Shear-Induced Assembly of λ-Phage DNA

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ABSTRACT Recombinant DNA technology, which is based on the assembly of DNA fragments, forms the backbone of biological and biomedical research. Here we demonstrate that a uniform shear flow can induce and control the assembly of λ-phage DNA molecules: increasing shear rates form integral DNA multimers of increasing molecular weight. Spontaneous assembly and grouping of end-blunted λ-phage DNA molecules are negligible. It is suggested that shear-induced DNA assembly is caused by increasing the probability of contact between molecules and by stretching the molecules, which exposes the cohesive ends of the otherwise undeformed λ-phage DNA molecules. We apply this principle to enhance the kinetics and extent of DNA concatenation in the presence of ligase. This novel approach to controlled DNA assembly could form the basis for improved approaches to gene-chip and recombinant DNA technologies and provide new insight into the rheology of associating polymers.

INTRODUCTION

Traditional DNA cloning methods are based on linking two fragments of DNA, an insert and a plasmid vector, by DNA ligase; the resulting ligated construct is introduced into an expression system such as *Escherichia coli*. Ligation between DNA fragments is typically slow. Because ligation is traditionally performed at low temperatures, ligation may take hours to be completed. Here we use uniform shear flows to induce linking of DNA molecules. Shear flows have been extensively used to break DNA molecules into smaller fragments of random length for genomic applications, including the shotgun cloning approach (Marziali et al., 1999; Oefner et al., 1996). For instance, the Point-sink Shearer (PtS), which forces DNA molecules through a small hole with a syringe pump (Thorstenson et al., 1998), generates reproducible distributions of randomly broken fragments that are more readily amenable to rapid sequencing (Hengen, 1998; Zeng and Kreitman, 1996). Processing of plasmid-based genes for gene therapy and DNA vaccination also involves shear flows, which may, however, accidentally degrade and break DNA molecules into smaller, nonfunctional fragments (Levy et al., 1999).

In contrast, this paper demonstrates that a uniform shear flow can induce and control the formation of multimers of DNA molecules. The DNA primarily used in this study is isolated from the genome of bacteriophage λ, which is one of the best characterized DNA molecules because of its widespread use in the preparation of genomic libraries (Kornberg and Baker, 1991). Lambda DNA (λ-phage DNA) is a linear double-stranded helix that contains 48,502 bp; its molecular mass is ~30.6 MDa, and its contour length is ~17.2 μm (Sanger et al., 1982). The termini of λ-phage DNA consist of 12-nucleotide-long, single-stranded, complementary overhangs, which serve as "sticky" or cohesive ends. Interactions between cohesive ends is mediated by hydrogen bonding and base stacking between complementary base pairs. Here we show that a uniform shear flow can induce end-to-end DNA assembly and that the rate of shear increases the length of the DNA "polymers" at low and moderate shear rates. Spontaneous assembly and the grouping of end-blunted λ-phage DNA molecules are negligible. At high shear rates, DNA multimers break back into DNA monomers without further fragmentation. The rate of shear, the ionic strength of the solution, and the nature of DNA termini have a strong influence on the molecular weight distribution of DNA under shear. Moreover, we show that shear greatly enhances the kinetics of DNA concatenation by ligase.

MATERIALS AND METHODS

λ-phage DNA solutions and application of controlled shear flows

A 0.5 mg/ml stock solution of λ-phage DNA (New England Biolabs, Beverly, MA) is diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) with 2 mM $MgCl_2$ to a final concentration of 0.05 mg/ml. At that concentration, DNA molecules are not entangled but may partially overlap (Mason et al., 1998). To linearize the concatemers and relax any preexisting entanglements, the solution is heated to 70°C for 10 min and immediately placed in a freezer at −20°C for 45 min (a quick chill to 0°C produced the same final results). In some experiments, we also use a shorter DNA fragment, the plasmid pBR322, a widely used cloning vector, which contains 4361 bp (contour length 1.49 μm; New England Biolabs) and 4-nucleotide-long overhangs generated by *Hin*dIII (New England Biolabs).

The DNA solution is thawed and placed in a shearing device, a strain-controlled rheometer described by Ma et al. (1999). This device consists of a 50-mm-radius fixed upper cone (angle 0.04 rad) and a 50-mm-radius rotating lower plate connected to a computer-controlled motor (Ares 100; Rheometrics, Newark, NJ). These upper and lower tools subject DNA molecules to steady, laminar shear flows of controlled rates of shear (see schematic in Fig. 1). The rate of shear (i.e., velocity gradient of the flow field) between the cone and plate of the rheometer, $dv/dy = \dot{\gamma}$, is constant. The temperature of the specimen is maintained at 21°C; the tools are

