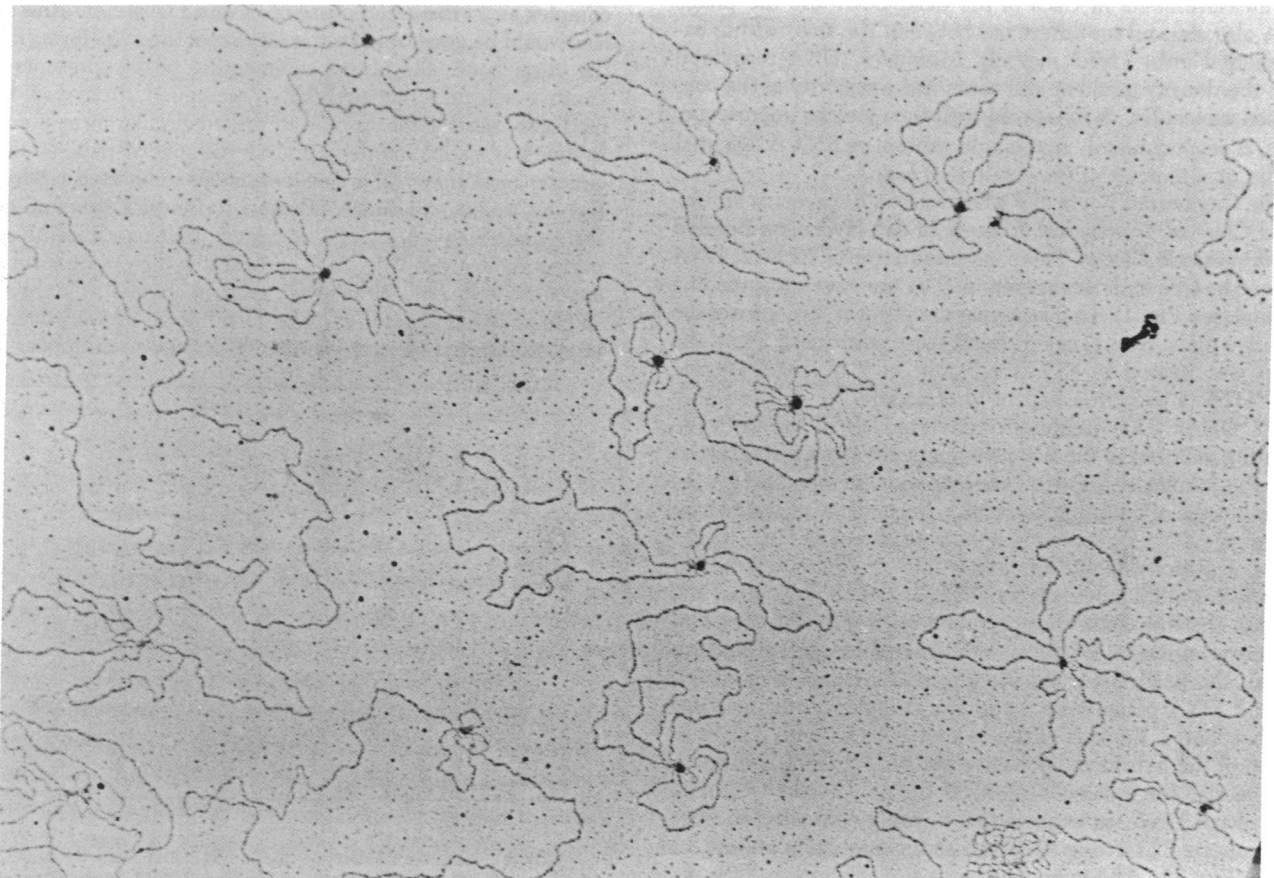


FIG. 1. Electron micrograph of DNA from tailless capsids of P2 *vir22 Lts37*.

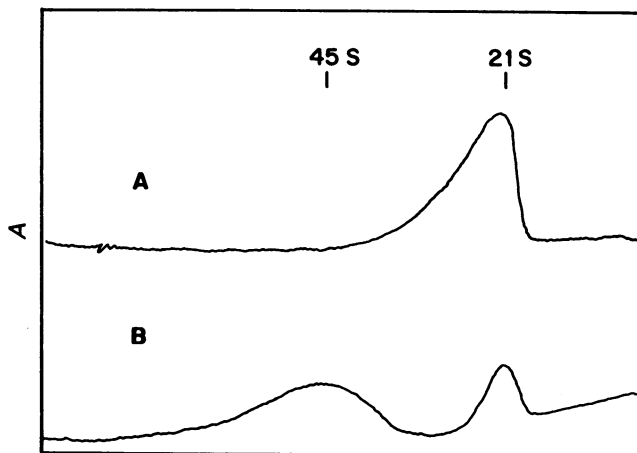


FIG. 2. Band sedimentation of P2 phage DNA and head DNA in neutral 3 M CsCl. The direction of sedimentation is to the left. Curves: upper, phage DNA; lower, head DNA. The trailing of the 21S species in the upper curve toward the bottom of the cell is due to the formation of some circles and concatemers.

