© 1991 Oxford University Press

Effects of temperature on excluded volume-promoted cyclization and concatemerization of cohesive-ended DNA longer than 0.04 Mb

Donna Louie and Philip Serwer
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78284-7760, USA

Received January 28, 1991; Revised and Accepted April 19, 1991

ABSTRACT

The 0.048502 megabase (Mb), primarily double-stranded DNA of bacteriophage λ has single-stranded, complementary termini (cohesive ends) that undergo either spontaneous intramolecular joining to form open circular DNA or spontaneous intermolecular joining to form linear, end-to-end oligomeric DNAs (concatemers); concatemers also cyclize. In the present study, the effects of polyethylene glycol (PEG) on the cyclization and concatemerization of λ DNA are determined at temperatures that, in the absence of PEG, favor dissociation of cohesive ends. Circular and linear λ DNA, monomeric and concatemeric, are observed by use of pulsed field agarose gel (PFG) electrophoresis. During preparation of λ DNA for these studies, hydrodynamic shear-induced, partial dissociation of joined cohesive ends is fortuitously observed. Although joined λ cohesive ends progressively dissociate as their temperature is raised in the buffer used here (0.1 M NaCl, 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA), when PEG is added to this buffer, raising the temperature sometimes promotes joining of cohesive ends. Conditions for promotion of primarily either cyclization or concatemerization are described. Open circular DNAs as long as a 7-mer are produced and resolved. The concentration of PEG required to promote joining of cohesive ends decreases as the molecular weight of the PEG increases. The rate of cyclization is brought, the first time, to values that are high enough to be comparable to the rate observed *in vivo*. For double-stranded DNA bacteriophages that have a linear replicative form of DNA (bacteriophage T7, for example), a suppression, sometimes observed here, of cyclization mimics a suppression of cyclization previously observed *in vivo*. The PEG, temperature effects on DNA joining are explained by both the excluded volume of PEG random coils and an increase in this excluded volume that occurs when temperature increases.

INTRODUCTION

The nonspecific effects that intracellular molecules have on intracellular processes (these effects include excluded volume[1] and osmotic pressure[2]) have been mimicked by adding either neutral polymers or non-reactive proteins to solutions of either DNA, protein or DNA-protein complex. The following effects have been observed: (a) stimulation of the end-to-end joining of DNA (either inter- or intramolecular) during either complementary base pairing[3-6] or blunt-ended ligation,[4-8] (b) stimulation of *in vitro* DNA replication,[9] (c) stimulation of *in vitro* packaging of DNA in bacteriophage capsids,[10] (d) stabilization of DNA-protein complexes,[11] multimeric protein complexes[12-14] and viruses,[15] and (e) raising the melting temperature of DNA.[16]

Bacteriophage λ DNA has 12 base pair, single-stranded, complementary (cohesive) ends that join in buffered solution (*in vitro*) at least one order of magnitude less rapidly than they do in λ-infected cells (*in vivo*).[17,18] Linear λ concatemers produced *in vitro* have been used as length standards during pulsed field agarose gel (PFG) electrophoresis.[19,20] Among the possible reasons for the comparatively high rate of joining of cohesive ends *in vivo* are: (a) excluded volume effects believed to be mimicked by polyethylene glycol (PEG) in the studies of refs. 3−8 (for theory, see also refs. 1, 21), and (b) unraveling of secondary structure in the cohesive ends,[18] by previously described (reviewed in ref. 22) DNA-binding proteins that have this activity. Thus, in the present study, to accelerate joining of cohesive ends, an attempt was made to mimic the effects of both these DNA-binding proteins and excluded volume. The procedure used for this purpose was raising the temperature in the presence of PEG. The rationale for use of this procedure included the assumption that premature melting of the DNA would be prevented by the PEG.[16] The desired effects were obtained; these effects were observed by use of a hybrid mode of PFG electrophoresis.[23] A detailed characterization of the concatemerization and cyclization of λ DNA at elevated temperatures and in the presence of PEG is presented here.

MATERIALS AND METHODS

Bacteriophage DNAs

To grow bacteriophage λ, a λ lysogen of *Escherichia coli*, SA-9, was grown at 30°C in 2×LB medium: 20 g tryptone, 10 g yeast extract, 5 g NaCl, 1000 ml H_2O. The lysogen was induced by exposure to elevated temperature.[24] Bacteriophage T7 was grown by use of lytic infection of *Escherichia coli* BB/1 in 2×LB

medium. Bacteriophages λ and T7 were purified by centrifugation in cesium chloride density gradients[23] and dialyzed against Tris/Mg buffer: 0.2 M NaCl, 0.01 M Tris-Cl, pH 7.4, 0.001 M $MgCl_2$. DNA was released from bacteriophage capsids immediately before use, by raising the temperature to 75°C for 15 min, after diluting by at least a factor of 100 into NPE buffer: 0.1 M NaCl, 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA. Unless otherwise indicated, the term, λ DNA, refers to the linear, monomeric DNA released from mature bacteriophage λ. When λ DNA was obtained by use of extraction with phenol,[24] the results obtained in selected experiments performed less than two weeks after extraction (for example, the experiment in Fig. 7a) did not significantly differ from those obtained by using DNA released by use of elevated temperature. Elevated temperature was used here to minimize the damage to cohesive ends that occurs during storage of λ DNA that has been released from its capsid.[19]

