Volume 13 Number 7 1985 Nucleic Acids Research

Macromolecular crowding accelerates the cohesion of DNA fragments with complementary termini

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Received 14 January 1985; Revised and Accepted 8 March 1985

ABSTRACT

Macromolecular crowding increases the rate of nonenzymatic cohesion of the complementary ends of λ DNA. Both λ DNA and DNA fragments bearing the cohesive ends of λ DNA are similarly affected. High concentrations of plasma albumin or Ficoll 70 increase the rate of cohesion by ca. 100-fold whereas high concentrations of polyethylene glycol 8000 cause >2000-fold stimulation in this rate. These results have implications for the mechanism of polymer-stimulated enzymatic ligation of DNA or RNA. In addition, these crowding effects may help to explain the rapid cohesion of λ DNA observed in vivo.

An improved procedure for the recovery of DNA fragments separated by agarose gel electrophoresis is also described.

INTRODUCTION

The concentration of macromolecules within living cells is enormous (1,2). At such high concentrations, excluded volume effects of the macromolecules can cause large changes in equilibria or rates of chemical reactions (3). As part of a program to assess the importance of these effects on systems involving nucleic acids, we have recently found that the in vitro rate at which T4 DNA ligase joins DNA fragments with blunt or short cohesive ends can be increased over 1000-fold by macromolecular crowding (2,4). High concentrations of a variety of different macromolecules such as serum albumin, glycogen, polyethylene glycol, or Ficoll stimulated the rates of these ligation reactions, apparently by increasing the effective concentration of the ends of the DNA molecules to be joined. If this basis for the stimulation is correct, we would expect that crowded conditions would also stimulate the rates of nonenzymatic cohesion of DNA duplexes with cohesive ends. We here provide evidence that macromolecular crowding causes such an effect.

Two systems are used, both based upon the naturally cohesive ends of λ DNA. This duplex DNA of ca. 49,000 base pairs length possesses protruding single-stranded termini which are 12 residues in length and which are comple-

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mentary to each other (5). These ends can overlap in a reversible noncovalent interaction which generates a cohered product with considerable stability in media of moderate or high ionic strength (e.g., Tm = 51° in 0.13 M Na^+) (6-11). We initially describe the effects of macromolecular crowding upon the rates of cohesion of two isolated fragments, 4590 and 6694 base pairs in length, each bearing one of the two λ DNA cohesive ends and one blunt-end. These fragments were isolated from Nru I nuclease digests of λ DNA. Only a single product is formed by cohesion of these DNA fragments. We then describe the effects of crowding on the more complex series of products formed by cohesion of λ DNA *per se*.

MATERIALS AND METHODS

Materials

λ DNA (New England Biolabs or Bethesda Research Labs) was digested with Nru I nuclease under the conditions described by the supplier, Bethesda Research Labs. PEG 200 and PEG 8000 were purchased from Baker and Ficoll 70 from Pharmacia. A 50% w/v solution of crystallized bovine plasma albumin (Miles) in deionized water was adjusted to pH 7.6 with 0.08 volume of 1 N NaOH.

Isolation of Cohesive-ended DNA Fragments

The 4.6 and 6.7 kbp fragments, each bearing one of the cohesive ends of λ DNA, were isolated from Nru I nuclease digests of λ DNA. The procedure of Dretzen *et al*. (12) was initially employed: the fragments were separated by agarose gel electrophoresis, stained with ethidium bromide, and the bands collected electrophoretically onto DEAE-cellulose paper strips from which the DNA was eluted by high salt concentrations. The yields of cohesive-ended fragments were low (< 15%), perhaps due to the presence of the short single-stranded regions at the DNA termini. A more serious problem was that a fraction of the fragments (varying from 5-40% in different preparations) showed anomalous, essentially instantaneous cohesion as if the two fragments were entangled with or cross-linked by a contaminant. This anomaly was clearly due to contaminants carried over from the isolation procedure (13) as demonstrated by effects on the cohesion behavior of unfractionated DNA due to the addition of the preparation of isolated fragments. The yield of the isolated fragments was increased to > 60% by using containers and glass wool treated with Sigmacote (Sigma) (14) and by isolating the 4.6 and 6.7 kbp fragments in the form of the cohered 11.3 kbp aggregate. The latter change also ensured a stoichiometric ratio of the 4.6 and 6.7 kbp fragments. The