condensed form seen under the electron microscope. Thus, the condensed form cannot be simply an artifact of the electron microscopic technique. To test whether the condensed form contains components other than DNA (such as protein and RNA), the head DNA was digested separately with Pronase, proteinase K, and RNase A. These treatments did not alter the sedimentation velocity of the DNA in neutral 3 M CsCl, nor was there any change in the amount or appearance of the condensed DNA form seen under the electron microscope. Buoyant density measurements in CsCl of the head DNA and the phage DNA also showed no difference between the two, within experimental error (± 0.2 mg/ml). Moreover, DNA recovered from the density gradient still contained a majority of the condensed molecules, as measured with the electron microscope. Thus, it seems unlikely that bound protein or RNA is essential for the maintenance of the condensed form.

The Condensed Form of P2 Head DNA Represents an Unusual Class of Topological Isomers of the Hydrogen-Bonded P2 Monomeric Circle. The condensed form of P2 head DNA showed no free ends when examined by the aqueous spreading method (see Fig. 1). To investigate the physical structure of the cohesive ends, several experiments were performed. First, the DNA was heated to 85°C for 5 min in 2 M NaCl/10 mM Na₃EDTA (a condition known to dissociate the cohered ends of P2 DNA). This treatment converted all of the fast-sedimenting material to the 21S form (data not shown), suggesting that the joining of the two cohesive ends is important for the maintenance of the condensed form. Second, we analyzed head and phage DNA by sedimentation in alkaline 3 M CsCl. Under these conditions, DNA from either source sedimented as a single species of P2 monomer length (data not shown), again suggesting that the ends are not joined covalently and that their joining is important for the maintenance of the condensed form. Third, the head DNA and the phage DNA were treated with *E. coli* DNA polymerase I and the four deoxyribonucleoside triphosphates. Because of the recessed 3'-OH end, nick translation of the hybridized cohesive ends of P2 DNA is expected to linearize the molecule (4). After the polymerase treatment, the phage DNA, as expected, is entirely converted to linear monomeric P2 DNA as shown by sedimentation in neutral 3 M CsCl (Fig. 3A). The same treatment also converted all the fast-moving material of P2 head DNA (45 S) to the linear form (21 S) (Fig. 3B). These results were also confirmed by electron

microscopy. We thus conclude that the joining of the two cohesive ends is essential for the maintenance of the condensed structure and that the condensed structure is most likely a topological variant of hydrogen-bonded simple P2 DNA circle.

The Topology of the Condensed Form Is Complex Knots. All the results obtained so far for the condensed form of P2 head DNA are consistent with the interpretation that the DNA is in a highly knotted topological state and that the circularity of the DNA maintains these complex knots. To confirm the knotted topology of the condensed form, we sealed its hybridized cohesive ends with DNA ligase and analyzed the products of random nicking with DNase I. After sealing the first nick of a P2 hybridized circle, a singly nicked circle is obtained, and sealing of the second nick gives covalently closed circular molecule. If singly nicked circular molecules are knotted, then alkaline sedimentation will show a band representing knotted single-stranded P2 DNA (23). As expected, when ligase-treated P2 head DNA was sedimented in alkaline 3 M CsCl, four major components were observed (Fig. 4). The slowest peak (≈ 24 S) represents monomeric linear and circular single-stranded DNAs, which separate from one another after further sedimentation. A peak that sediments at ≈ 93 S represents covalently closed double-stranded molecules (22). A broad peak at ≈ 125 S and the remaining peak at ≈ 45 S most likely represents knotted double-stranded circles and knotted single-stranded circles, respectively.