Association and dissociation of cohesive ends

To either associate or dissociate the cohesive ends of λ DNA, this DNA was diluted in NPE buffer to the indicated concentration and incubated as indicated. The effects of PEG were determined by adding 9 parts of PEG in NPE buffer (concentration, C, in percentage weight per final volume) to 1 part of DNA in NPE buffer. Final percentages of PEG, calculated by assumption of additive volumes, are indicated. During incubation, temperature was controlled ±0.3°C, by use of the circulated water of a circulating constant temperature water bath. The chamber containing the DNA was covered and the DNA, in a 0.5 ml, siliconized, polypropylene microfuge tube, was tightly capped. Nonetheless, significant variability in the results was caused by evaporation at temperatures above 45°C, unless the solution of DNA was covered with mineral oil (60 μl was used). As described in the Results Section, some results were so dependent on PEG concentration that concentrations had to be adjusted by use of refractive index. For PEG with a mean molecular weight of 6000, PEG-6000 (mean molecular weight, determined by the manufacturer, will be indicated after PEG), the dependence of refractive index at 25°C (η^{25}) on C was linear and followed the relation: $\eta^{25} = 1.3684\ C \cdot 10^{-3} + 1.3332$. PEG-6000 was obtained from Fisher Scientific (Fair Lawn, New Jersey). PEGs 200, 586, 960, 1500, 4000, 5000, 9000, 15,000 and 21,200 were obtained from Polysciences (Warrington, Pennsylvania).

Analysis by gel electrophoresis

To stop both the association and dissociation of cohesive ends before analysis, samples were cooled by placing in an ice bath. For samples without PEG, after adding a 1/20× volume of 55% sucrose, 400 μg/ml bromphenol blue in 0.1 M sodium phosphate, pH 7.4, 0.01 M EDTA, the DNA was layered in the sample well of a 1.0% agarose (Seakem LE, FMC Bioproducts, Rockland, Maine) gel cast in 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA (electrophoresis buffer). For samples with PEG, after adding 5 μl of this sucrose-containing mixture to the bottom of sample wells, the DNA was layered above the 5 μl layer. Within 2 min after cooling, the DNA was subjected to invariant field electrophoresis at 0.5 V/cm for 30−60 min, followed by the PFG electrophoresis described in the next paragraph.

PFG electrophoresis was used to separate from each other: (a) monomeric, linear λ DNA and the linear concatemers of monomeric λ DNA, and (b) monomeric open circular λ DNA and the open circular concatemers of λ DNA. PFG electrophoresis was performed at 20°C, 2 V/cm, in the presence of an electrical field that was made to have a variable field-gel angle by rotation of the gel. Both the field-electrophoresis apparatus angle and the magnitude of the field were constant. A stepping motor-driven, circular disk in the bottom of a conventional apparatus for submerged, horizontal agarose gel electrophoresis was used to rotate the gel according to a program that was repeated throughout the electrophoresis (one version of this apparatus is described in ref. 23). The program for rotation was: no rotation for a time, t_s, at an angle of 0.3 π radians relative to the direction of net electrophoresis, followed by rotation through 1.4 π radians at a speed of 0.7 π radians/sec, to an angle of −0.3 π radians relative to the direction of net electrophoresis, followed by no rotation for a time, t_s, followed by reversal of the direction of rotation and rotation through 1.4 π radians at a speed of 0.7 π radians/sec. During the periods of stationary gel, this program produces the sequence of field-gel angles that has previously been used to separate by length linear λ DNA concatemers as long as a 30-mer (reviewed in refs. 19,25). During the periods of rotation, the open circular DNA is released from entanglements that prevent separation by DNA length to form sharp bands.[23]

After PFG electrophoresis, discrimination between linear and open circular λ DNA was made by use of a second dimension electrophoresis, orthogonally oriented to the first. Unless otherwise indicated, during the second dimension electrophoresis, an invariant field (without rotation of the gel) of 6 V/cm was used for the time indicated. This field caused complete arrest of all open circular DNA and caused linear DNA to move at a speed independent of DNA length.[23] For some of the separations made here, all detectable linear DNAs were separated from the detectable open circular DNAs even during the PFG electrophoresis performed without the second dimension. However, all identifications of bands formed by DNA during PFG electrophoresis were confirmed by use of the second dimension. After electrophoresis, DNA was stained by use of ethidium bromide and photographed by use of procedures previously described.[23] Images of stained gels were collated and marked by digitization of photographic negatives,[26] followed by use of the program, IMAGE.[27] If two images were obtained from separate gels, they are separated in Figures by a vertical region that is blank (white). Because of experimental logistics in determining the effects of temperature, separate gels were sometimes used for a single series of temperatures. During collation, contrast, background and sometimes lane order of images was altered; no other changes were made.

In Figures, the position of bands formed by linear DNA and open circular DNA are indicated by L and C, respectively, sometimes followed by the number of monomeric DNA molecules present per concatemeric DNA molecule.

RESULTS

Assay for Joining

The use of PFG electrophoresis for analyzing linear and open circular bacteriophage λ DNA monomers and concatemers is based on the assumption of no interconversion among these DNAs during electrophoresis. To test this assumption, PFG electrophoresis (t_s = 20 sec) was performed with λ DNA for which joining of cohesive ends had been performed by use of a procedure found (details are in a subsequent section) to yield only limited joining. These conditions of electrophoresis resolve