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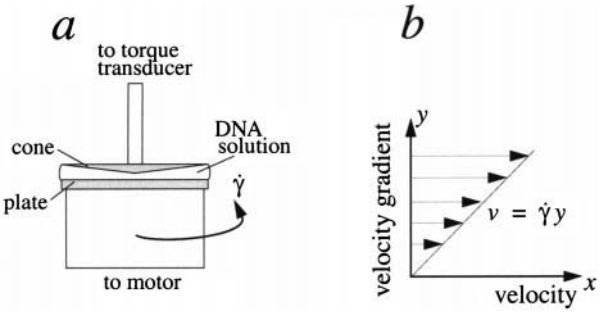

FIGURE 1 Schematic of a strain-controlled rheometer used here to shear DNA solutions. The lower, circular tool is connected to a computer-controlled motor; the upper tool is fixed and has a conical shape. These upper and lower tools create a laminar, shearing flow, which has a uniform rate of shear across the space between the outer edge and the center of symmetry of the tools. (*a*) Rheometer geometry. (*b*) Shear-flow field of the constant velocity gradient, $dv/dy = \dot{\gamma}$, generated by the cone and plate of the rheometer.

enclosed in a vapor trap, which prevents buffer evaporation. A volume of 180 μl of the 0.05 mg/ml solution of DNA molecules is sheared at a fixed shear rate for 2 min in the rheometer.

Gel electrophoresis

After each shear run, 10 μl of sample is transferred to an electrophoresis unit (Chef-Dr II; BioRad, Santa Barbara, CA) for the determination of the molecular weight distribution of the sheared DNA molecules. Each aliquot is mixed with 1% agarose solution in 0.5× TBE buffer (44.5 mM Tris, 1.25 mM EDTA, 44.5 mM boric acid, pH adjusted to 8.3). Two unsheared samples are also loaded into the electrophoretic gel before and after the shearing test; a lambda ladder (New England Biolabs) serves as a molecular weight marker. Unless stated otherwise, for the best resolution of the molecular weight distribution, electrophoretic gels are run for 15 h at 10°C at 6 V/cm with a ramped pulse of 1–12 s. Gels are stained with 0.5 μg/ml ethidium bromide, then destained with distilled water for 1.5 h before being transferred to a UV illuminator (Eagle Eye Still Video System; Stratagene, Palo Alto, CA) for densitometric analysis, which is performed using the software National Institutes of Health Image (Bethesda, MD).

Light microscopy

To prepare DNA molecules for fluorescence microscopy, we use the intercalating fluorescent dye YOYO-1 (Molecular Probes, Eugene, OR), mixed with Tris-EDTA buffer and an antiphotobleaching solution (Matsumoto et al., 1992). DNA monomers and multimers are visualized in a custom-built chamber mounted on an upright microscope (Eclipse E-600; Nikon, Tokyo, Japan), with a 100× oil-immersion lens (N.A. 1.30; Nikon).

RESULTS

Shear-induced λ-phage DNA assembly

The effect of shear flow on the architecture of λ-phage DNA molecules is examined using a combination of quantitative gel electrophoresis and fluorescence microscopy. Fig. 2 displays electrophoretic gels obtained for sheared solutions of DNA with cohesive ends. This figure shows