If our interpretation of the alkaline sedimentation pattern is accurate, then the disappearance of the fastest sedimenting form (125 S, knotted duplex rings) by nicking with DNase I should follow one-hit kinetics. The kinetics for the disappearance of the 45S species (knotted single-stranded rings) during the course of digestion with DNase I would, however, be more complex as, in the initial phase of DNase I treatment, this species would be generated by the nicking of the 125S form. If, on the other hand, the fastest sedimenting peak represents unknotted molecules that are highly supercoiled, these would immediately assume the unknotted nicked circular form after the first nick is made, and denaturation will give a single-stranded circular monomer and a single-stranded unknotted circle. To test our model, we added DNase I to the ligase-treated head DNA and removed samples at various times for alkaline sedimentation analysis (Fig. 5). The fastest sedimenting form disappeared with a half-time of ≈ 1 min. In contrast, the putative single-stranded knotted circular form (45 S) did not decrease at all in the first several minutes. It started to disappear only

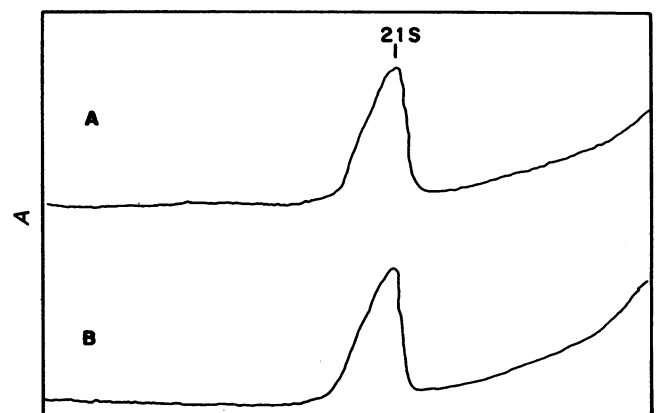


FIG. 3. Nick translation of phage DNA and head DNA at the cohesive ends. Phage DNA (curve A) and head DNA (curve B) were nick translated by *E. coli* polymerase I and sedimented in neutral 3 M CsCl. The direction of sedimentation is to the left.

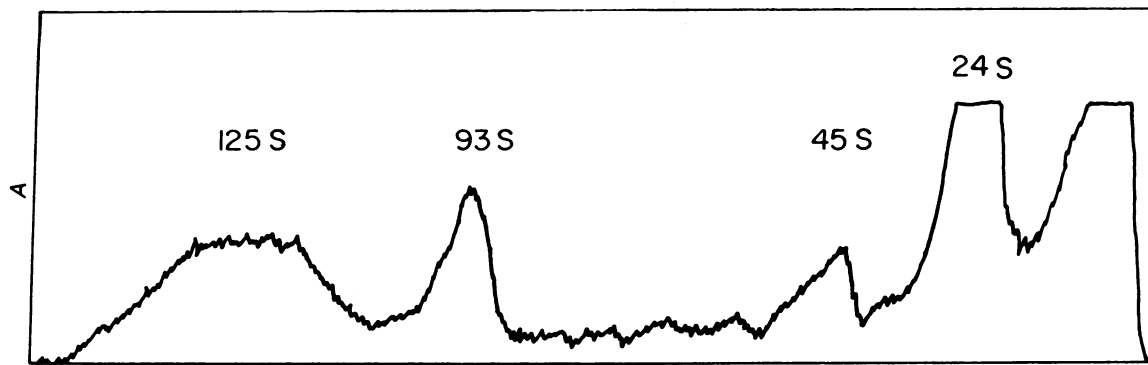


FIG. 4. Alkaline sedimentation of P2 *vir22 Lts37* head DNA after treatment with DNA ligase. The direction of sedimentation is to the left.

when the double-stranded knotted molecules (125 S) had nearly disappeared (Fig. 5). In summary, ligase closure and subsequent DNase cleavage gives results consistent with the idea that the condensed form of P2 head DNA is knotted. The high rate of sedimentation of both the knotted double-stranded circles (125 S) and the knotted single-stranded circles (45 S) further suggests that the knots must be exceedingly complex.

DISCUSSION

The Nature of the Knots in P2 Head DNA. Our results indicate that the P2 DNA extracted from tailless capsids is a topological variant of the P2 DNA extracted from mature phage. P2 head DNA is a monomeric circular form in which the two cohesive ends are hydrogen bonded, and it contains complex knots. The sedimentation rate of knotted single-stranded DNA has been measured previously for fd phage DNA and for ϕ X174

phage DNA knotted by *E. coli* DNA topoisomerase I (23). DNA with the most complex knots produced by topoisomerase treatment sedimented not faster than 1.5 times the rate of the unknotted form (23). In contrast, the knotted P2 head DNA sedimented about twice as fast as the unknotted form, both in neutral 3M CsCl (45 S vs. 21 S) and in alkaline 3M CsCl (45 S vs. 24 S). The complexity of the topological knots, however, does not seem to be reflected by the electron micrographs. We believe that the central dark region represents very complex overlapping of DNA double-strands, due to knotting. This is consistent with the result of the contour length measurements, which show that the central dark spot contains 20% of the total DNA. Such a looped structure with central overlapping might result from the spreading force during DNA fixation for electron microscopy (20). Alternatively, the highly knotted topology might induce structural changes of the DNA to give a compact core of yet unknown nature.