linear DNA as long as an 8-mer of λ DNA. To test for in-gel change in the state of association of cohesive ends, after the first electrophoresis, the gel was allowed to incubate in electrophoresis buffer, for 66 hr, but without electrophoresis. Subsequently, orthogonally-oriented electrophoresis (i.e., a second dimension) was performed in two successive stages: the invariant field electrophoresis (6 V/cm for 15 min) used to separate linear from open circular DNA (Materials and Methods), followed by PFG electrophoresis for 8 hr with t_s lowered to 10 sec. The change in t_s from 20 sec to 10 sec improved resolution of 1-mer through 4-mer, linear DNA, but decreased the resolution of longer, linear DNA. During the second, but not the first, stage of the second dimension, open circular DNA migrated. The result of this experiment was the finding that more than 90% of the DNA that behaved as linear, monomeric DNA in the first dimension behaved as linear, monomeric DNA in the second dimension (L1 in the profile from Fig. 1), even though separate experiments (not shown) revealed all DNA capable of concatemerization (see Figs. 5,6 in a subsequent section). However, a comparatively small amount of DNA formed a band at the position of DNA that was linear and monomeric in the first dimension, but was immobile during both stages of the second dimension (white arrow number 1 in Fig. 1). In contrast to the DNA indicated by white arrow number 1 in Fig. 1, DNA that behaved as open circular 1-mers and 2-mers in the first dimension migrated in the second dimension. The positions of open circular 1-mers and 2-mers in Fig. 1, obtained from a separate region of the same gel, are indicated by white lines labeled C1 and C2 in Fig. 1. Thus, the conclusion is drawn that the DNA indicated by white arrow number 1 in Fig. 1 was linear 1-mer after the first dimension, and, during the 66 hr incubation, cyclized around agarose fibers. That is, this latter DNA became topologically linked to the gel (see also ref. 28). A smaller amount of 1-mer cyclized without linkage to the gel, during the 66 hr incubation (white arrow number 2 in Fig. 1). In some experiments (not shown), an even weaker band was observed between the two bands indicated by white arrows in Fig. 1; the reason for the existence of this latter band is not known. The association of cohesive ends that is represented by the DNA indicated by white arrows in Fig. 1 will be neglected in future sections. No other effect of in-gel incubation was observed in Fig. 1.

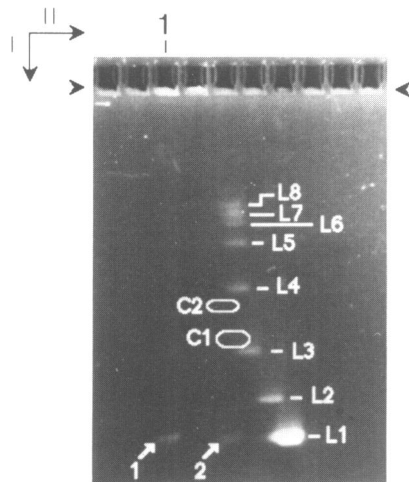

Figure 1. Test of the procedure for assay. A collection of λ DNA molecules that contained some linear concatemers, and a comparatively large amount of linear 1-mer was obtained by incubating 4 μg/ml DNA in 9.0% PEG-6000 at 75°C for 15 min. The rationale and details for this procedure are described in subsequent sections: Figs. 5 and 6. A collection that contained open circular 1-mers and 2-mers was made by incubating 3.2 μg/ml in NPE buffer, without PEG, at 45°C for 120 min. Both collections were subjected to a first dimension PFG electrophoresis for 28 hr, with ts = 20 sec. After completion of the first dimension, the gel was, first, incubated at room temperature (23 ± 3°C) for 66 hr and then rotated by 0.5 π radians from its average direction during the first electrophoresis. Subsequently, the gel was subjected to further electrophoresis in two stages: 6 V/cm for 0.25 hr with an invariant field, followed by PFG electrophoresis for 8 hr, with ts = 10 sec. The profile of the collection of linear DNAs is shown. The position of open circular 1-mer and 2-mer, obtained from the profile of the collection with circular DNAs fractionated in the same gel (this latter profile is not shown), is indicated by a white tracing of the bands' profiles. The arrowheads indicate the origins of electrophoresis; the black arrows indicate the direction of the first (I) and second (II) electrophoresis.

Dilution of Concatemers

During preparation of λ concatemers for analysis of the effects of temperature and PEG concentration, dilution and mixing of concatemers was found to alter the profile of concatemers observed. For example, when λ DNA was concatemerized at 40 μg/ml in NPE buffer and then diluted at 0°C to 4 μg/ml in NPE buffer, the profile of linear concatemers depended on the number of times that the tube for dilution was mixed. After dilution without mixing into a 9×volume of NPE buffer, linear concatemers as long as a 16-mer were observed by use of PFG electrophoresis (Fig. 2, lane 1). However, when this same dilution was accompanied by mixing induced by tapping the DNA-containing tube, either 2, 5, 10, 30 or 50 times, the longer concatemers were found progressively converted to either smaller concatemers (2-mer, 3-mer) or monomeric, linear λ DNA (Fig. 2, lanes 2−6, respectively). Separate experiments (not shown) have revealed no difference between the profile of undiluted DNA and DNA diluted, but not mixed. Thus, most, possibly all, of the depolymerization observed in Fig. 2 required the process of mixing.

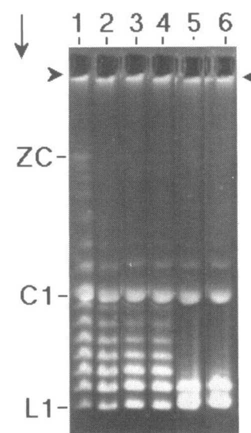

Figure 2. Effects of diluting concatemers. Bacteriophage λ DNA was concatemerized by incubating 40 μg DNA/ml in NPE buffer, at 45°C, for 5 hr. Subsequently, 3 μl of the concatemerized DNA was diluted into 27 μl of NPE buffer. The DNA was subjected to PFG electrophoresis (ts = 80 sec) for 29 hr, starting 0.25 hr after dilution either without mixing of the diluted DNA (lane 1) or with mixing achieved by tapping the DNA-containing tube. The number of taps was (lane number, followed by number of taps): (1) 0, (2) 2, (3) 5, (4) 10, (5) 30, (6) 50. The arrowheads indicate the origins of electrophoresis; the arrow indicates the direction of electrophoresis.