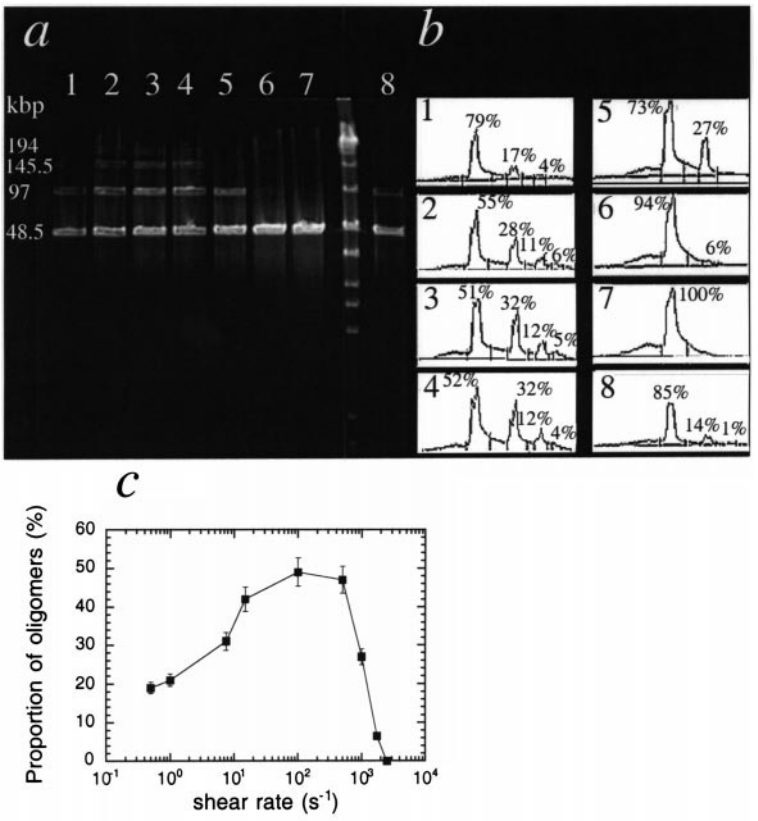

FIGURE 2 Resolution of λ-phage DNA (cl857*ind*1 *Sam*7) by pulsed-field gel electrophoresis (PFGE), run for 15 h at 6 V/cm, with a pulse time ramping from 1 to 12 s in a 1% agarose gel at 15°C. *Lanes 1* and *9*: the unsheared samples before and after the experiment. *Lanes 2–7*: DNA sheared at 1, 100, 500, 1000, 1750, and 2500 s^{-1}, respectively. *Lane 8*: Low Range PFG Marker (New England Biolabs). (*a*) Electrophoretic gel. (*b*) Densitometric analysis of the gel. (*c*) Proportion of oligomers in solution (dimers, trimers, etc.) as a function of shear rate for λ-phage DNA.

that multimers made of the starting DNA "monomers" are readily created under shear (*lanes 2–7* in Fig. 2). The rate of shear is a factor controlling the molecular weight distribution because the proportion of dimers, trimers, and even tetramers greatly increases for increasing shear rates (Fig. 2). However, past a threshold shear rate of $\sim 500\ s^{-1}$, longer macromolecules start vanishing and only smaller multimers of DNA are formed. At $\sim 1750\ s^{-1}$, only monomers of DNA are detected (Fig. 2).

Over the wide range of shear rates probed here, minimal fragmentation occurs, shown by well-defined, narrow bands and by the absence of DNA fragments migrating faster than monomeric λ-phage DNA: DNA monomers remain largely intact, even at high shear rates. We also find that, at room temperature, if the λ-phage DNA solution is first sheared at a high shear rate but is still lower than $500\ s^{-1}$ (see Fig. 2 *c*) and is then sheared at a lower shear rate, the DNA molecular mass distribution corresponds to that generated at the initial higher shear rate. Vice versa, if the DNA solution is first sheared at a low shear rate (lower than $100\ s^{-1}$) and subsequently sheared at a higher shear rate, the structures generated correspond to the latter, higher shear rate (Fig. 2). At high temperatures ($45°C < T < 70°C$), concatenation is shorter-lived and becomes reversible (data not shown). Together, these results suggest that the shear rate of the flow, which is the unique variable in the system, induces and controls the molecular weight distribution of DNA molecules with cohesive ends. Similar results are obtained for much shorter DNA fragments with short cohesive ends. Note that our results predict that increasing shear rates should decrease the number of circles (self-pairing DNA molecules). Using molecular combing and fluorescence microscopy (see below), we are currently testing this prediction.

Spontaneous DNA assembly and shear of end-blunted DNA

To ensure that the formation of multimers is enhanced by the applied flow field alone and that the flow shear rate is a controlling parameter, we conduct two control experiments. In the first control experiment, we investigate the possible spontaneous assembly of DNA over long periods of time in the absence of shear. Lanes 1 and 8 in Fig. 2 correspond to the state of DNA "polymerization" after 5 min and 1 h, respectively. The presence of small DNA concatemers can be attributed to the combined effects of nonzero probability of interactions between DNA ends and unavoidable shearing of DNA upon pipetting (we used wide-bore tips from Bio-Rad; estimated shear rate $0.5\ s^{-1}$). Therefore, spontaneous assembly of DNA molecules with cohesive ends is unfavorable, at least at small DNA concentrations.

We also tested to determine whether DNA association is promoted even when the cohesive ends are replaced by blunt ends. For that purpose, we enzymatically generate the complementary base pairs of the overhangs, which "fill in" the λ-phage DNA overhangs and render them noninteracting. The cohesive ends of λ-phage DNA can be eliminated by pairing each nucleotide of the overhang with its complementary base generated with the enzyme DNA polymerase I (Kleenow; New England Biolabs). The Ecopol buffer used for the enzymatic reaction contains 5 mM $MgCl_2$. The reaction is stopped by adding EDTA to a 10 mM final concentration and subsequently heating at 75°C for 10 min. Fig. 3 shows the electrophoretic gels obtained with solutions of DNA with blunt ends subject to shear flows of increasing shear rates. These gels do not display any band outside the monomer at all tested shear rates. Therefore, not only is shear essential to the promotion of λ-phage DNA association (Fig. 2), but so is the presence of cohesive ends (Fig. 3). This control experiment also supports the fact that DNA assembly is not lateral but linear, because, in the absence of cohesive ends, no aggregation is observed. The use of shear-induced DNA assembly was extended to molecules with shorter cohesive ends. Using restriction endonucleases, which create staggered ends with a specific number of complementary nucleotides, we found that slightly longer shearing times (yet much shorter than for ligase alone; see below) were necessary to induce the assembly of λ-phage DNA with shorter cohesive ends (data not shown here).