cohered 11.3 kbp aggregate was formed by incubating phenol-treated and ethanol-precipitated Nru I nuclease digests of λ DNA for 5 hours at 45° in 0.1 M NaCl - 0.1 mM EDTA at 0.6 mg/ml of DNA. The 11.3 kbp aggregate was subsequently isolated on 0.8% agarose gels as ref. 12. While the yields were improved by the changes mentioned, the anomalous cohesion behavior was not. The contaminants responsible were not removed by centrifugation or ethanol precipitation nor by repetitions of the n-butanol extractions used to remove ethidium bromide. Phenol or phenol-$CHCl_3$ extractions (13) did remove the contaminant but caused an almost total loss of the DNA in the process. The DNA was apparently being trapped with the contaminants in the visible accumulation at the phenol-water interface. Phenolization was therefore carried out in a number of media to attempt to release the DNA into the aqueous phase. Tris-borate-EDTA (13) or 2 M NaCl solutions were ineffective. However, inclusion of 1.3% SDS allowed full partition of the DNA into the aqueous phase and the concomitant removal of the contaminant. The extraction with phenol in the presence of the SDS was followed by extractions with phenol-$CHCl_3$ (1:1) and $CHCl_3$. The 11.3 kbp aggregate so obtained was finally ethanol-precipitated and washed, redissolved in 10 mM Tris·Cl (pH 8.0) - 0.1 mM EDTA and, shortly before use, the 11.3 kbp fragment was dissociated into 4.6 and 6.7 kbp fragments by heating for 10 min at 65° and quenching in ice water. The artefactual instantaneous cohesion of the 4.6 and 6.7 kbp fragments did not occur with material purified as described. The kinetics of cohesion of the purified fragments and of the appropriate fragments in the total Nru I nuclease λ DNA digest were indistinguishable.

RESULTS AND DISCUSSION

The effect of changes in the concentration of several background macromolecules on the rate of cohesion of the Nru I nuclease terminal fragments of λ DNA is shown in Figures 1-3. At relatively high concentrations of PEG 8000, Ficoll 70, or bovine plasma albumin there is a dramatic increase in the fraction of cohered DNA. The half-time for cohesion in the absence of crowding is several days (Figure 3). This rate is increased over 2000-fold in 15% w/v PEG 8000 (avg. mol. weight = 7000-9000) (Figure 1; cf. lane 11 of Figure 3) and about 100-fold in either 30% w/v bovine plasma albumin (Figure 2) or 50% w/v Ficoll 70 (avg. mol. weight ca. 70,000) (Figure 3). As expected, the cohesion was fully reversed by mild heating (5 min at 65°) if the background polymer concentration had been lowered by dilution (data not shown) (6). Two low molecular weight analogues of the polymers were tested. Sucrose,