The dilution-mixing-induced depolymerization of Fig. 2 was also observed during dilution into solutions of PEG (not shown) and produced a collection of DNA molecules, circular and linear, that had an intermediate degree of polymerization. For determining the effects of elevated temperature on the distribution of dissociated and undissociated cohesive ends, such a collection is useful. The sample marked Con in future experiments has the starting (control) DNA and was obtained by the use of procedure of Fig. 2, lane 4.

Effects of Temperature and PEG-6000

To determine the effects of elevated temperature in the presence of PEG, separate portions of control DNA that had been diluted into 12.6% PEG-6000 were incubated for 30 min at temperatures between 37° and 95°C. All incubated portions were analyzed by use of PFG electrophoresis (ts = 80 sec; linear concatemers as long as a 16-mer were resolved). Incubation at 37°–80°C stimulated joining of cohesive ends. At 37°–55°C, DNAs at the positions expected of both open circular monomeric DNA and open circular concatemers were the primary products; at 57.5°–80°C, DNAs at the positions of linear concatemers were the primary product (Fig. 3a; the temperature used is indicated at the top of a lane). To more rigorously identify linear and open circular DNA for samples incubated at temperatures between 50.0 and 57.5°C, DNA incubated at these temepratures was subjected to two-dimensional agarose gel electrophoresis (Materials and Methods). The identification of linear and open circular DNA made for Fig. 3a was confirmed and the temperature-caused transition from open circular DNA to linear DNA observed in Fig. 3a was reproduced (Fig. 4). When the temperature in Fig. 3a was above 80°C, all detected DNA was found at the origin of electrophoresis. Comparatively small amounts of this origin-associated DNA were also observed at lower temperatures.

Six bands of open circular λ DNA are seen in the 50°C lane of Fig. 4 and an additional band of open circular DNA is seen in the original negative. The assumption is made that these bands are formed by open circular 2-mers through 7-mers, the length increasing as the distance from the origin decreases.

When the experiment of Fig. 3a was repeated with 7.2% PEG-6000 (instead of 12.6% PEG-6000), stimulation of cyclization again occurred at 37°–55°C (Fig. 3b). However, the stimulation of concatemerization observed at higher

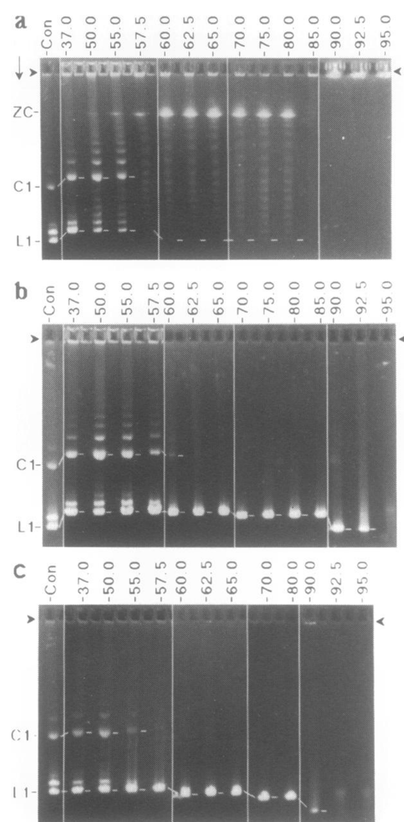

Figure 3. Joining of cohesive ends as a function of both temperature and concentration of PEG-6000. Control DNA in either: (a) 12.6% PEG-6000, (b) 7.2% PEG-6000 or (c) 0.0% PEG-6000 was placed in a water bath and equilibrated for 30 min to the temperature indicated at the top of a lane. After quenching, analysis was performed by use of PFG electrophoresis for 29 hr (ts = 80 sec). The arrowheads indicate the origin of electrophoresis; the arrow indicates the direction of electrophoresis.

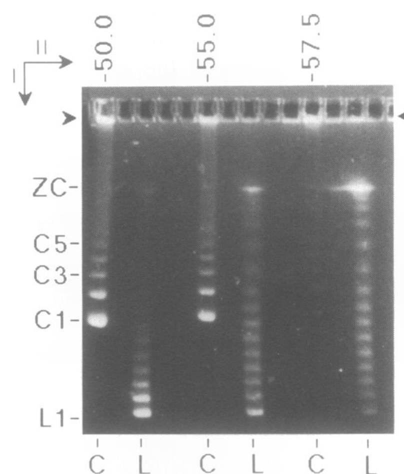

Figure 4. Two-dimensional electrophoresis. Control DNA in 12.6% PEG-6000 was equilibrated for 30 min to the temperature indicated at the top of a lane. After quenching, analysis was performed by use of two-dimensional electrophoresis. The ts and time of electrophoresis for the first dimension were 80 sec and 29 hr, respectively. The time of the second dimension was 0.5 hr. The arrowheads indicate the origins of electrophoresis; the arrows indicate the direction of the first (I) and second (II) electrophoresis.