Origin of the Knots. The fact that P2 head DNA contains circular knotted molecules indicates that, in a P2 head, the cohesive ends either are free to join or are in close proximity, so that their joining occurs rapidly on disruption of the capsids by phenol. In either case, the complexity of the knots must reflect the way the DNA is packaged in the phage head. DNA in phage heads is currently thought to be arranged as in a spool of thread (24-26), and the position of the DNA ends has not been established. The ends may not have a fixed position and may meet and hybridize after a random walk that occurs after packaging is complete. Although random cyclization of P2 DNA has been calculated to produce only $\approx 28\%$ knotted rings with low complexities (27), random cyclization of P2 DNA in the phage capsid may produce more knotted rings with high complexity, due to the confined space. It is also possible that the topology of the knots observed bears little relationship to the DNA topology in the capsid. The knots may be generated as a result of random diffusion and subsequent joining of the cohesive ends during or shortly after disruption of the phage heads by phenol, while DNA is decondensing.

The lack of knots in DNA from mature phage is not surprising. After the tail is joined to the head, a unique end of the DNA can be crosslinked to the head-proximal end of the phage tail (6), indicating that at least one DNA end has a fixed position in mature phage. The fixation of one DNA end to the phage collar presumably accounts for the lack of circularization of DNA in mature phage.

A Substrate for Type II Topoisomerase. Highly knotted DNA is a convenient substrate for studies of type II DNA topoisomerases (28). These enzymes catalyze the breakage and rejoining of DNA backbone bonds in a double-stranded fashion to permit the passage of one double-stranded DNA segment

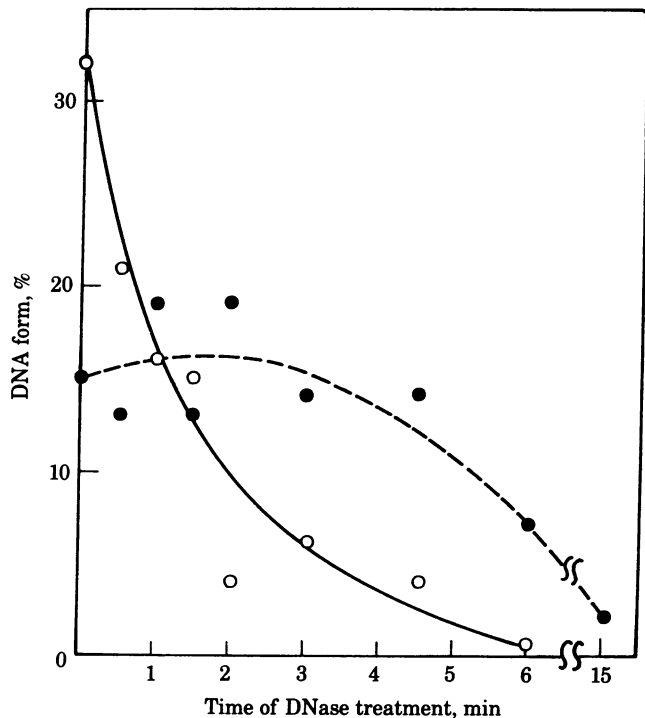


FIG. 5. Effect of pancreatic DNase I treatment on ligase-closed knotted P2 head DNA. P2 *vir22 Lts37* head DNA treated with DNA ligase was subsequently treated with DNase I. Samples were withdrawn at the indicated times and analyzed by sedimentation in alkaline 3 M CsCl (see Fig. 3). The relative amount of each DNA form was estimated from the areas under each peak. ○, Double-stranded knotted DNA (125 S); ●, single-stranded knotted DNA (45 S).

through another (28–30). As a result, knotted duplex DNA rings can be converted to rings without knots by these enzymes (28, 30). We have demonstrated that the complex knots of P2 head DNA are quickly removed by type II DNA topoisomerases (31). The type I DNA topoisomerases, whether from eukaryotic or prokaryotic sources, do not change the knotted topology of P2 head DNA efficiently although, with the prokaryotic enzymes, some unknotting is detectable if the DNA is first nicked with DNase I (32, 33).

The most convenient source of substrate for the assay of type II DNA topoisomerases appears to be the DNA extracted from the heads of satellite phage P4. P4 DNA contains only 11 kilobases (34) and has the same cohesive ends as P2 (35). More than 95% of the DNA from P4 heads is highly knotted. P4 head DNA has been used successfully as a substrate for assaying eukaryotic type II topoisomerase in crude cell extracts containing excess type I DNA topoisomerase (33).