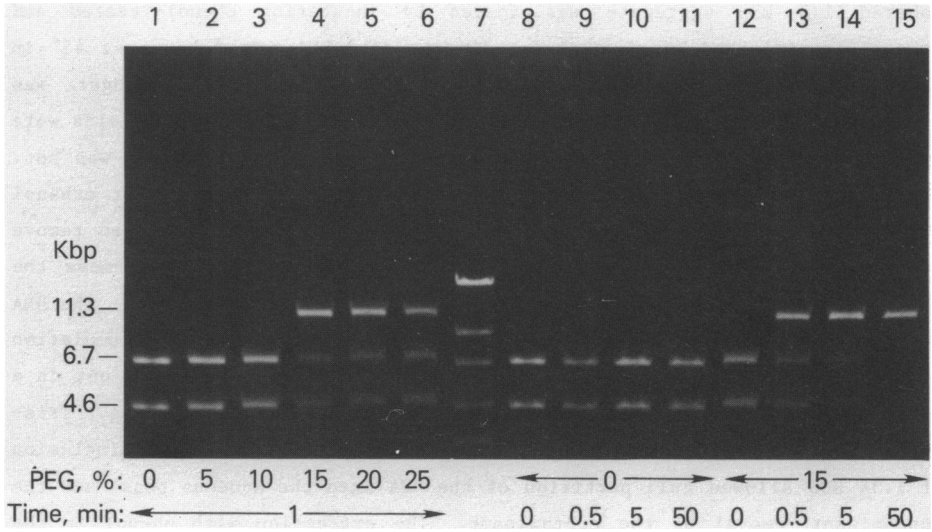

Figure 1. Effect of PEG 8000 on the cohesion of terminal fragments from λ DNA. The assay measures the cohesion of the 4.6 and 6.7 kilobase pair (kbp) fragments, each bearing one of the cohesive ends of λ DNA, to form an 11.3 kbp aggregate. Reaction mixtures (10 µl) containing a total of 30 ng of DNA as an equimolar mixture of 4.6 and 6.7 kbp fragments in 50 mM Tris•Cl (pH 7.8) - 5 mM $MgCl_2$ and the indicated (w/v) concentrations of PEG 8000 were incubated at 20° for the times indicated. The assay reaction was stopped by addition of 1.9 µl of 45% v/v glycerol - 4.5% SDS - 0.05% bromthymol blue - 36 mM EDTA. Aliquots (7 µl) were applied to agarose minigels (0.8% agarose (Sigma Type II, medium EEO) in 89 mM Tris - 89 mM boric acid - 2.5 mM EDTA). After 90 min at 60 v (Mini-cell, Bio-Rad) the gel was stained in 1 µg/ml of ethidium bromide, destained, and photographed under ultraviolet illumination. Lane 7: Nru I nuclease-digested λ DNA with 23.5, 9.4, 6.7, 4.6, and 3.7 kbp bands.

the monomer of Ficoll, at 30, 50 or 70% w/v did not significantly stimulate cohesion and PEG 200 (avg. mol. weight = 190-210) at 15% w/v had a 100-fold lower effect than did the same concentration of PEG 8000 (data not shown). The requirements for relatively high concentrations of high molecular weight materials and the lack of specificity for the nature of the polymers is fully consistent with a volume-exclusion basis for the stimulation of the cohesion reaction (3). In addition, the strong dependence of the stimulation in rate upon the concentration of background molecules is expected for crowding effects: the probability of finding an unoccupied volume of solution which can accommodate the DNA molecules should decrease precipitously as the occupancy increases.

Several factors were tested for effects on the rate of cohesion of these

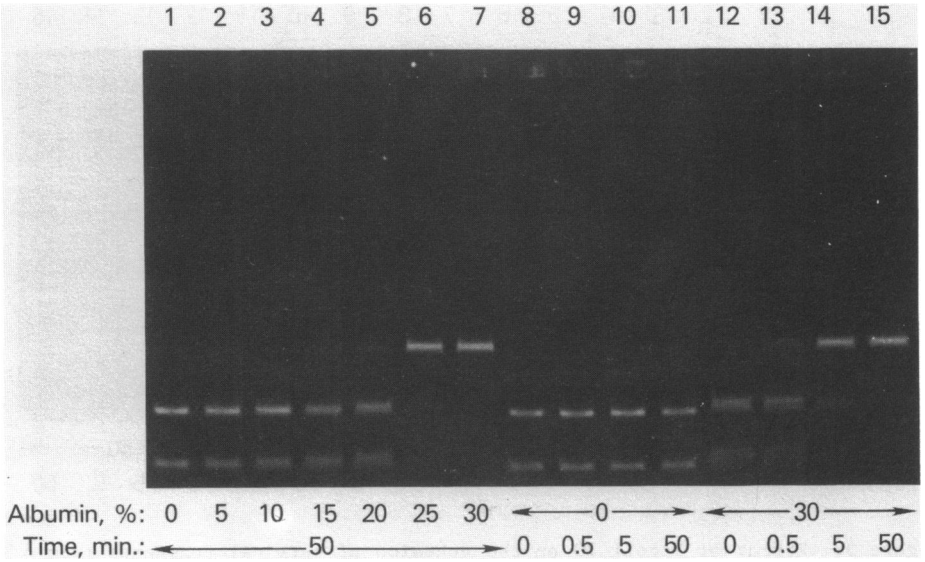

Figure 2. Effect of bovine plasma albumin on the cohesion of terminal fragments from λ DNA. To minimize distortion of the DNA gel pattern by the albumin, after incubation at 20° for the times indicated the reaction mixtures (6 µl containing 30 ng of DNA as an equimolar mixture of 4.6 and 6.7 kbp terminal fragments of λ DNA, in 50 mM Tris·Cl (pH 7.8) - 5 mM $MgCl_2$ and the indicated (w/v) concentrations of bovine plasma albumin) were diluted with 11 µl of 10% v/v glycerol - 2% SDS - 0.01% bromthymol blue - 8 mM EDTA; 8 µl aliquots were applied to a 0.6% agarose gel containing 0.1% SDS in 89 mM Tris - 89 mM boric acid - 2.5 mM EDTA - 0.1% SDS buffer. After 90 min at 60 v, the gel was soaked for 1 hr in gel buffer lacking SDS and then stained, etc., as for Figure 1. The faint band between the 11.3 kbp and 6.7 kbp bands in Figures 2 and 3 is contaminating blunt-ended 9401 kbp fragment from the Nru I nuclease digest of λ DNA.