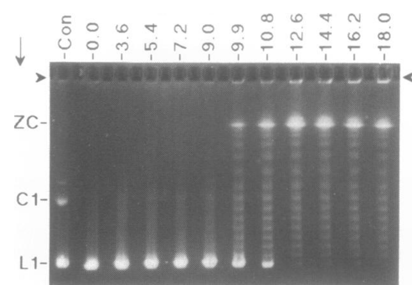

Figure 5. Effect of the concentration of PEG-6000. Control DNA was incubated at 65°C, for 30 min, in the presence of the percentage of PEG-6000 that is indicated at the top of a lane. After quenching, analysis was performed by use of PFG electrophoresis for 29 hr (ts = 80 sec). The arrowheads indicate the origins of electrophoresis; the arrow indicates the direction of electrophoresis. DNA for the Con lane was prepared by dilution into NPE buffer without PEG.

temperatures in the presence of 12.6% PEG-6000 was not observed in the presence of 7.2% PEG-6000 in Fig. 3b. Instead, at 60° −62.5°C the cohesive ends dissociated, thereby leaving all detected DNA as a linear monomer for 60.0° −90.0°C, in Fig. 3b. At 92.5° −95.0°C the DNA formed a broad band and was found (data not shown) to be selectively sensitive to digestion with nuclease S1. Thus, at the highest temperatures, the λ DNA was denatured.

The results in Figs. 3a and 3b are in contrast to the progressive dissociation of cohesive ends that was observed in the absence of PEG, as temperature increased from 55°C to 57.5°C (Fig. 3c). When the temperature of Fig. 3c was increased from 57.5°C, no further change in profile was observed until denaturation occurred at 90°C. The results are in Fig. 3c are in agreement with those previously obtained[18] by use of centrifugation to detect joining of cohesive ends. Thus, all of the stimulation of both cyclization and concatemerization observed in Figs. 3a and 3b required the presence of PEG

Effect of the Concentration of PEG

To determine the minimal concentration of PEG-6000 required to observe stimulation of concatemerization, analysis by use of PFG electrophoresis was performed for control DNA after incubation at 65°C, for 30 min, as a function of the concentration of PEG-6000. For concentrations as high as 9.0%, extensive concatemerization was not detected (Fig. 5; the lanes are labeled by the percentage of PEG used). However, for 9.9% PEG, concatemerization beyond a 16-mer was observed in Fig. 5. As the concentration of PEG-6000 increased from 9.9% to 14.4%, the extent of concatemerization increased in Fig. 5. Further increase of PEG concentration to 18.0% did not detectably further increase the extent of concatemerization. For 12.6% −18.0% PEG-6000, the zone of compression in Fig. 5 became, for reasons not known, a doublet.

Kinetics of Concatemerization

Initially, attempts were made to determine the kinetics of concatemerization by incubation of DNA with 12.6% PEG-6000 at 65°C, without mineral oil to prevent evaporation. However, the evaporation that occurred (usually 5−10% of the liquid in 90 min) was sufficient to detectably distort kinetics. The use of mineral oil above the solution of DNA and PEG (Materials and Methods) reduced evaporation to levels undetectable by use of refractive index. However, the rate of concatemerization was still too high to obtain reproducible results. To improve reproducibility, the concentration of PEG-6000 was reduced to 9.9% and concentrations were adjusted by use of refractive index (Materials and Methods).

Five min after placing control DNA at 65°C, the cohesive ends of the open circular DNA present had dissociated; most DNA was linear and monomeric (Fig. 6; lanes are labeled by the time [min] of incubation at 65°C). However, as the time increased from 10 to 75 min, the DNA progressively concatemerized in Fig. 6. No further concatemerization was observed between 75 and 90 min, even though roughly one-half of the λ DNA remained monomeric. This monomeric DNA was capable of further joining because increase in the concentration of PEG caused concatemerization of the monomeric DNA (data not shown; see Fig. 5). The comparatively large amount of monomeric DNA in the presence of concatemers longer than a 16-mer indicates non-random joining of cohesive ends, at least when a monomeric DNA is one of the two molecules involved.

In Fig. 6, the dissociation of open circular DNA, followed by extensive polymerization, could be caused by either: (a) stability of open circular DNA lower than the stability of linear concatemers, or (b) a time-dependent change in the solution of PEG. An example of the latter possibility is cleavage (perhaps free radical-induced) at nicks in open circular DNA, followed by non-cohesive ended joining. This latter possibility is excluded by the observation that the blunt-ended, 0.04 Mb mature DNA of bacteriophage T7 stays monomeric, with unaltered profile, when used for the experiment of Fig. 6 (data not shown). To further test for changes in the PEG solution, the experiment of Fig. 6 was repeated with a PEG solution that had been incubated at 65°C for 40 min, immediately before use. The results were indistinguishable from those in Fig. 6 (data not shown).

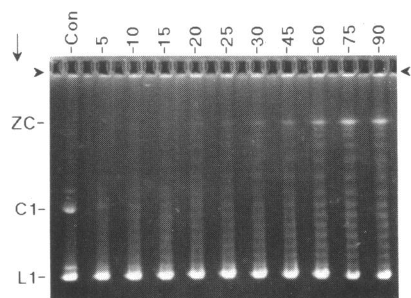

Figure 6. Kinetics of concatemerization. Control DNA was incubated at 65°C, in 9.9% PEG-6000 and samples were taken for analysis by PFG electrophoresis at the times (min) indicated at the top of a lane. The ts and time of electrophoresis were 80 sec and 29 hr, respectively. The arrowheads indicate the origins of electrophoresis; the arrow indicates the direction of electrophoresis.