We are grateful to Gail Pruss for advice. This investigation was supported by Grants GM 27731 and GM 24544 from the National Institute of General Medical Sciences, AI 08722 from the National Institute of Allergy and Infectious Diseases, and PCM 78-05892 from the National Science Foundation.

1. Mandel, M. (1967) *Mol. Gen. Genet.* **99**, 88–96.
2. Inman, R. B. & Bertani, G. (1969) *J. Mol. Biol.* **44**, 533–549.
3. Murray, K. & Murray, N. E. (1973) *Nature (London) New Biology* **243**, 134–139.
4. Padmanabhan, R., Wu, R. & Calendar, R. (1974) *J. Biol. Chem.* **249**, 6197–6207.
5. Bertani, G., Ljungquist, E., Jagustyn-krynicka, K. & Jupp, S. (1978) *J. Gen. Virol.* **38**, 251–261.
6. Chatteraj, D. K. & Inman, R. B. (1974) *J. Mol. Biol.* **87**, 11–22.
7. Sasaki, I. & Bertani, G. (1965) *J. Gen. Microbiol.* **40**, 365–376.
8. Sunshine, M. G., Thorn, M., Gibbs, W., Calendar, R. & Kelly, B. (1971) *Virology* **46**, 691–702.
9. Bertani, L. E. (1957) *Virology* **4**, 53–71.
10. Bertani, G. (1975) *Mol. Gen. Genet.* **136**, 107–137.
11. Pruss, G. & Calendar, R. (1978) *Virology* **86**, 454–467.
12. Chatteraj, D. K. & Inman, R. B. (1972) *J. Mol. Biol.* **66**, 423–434.
13. Lindahl, G. (1971) *Virology* **46**, 620–633.
14. Lengyel, J., Goldstein, R. N., Marsh, M., Sunshine, M. G. & Calendar, R. (1973) *Virology* **53**, 1–23.
15. Bertani, G. (1951) *J. Bacteriol.* **62**, 293–300.
16. Thomas, C. A., Jr. & Abelson, J. (1966) in *Procedures in Nucleic Acid Research*, eds. Cantoni, G. L. & Davies, D. R. (Harper & Row, New York), pp. 553–561.
17. Jovin, T. M., Englund, P. T. & Bertsch, L. L. (1969) *J. Biol. Chem.* **244**, 2996–3008.
18. Modrich, P., Anraku, Y. & Lehman, I. R. (1973) *J. Biol. Chem.* **248**, 7495–9501.
19. Vinograd, J., Radloff, R. & Bruner, R. (1965) *Biopolymers* **3**, 481–489.
20. Davis, R. W., Simon, M. & Davidson, N. (1971) *Methods Enzymol.* **21**, 413–428.
21. Koller, T., Sogo, J. M. & Bujard, H. (1974) *Biopolymers* **13**, 995–1009.
22. Lindqvist, B. H. (1971) *Mol. Gen. Genet.* **110**, 178–196.
23. Liu, L. F., Depew, R. E. & Wang, J. C. (1976) *J. Mol. Biol.* **106**, 439–452.
24. Earnshaw, W. C. & Casjens, S. R. (1980) *Cell* **21**, 319–331.
25. Richards, K. E., Williams, R. C. & Calendar, R. (1973) *J. Mol. Biol.* **78**, 255–259.
26. Earnshaw, W. C. & Harrison, S. C. (1977) *Nature (London)* **268**, 598–602.
27. Frank-Kamenetskii, J. D., Lukashin, A. V. & Vologodskii, A. V. (1975) *Nature (London)* **258**, 398–402.
28. Liu, L. F., Liu, C. C. & Alberts, B. M. (1980) *Cell* **19**, 697–707.
29. Brown, P. O. & Cozzarelli, N. R. (1979) *Science* **206**, 1081–1083.
30. Mizuuchi, K., Fisher, L. M., O'Dea, M. & Gellert, M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1847–1851.
31. Miller, K. G., Liu, L. F. & Englund, P. T. (1981) *J. Biol. Chem.*, in press.
32. Liu, L. F. & Miller, K. G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3487–3491.
33. Liu, L. F., Davis, J. L. & Calendar, R. (1981) *Nucleic Acids Res.*, in press.
34. Inman, R. B., Schnos, M., Simon, L. D., Six, E. W. & Walker, D. H. (1971) *Virology* **44**, 67–72.
35. Wang, J. C., Martin, K. V. & Calendar, R. (1973) *Biochemistry* **12**, 2119–2123.