DNA fragments in a 50% w/v Ficoll 70 medium at 20°: Addition of a 5-fold excess (weight basis) of an unrelated DNA of similar molecular weight with blunt ends (Pvu II nuclease-digested pBR322 DNA) had no effect on the rate of cohesion. The rate decreased by at least 30-fold in the absence of Mg^{++}. Finally, the rate at 37° in a medium containing 50% w/v Ficoll 70 was ca. 2-fold higher than that at 20° in this medium.

Attempts to measure the effects of crowding on the equilibrium for cohesion of the Nru I fragments by shifts in the Tm were unsuccessful. In concentrated Ficoll 70 solutions, heating converted the Nru I fragments to a form which did not enter agarose gels whereas in concentrated PEG 8000 solutions, the rates of reassociation of the 6.7 and 4.6 kbp fragments were so great that we were unable to adequately freeze the reaction.

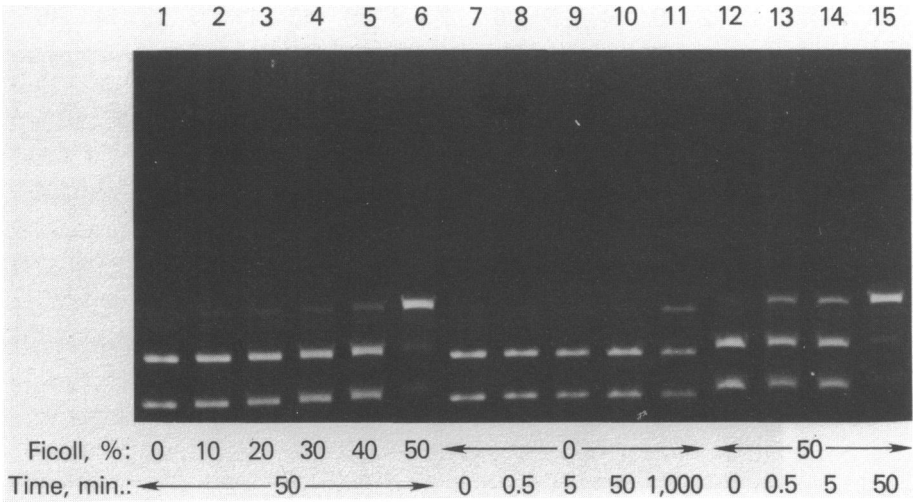

Figure 3. Effect of Ficoll 70 on the cohesion of terminal fragments from λ DNA. Procedure as for Figure 1 except for the indicated (w/v) concentrations of Ficoll 70.

The effects of macromolecular crowding upon the cohesion of λ DNA per se were similar in many respects to the effects on the cohesion of the Nru I fragments of λ DNA. The magnitudes of the stimulation in rate were generally similar for the two substrates (data not shown). The concentrations of the

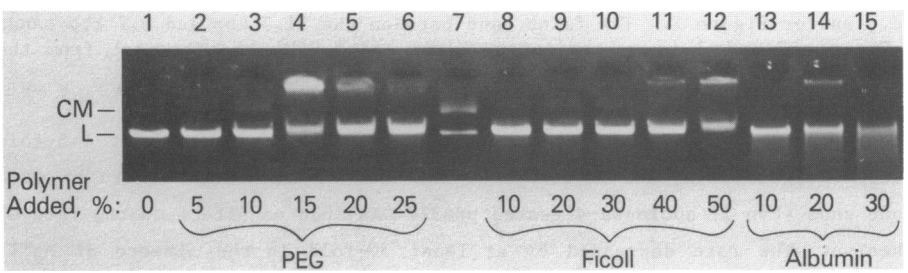

Figure 4. Effect of PEG 8000, Ficoll 70 or bovine plasma albumin concentrations on the cohesion of λ DNA. Reaction mixtures (20 µl) contained 60 ng of λ DNA in 50 mM Tris•Cl (pH 7.8) - 5 mM $MgCl_2$ and the indicated concentrations (w/v) of PEG 8000, Ficoll 70, or bovine plasma albumin. After incubation at 20° for the indicated times, 7.5 µl of 23% v/v glycerol - 4.5% SDS - 0.023% bromthymol blue - 18 mM EDTA was added. Aliquots (7 µl) were electrophoresed (20 v, 2 hrs) on an 0.8% agarose minigel in the buffer of Serwer and Allen (15). Lane 7 contains a mixture of H-bonded circular monomers (CM) and linear (L) λ DNA prepared by cohesion at 2 µg/ml of DNA as in ref. 15.