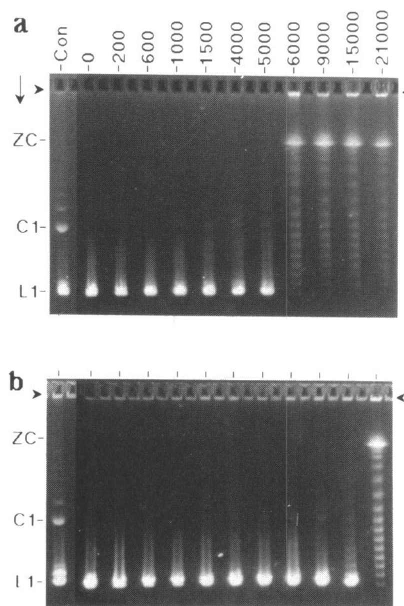

Figure 7. Dependence on the molecular weight of PEG. Control DNA was incubated for 30 min at 65°C in either: (a) 12.6% PEG or (b) 7.2% PEG. The lanes are labeled by the molecular weight of the PEG used. After quenching, the samples were subjected to PFG electrophoresis (ts = 80 sec) for 29 hr. The arrowheads indicate the origins of electrophoresis; the arrow indicates the direction of electrophoresis. DNA for the con lane was prepared by dilution into NPE buffer without PEG.

To determine whether the rate of λ DNA cyclization in solutions of PEG could be made comparable to the rate observed *in vivo*, an approximate measurement of cyclization rate in 12.6% PEG-6000 at 55°C was made. Up to 80–90% cyclization was observed after 15 min (data not shown). This rate is equal, within experimental error, to that previously observed[17] *in vivo*.

Dependence on the Molecular Weight of PEG

To determine the dependence of the effects of PEG on the molecular weight of the PEG, control DNA was incubated at 65°C, for 30 min with 12.6% PEG of several different molecular weights. For PEG with molecular weight of either 5000 or below, concatemerization was not observed (Fig. 7a; the lanes are labeled by the molecular weight of the PEG). However, for molecular weight of either 6000 or above, extensive concatemerization was observed in Fig. 7a. When the concentration of PEG was reduced to 7.2%, the lowest PEG molecular weight that yielded extensive concatemerization was increased to 21,000 (Fig. 7b). Thus, the minimum PEG concentration effective in promoting extensive concatemerization decreases as the molecular weight of the PEG increases.

DISCUSSION

Techniques Used

The technique of PFG electrophoresis used here was designed to separate both linear and open circular DNA. The longest open circular DNA resolved, a λ DNA 7-mer (0.339 Mb), is, to the authors' knowledge, the longest open circular DNA yet resolved. Studies designed to determine whether even longer open circular DNA is resolvable have not yet been undertaken. The conditions shown here to promote the formation of open circular λ DNA concatemers are useful for the production of length standards for open circular DNA.

When DNA is incubated in an agarose gel after electrophoresis, the comparatively small amount of change found (Fig. 1) in the association of cohesive ends is in contrast to the extensive concatemerization found when λ DNA is gelled in an agarose plug.[19,20] Although the reasons for this contrast have not been investigated in detail, they probably include: (a) lower DNA concentration used for Fig. 1; the maximum concentration of DNA in a band in Fig. 1 is 3–5 μg/ml, and (b) temperature lower than that present during gelation in an agarose plug. In any case, for a period of time three times longer than that usually used here for analysis by PFG electrophoresis, the maximal distortion in band intensity caused by in-gel association of cohesive ends was found to be 10% for monomeric λ DNA. Because the rate of λ DNA cyclization decreases as DNA length increases,[18] distortion from association should be even less for DNA longer than a 1-mer.

Because the λ concatemers that depolymerized during dilution-mixing were subsequently repolymerized in the experiments performed for Figs. 3–7, the dilution-mixing-induced depolymerization must have occurred at cohesive ends. Hydrodynamic shear has previously been found to nonspecifically shorten double-stranded DNA,[29,30] and is assumed to be the cause of the mixing-induced, cohesive end-specific depolymerization of λ concatemers. Selective, shear-induced breakage at staggered nicks, such as those at joined cohesive ends, has, apparently, not previously been reported. This cohesive end-specific depolymerization was useful for preparing a sample that consisted primarily of 1-mers through 3-mers of both linear and open circular DNA. Further investigation of depolymerization has not been performed.

Concatemerization vs. Condensation

When the concentration of sodium chloride used for buffers was increased to either 0.3 M or higher, in the presence of 10% PEG-6000, rate zonal centrifugation reveals that 0.04–0.17 Mb long, linear double-stranded DNA undergoes both intramolecular and intermolecular condensation that is independent of end-to-end cohesion.[31] In the present study, to minimize this condensation, the sodium chloride concentration was reduced (to 0.1 M). Nonetheless, DNA arrested at the origin was sometimes observed here in the presence of PEG and became the major form of DNA when the temperature was raised above 80°C in 12.6% PEG-6000. Presumably, the latter DNA has condensed (precipitated). However, for the other conditions used, most DNA formed a band of either linear or open circular DNA. Whether or not cohesive end-independent condensation, as defined in ref. 31, is occurring before PFG electrophoresis, the conclusion is drawn that, at temperatures of 80°C or less, most concatemers formed in the presence of PEG migrate through the gel as single molecules. If condensation does occur, then the molecules must be decondensed during entry into the gel.

The linear concatemers produced here extend beyond a 16-mer. Although detailed investigation of the maximum length achieved has not been performed, the intensity of the zone of compression and the distribution of concatemers formed at the higher percentages of PEG (for example, Fig. 5) indicate the presence of concatemers longer than a 32-mer. The length of concatemers produced after a known time (30 min was usually used here, but other times can be used) can be controlled by altering either the temperature, concentration of PEG or molecular weight of PEG. Thus, length standards of more precisely and flexibly defined length range than those produced in agarose plugs[19,20] can be made in liquid solution, in the presence of PEG. If the concatemers are not diluted before loading for electrophoresis performed according to procedures in the Materials and Methods, problems with either cohesive end-specific or nonspecific, shear-induced shortening of concatemers have not yet been detected. The bands formed by λ concatemers loaded in solutions of PEG are more uniform than bands of λ concatemers previously[23] formed by DNA loaded in solutions of buffer and sucrose. Although the PEG-induced concatemerization of the Results was initiated with DNA that already had undergone some joining of cohesive ends, this concatamerization can also be initiated with unjoined λ DNA. After 30 min of concatemerization, this difference in the initial DNA does not cause a detectable difference in the extent of concatemerization (data not shown).

Mechanisms

Because the melting temperature of cohesive ends, in the absence of PEG is lower than it is in the presence of PEG (at least 20°C lower in the presence of 12.6% PEG-6000), the PEG and temperature-induced cohesive end-specific stimulation observed here for both cyclization (for example: 37°–55°C in 12.6% PEG-6000) and concatemerization (for example: 57.5–80°C in 12.6% PEG-6000) must be caused, at least in part, by shifting the equilibrium toward joined cohesive ends. Increased rate of joining caused by elevated temperature-induced unraveling of secondary structure previously found[18] in the separated cohesive ends is also likely. A quantitative study of kinetics is in progress.

The cause of polymer-induced changes in the kinetics and thermodynamics of the joining of DNA has, in the past, been assumed to be steric exclusion (excluded volume effect) of DNA by the molecules of polymer.[3-7] Tests of this assumption have not been made, presumably because the enzymatic ligation usually required to promote joining complicates the analysis. The steric exclusion proposed, sometimes also called macromolecular crowding, is known to explain the following effects on propolymer-induced presuma decrease in solubility, exclusion from gelled beads during molecular sieve chromatography, differential in partitioning between polymer-containing and non-polymer-containing solutions and polymer-induced increase in osmotic pressure.[1,21,32] In the case of these latter studies of proteins, excluded volume per mass of solute was found to increase as the size of both the polymer and the protein excluded increased. This observation is expected theoretically.[33,34] Qualitatively, this same observation was made here for the polymer-induced joining of cohesive ends of DNA. That is, as the molecular weight of the PEG increased, the concentration of PEG needed to induce concatemerization decreased. In contrast to this correlation with excluded volume, the following observation indicates that the water activity of solutions of PEG is not correlated with the results observed: At a concentration of 12.6%, solutions of PEG with molecular weight between 1500 and 20,000 are so non-ideal that osmotic pressure differences are not detected (± 10%) among them (data not shown; see also ref. 2). Thus, bulk osmotic pressure is not the primary cause of the effects observed here. In addition, no evidence of correlation with a macroscopic phase separation was observed (turbidity, more than one liquid layer).

An additional observation capable of testing mechanisms is the shift from cyclization to concatemerization that occurs when the temperature increases by 2.5–5.0°C; this is an observation apparently without precedent. Use of excluded volume to explain this effect of temperature yields the following predictions: (a) The excluded volume of PEG 6000–21,200 increases as temperature increases. (b) If the concentration of DNA is progressively increased by increasing excluded volume, then a steep transition from circular to linear DNA occurs. To test the accuracy of these predictions, the finding, made by use of rate zonal centrifugation, that PEG with at least a molecular weight of 6,000 is randomly coiled in solution[32] will be used. Assuming that the random coils of both λ DNA and its concatemers do not penetrate the much smaller random coil of PEG, then expansion of the random coil of PEG will increase its excluded volume. In theory, all random coils expand when temperature increases, if, as found for PEGs,[35] their critical miscibility temperature is positive.[36] Thus, the conclusion is drawn that raising temperature expands random coils of PEG. The current data do not quantify the expansion. If the (reasonable) assumption of noninterpenetration of random coils is made, then prediction (a) is correct. That prediction (b) is, in theory, correct has previously been shown.[37] That is, as the concentration of DNA increases, eventually a phase change-like transition from circular to linear DNA is a consequence of the theory. Thus, excluded volume qualitatively explains at least part of the elevated temperature-induced stimulation of the joining of cohesive ends that is observed here in the presence (but not in the absence) of PEGs.

Three aspects of the data presented here make the data, thus far, unsuitable for a quantitative analysis of kinetics and mechanism: (a) The concept of excluded volume is not yet sufficiently developed to introduce in analysis of kinetics (see also ref. 1); differences in excluded volume that depend on the length of the large number of different types of concatemer observed here thwart attempts at a simple analysis. (b) The rate of joining becomes too rapid for accurate quantification when the concentration of PEG is above the minimum for stimulation of concatemerization. (c) Detailed characterization of the PEG random coil, for the conditions used here, has not yet been performed. For example, both improved understanding of excluded volume and more detailed characterization of PEG will probably be needed to explain the observation that, in the presence of PEG, roughly equal amounts of 2-mers through 16-mers are sometimes found together with an excess of 1-mer (i.e., Figs. 5 and 6). This observation is not expected if random selection of cohesive ends occurs during joining.[38]

Implications for Events that Occur In Vivo

When λ DNA is injected at 37°C into an immune host, 90% of the DNA is cyclized within 15 min.[17] An order of magnitude slower rate is achieved in the absence of polymer at 37°C.[18] Apparently for the first time, the *in vivo* rate of cyclization has been achieved here in the presence of PEG. Thus, two-dimensional diffusion of cohesive ends, previously proposed,[18] is not necessary to explain the rate of cyclization observed *in vivo*. Excluded volume effects, coupled with protein-induced (rather than elevated temperature-induced) unraveling of secondary structure in cohesive ends is a possible alternative explanation.