three polymers which gave large stimulations (Figure 4) were similar to those described earlier for the Nru I fragments. As with the terminal fragments, the reaction with λ DNA in Ficoll 70 solution was dependent on the presence of Mg^{++} and increased about 2-fold at 37° versus 20°. Neither sucrose nor PEG 200 substantially affected the rate of λ DNA cohesion. The products of cohesion of λ DNA are both potentially and actually more complex than those from the Nru I nuclease DNA fragments. At lower concentrations of background polymer, small amounts of product migrating like monomeric circles ("Hershey circles") were seen (lanes 2-3 and 8-10, Figure 4). A similar small stimulation in the rate of formation of circular products has also been noted at relatively low background polymer concentrations with reactions catalyzed by T4 DNA ligase (4) or T4 RNA ligase (16). However, just as in those systems, under all conditions where we find sizeable increases in the rates of reaction, the products are neither monomeric circles nor small linear aggregates. The products move much more slowly on agarose gels than do either of these forms and are presumably large aggregates, perhaps containing catenanes or knotted molecules.

The large increases in rate of cohesion of complementary ends of λ DNA or λ DNA fragments that arise due to crowding are reasonably ascribed to increases in the effective local concentration of the ends, i.e. to increases in the concentration of ends near other ends (10). The particularly large stimulation due to the presence of PEG 8000 is probably a result of the formation of a condensed form of the DNA, the ψ form of DNA described by Lerman (17). Minton has discussed theoretical effects of crowding upon equilibria and rates of reaction (3). In the case of association reactions, he predicted an increased rate of association due to crowding effects on the entropy of activation, whereas the rate of dissociation is predicted to be relatively unchanged by crowding. Our results are consistent with the predicted increase in association rate; we have no data on changes in rates of dissociation or equilibrium constants (see comment above).

It is known from earlier studies of Wetmur and colleagues that macromolecular crowding can substantially increase the rate of reassociation of high molecular weight DNA strands. Effects of nonionic polymers were relatively small in this system (18), whereas anionic polymers caused larger effects (19). Both in their studies and in the present case, the effects of background polymers on reassociation are interpreted in terms of volume exclusion leading to increased effective concentrations of DNA. There is, however, a fundamental difference in the substrates employed. In Wetmur's

studies, the dissociated DNA was in the form of high molecular weight single strands. In the present case, < 1% of the dissociated DNA is single-stranded, and that portion is confined to the termini. The choice of such a substrate was made to study as directly as possible the effect of crowding upon the frequency with which the termini of double-stranded DNA come within a relatively small separation of each other. Such information was desired both for its possible application to the crowded, in vivo condition as well as to help understand the mechanism by which crowding causes the effects on enzymatic ligation noted in earlier studies.

Those earlier experiments (2,4) suggested that the increase in effective concentration of DNA ends due to crowding was responsible for most or all of the very large stimulating effects that macromolecular crowding can have on the enzymatic ligation of DNA fragments with blunt or short cohesive ends. We tested that suggestion in the following experiment: The ends of λ DNA were precohered in 15% w/v PEG 8000 and then the PEG 8000 concentration was lowered to below the polymer level which stimulates ligation. Such precohered DNA was ligated by T4 DNA ligase at a ca. 100-fold higher rate than was DNA that had not been precohered. Further, the presence of 15% w/v PEG 8000 made little difference in the subsequent rate of ligation of such precohered DNA. A similar result was obtained with the isolated terminal fragments of λ DNA. Hence, it is the rapid cohesion of the DNA termini of either λ DNA or of the Nru I nuclease fragments of λ DNA that is responsible for at least most of the 1000-fold stimulation of ligation of λ DNA termini that can be obtained in the presence of 15% w/v PEG 8000. The stimulation of ligation of blunt or short cohesive ends which we previously observed is likely to be due to a similar effect of crowding on the apposition of those termini.

These crowding effects may be relevant to the rate of cohesion of λ DNA in vivo. Cyclization of this DNA under a variety of dilute solution conditions is at least 1-2 orders of magnitude slower than that in vivo (11,20). The large stimulations in rates of cohesion in crowded media suggest that the high polymer concentrations in vivo may be involved in the rapid cohesion of the ends of the λ DNA.

ACKNOWLEDGEMENTS

We thank Martin Gellert for his comments, and Betty Canning and Linda Tripp for their expert assistance with the manuscript.

ABBREVIATIONS

PEG, polyethylene glycol; SDS, sodium dodecylsulphate; kbp, kilobase pair.

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