The open circular DNA-to-linear DNA transition observed here when temperature is raised shows that essentially complete dominance of linear over circular molecules can be achieved without any biological mechanism evolved for this purpose. This dominance mimics the dominance of linear concatemers in bacteriophage T7-infected cells.[39] In contrast, joining of cohesive ends in extracts of T7-infected cells produces some circular DNA[40] and the relative amount of circular DNA increases as the concentration of DNA decreases (M. Son and P. Serwer, unpublished observations). Such mimicking of processes that occur *in vivo* does not prove identity of mechanism. Further tests of *in vivo* mechanisms will include attempts to mimic *in vivo* processes in *in vitro* systems that include more of the components present *in vivo* (DNA-binding proteins, for example) than does the system used here.

ACKNOWLEDGEMENTS

The authors thank Marjatta Son for preparing T7 DNA, Gary A. Griess for assistance with the digital imaging, C. S. Raman for discussion and Linda C. Winchester for typing this manuscript. Support was received from the U. S. Public Health Service (GM-24365), the Texas Coordinating Board (004), and the Robert A. Welch Foundation (AQ-764).

REFERENCES

1. Minton, A. P. (1983) Mol. and Cell. Biochem. *55*, 119–140.
2. Parsegian, V. A., Rand, R. P., Fuller, N. K., and Rau, D. C. (1986) Methods in Enzymol. *127*, 400–416.
3. Zimmerman, S. B. and Harrison, B. (1985) Nucleic Acids Res. *13*, 2241–2249.
4. Pheiffer, B. H. and Zimmerman, S. B. (1983) Nucleic Acids Res. *11*, 7853–7871.
5. Hayashi, K., Nakazawa, M., Ishizaki, Y., Hiraoka, N., and Obayashi, A. (1985) Nucleic Acids Res. *13*, 3261–3271.

6. Hayashi, K., Nakazawa, M., Ishizaki, Y., Hiraoka, N., and Obayashi, A. (1986) Nucleic Acids Res. *14*, 7617–7631.
7. Zimmerman, S. B. and Pheiffer, B. H. (1983) Proc. Natl. Acad. Sci. USA *80*, 5852–5856.
8. Louie, D. and Serwer, P. (1990) Nucleic Acids Res. *18*, 3090.
9. Zimmerman, S. B. and Harrison, B. (1987) Proc. Natl. Acad. Sci. USA *84*, 1871–1875.
10. Son, M., Hayes, S. J., and Serwer, P. (1989) Gene *82*, 321–325.
11. Jarvis, T. C., Ring, D. M., Daube, S. S., and von Hippel, P. H. (1990) J. Biol. Chem. *265*, 15160–15167.
12. Prouty, M. S., Schechter, A. N., and Parsegian, V. A. (1985) J. Mol. Biol. *184*, 517–528.
13. Minton, A. P. and Wilf, J. (1981) Biochemistry *20*, 4821–4826.
14. Wilf, J. and Minton, A. P. (1981) Biochim. Biophys. Acta *670*, 316–322.
15. Serwer, P., Masker, W. E., and Allen, J. L. (1983) J. Virol. *45*, 665–671.
16. Laurent, T. C., Preston, B. N., and Carlsson, B. (1974) Eur. J. Biochem. *43*, 231–235.
17. Bode, V. C. and Kaiser, A. D. (1965) J. Mol. Biol. *14*, 399–417.
18. Wang, J. C. and Davidson, N. (1968) Cold Spring Harbor Symp. Quant. Biol. *33*, 409–415.
19. Anand, R. (1986) Trends in Genetics *2*, 278–283.
20. Waterbury, P. G. and Lane, M. J. (1987) Nucleic Acids Res. *15*, 3930.
21. Laurent, T. C. (1968) in Quintarelli, G. (ed.) The Chemical Physiology of Mucropolysaccharides, Little, Brown and Co., Boston, pp. 153–170.
22. Chase, J. W. and Williams, K. R. (1986) Ann. Rev. Biochem. *55*, 103–136.
23. Louie, D. and Serwer, P. (1989) Appl. Theor. Electrophor. *1*, 169–173.
24. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Cantor, C. R., Smith, C. L., and Mathew, M. K. (1988) Ann. Rev. Biophys. Biophys. Chem. *17*, 287–304.
26. Griess, G. A. and Serwer, P. (1991) in Cantor, C. R. and Lim, H. A. (eds.) Electrophoresis, Supercomputing and the Human Genome, World Scientific, Teaneck, New Jersey, in press.
27. O'Neill, R. R., Mitchell, L. G., Merril, C. R., and Rasband, W. S. (1989) Appl. Theor. Electrophor. *1*, 163–167.
28. Fuke, M. and Thomas, C. A. (1970) J. Mol. Biol. *52*, 395–397.
29. Adam, R. E. and Zimm, B. H. (1977) Nucleic Acids Res. *4*, 1513–1537.
30. Zimm, B. H. and Reese, H. R. (1990) Nucleic Acids Res. *18*, 4469–4470.
31. Lerman, L. S. (1971) Proc. Natl. Acad. Sci. USA *68*, 1886–1890.
32. Polson, A. (1977) Prep. Biochem. *7*, 129–154.
33. Ogston, A. G. (1958) Trans. Faraday Soc. *54*, 1754–1757.
34. Hermans, J. (1982) J. Chem. Phys. *77*, 2193–2203.
35. Ataman, M. (1987) Colloid and Polymer Sci. *265*, 19–25.
36. Flory, P. J. (1953) Principles of Polymer Chemistry, pp. 509–600, Cornell University Press, Ithaca.
37. Jacobson, H. and Stockmayer, W. H. (1950) J. Chem. Phys. *18*, 1600–1606.
38. Tanford, C. (1963) Physical Chemistry of Macromolecules, Wiley, New York.
39. Serwer, P., Watson, R. H., and Hayes, S. J. (1987) J. Virol. *61*, 3499–3509.
40. Son, M., Hayes, S. J., and Serwer, P. (1988) Virology *162*, 38–46.