Effect of divalent cations

Divalent cations serve as "gatekeepers" of shear-induced assembly of DNA (Hagerman, 1988; Kuhn et al., 1999). In the absence of divalent ions such as Mg^{2+} and Ca^{2+}, no association occurs, even at higher shear rates and large DNA concentrations (data not shown). When the Mg^{2+} concentration reaches 1 mM, shear-controlled assembly of DNA is triggered (Revet and Fourcade, 1998). However, 2 mM Mg^{2+} alone (and no shear) does not induce DNA

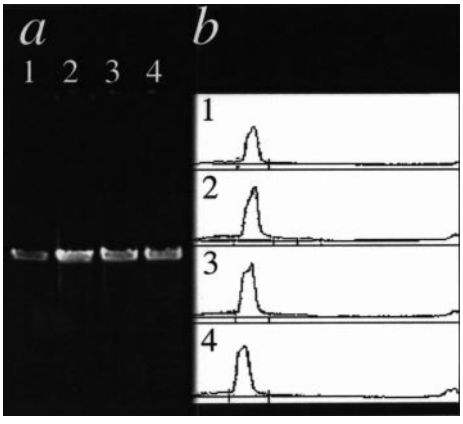

FIGURE 3 Resolution of blunt-ended λ-phage DNA that has been filled in by DNA polymerase. The PFGE conditions are identical to those in Fig. 2. *Lanes 1* and *4*: Unsheared samples before and after the experiment. *Lanes 2* and *3*: DNA sheared at 100 and $1000\ s^{-1}$, respectively.

assembly (Fig. 2). These results are qualitatively confirmed by atomic force microscopy (data not shown here) and fluorescence microscopy (Fig. 4). We derivatize the surface of a glass coverslip with a self-assembled monolayer of an amino silane group (aminopropyl triethoxysilane; Pierce) to promote the adhesion of DNA molecules (Hu et al., 1996). A droplet of a 0.01 mg/ml solution of DNA is deposited, and DNA molecules are elongated on the coverslip with the method of "molecular combing" (Michalet et al., 1997; Parra and Windle, 1993; Wang et al., 1998). This method consists of placing another silinazed coverslip on top of the DNA droplet; the weight of the coverslip, in addition to some compression and the receding meniscus during buffer evaporation, causes the sandwiched solution to spread and the DNA to align. After 3 min, the top coverslip is removed and the substrate is rinsed, dried, and fluorescently labeled for visualization. No multimers appear in the absence of Mg^{2+}, even when subject to high shear rates; instead, multimers are formed in the presence of Mg^{2+} (Fig. 4 *b*). Hence Mg^{2+} and shear are both necessary and sufficient to induce the formation of long multimers of DNA.

Combined effects of shear and ligase

Fragments of DNA are traditionally concatenated using ligase, an enzyme that has become widely used in the assembly of recombinant DNA. However, the efficiency, yield, and rate of reaction of the ligase-based assay, run in quiescent conditions, are typically poor (Dugaiczyk et al., 1974), particularly at room temperature. We now apply our new method of shear-induced DNA assembly to expedite the concatenation of DNA molecules by ligase at room temperature. Ligation of DNA molecules involves the formation of new bonds between the phosphate residues located at 5′ termini and adjacent 3′-hydroxyl moieties. Our work suggests that shearing DNA in the presence of ligase can enhance the assembly of DNA fragments. To test this hypothesis, we characterize the separate effects of ligase, ligase in the presence of shear, and shear alone on the assembly of DNA.

We find that, while ligase produces multimers of DNA as expected, shear dramatically enhances the relative populations of multimers and produces longer oligomers than ligase alone (Fig. 5). The content of stained material in the wells is greatly increased when shear and ligase are combined (see top of *lanes 2* and *3* in Fig. 5), which suggests that polymers of DNA longer than five "monomers" are also produced. The presence of very long oligomers was not resolved in the conditions used in our experiments. Shorter shear times and/or lower ligase concentration and/or different electrophoretic conditions may help address this possibility, which will be tested in future work. Ligase produces long oligomers only when it is incubated with DNA for much longer periods of time; here shear requires much less time to accomplish the same or a superior result (Fig. 5). Therefore, the combination of shear-induced assembly and ligase constitutes the most effective method for concatenating DNA molecules.

DISCUSSION

Possible mechanism of DNA assembly

What is the mechanism by which shear induces gene assembly? Shear-induced assembly of DNA molecules originates from the enhanced probability that two ends of different molecules will interact in the presence of shear. Shear allows the DNA molecules to overcome the unfavorable

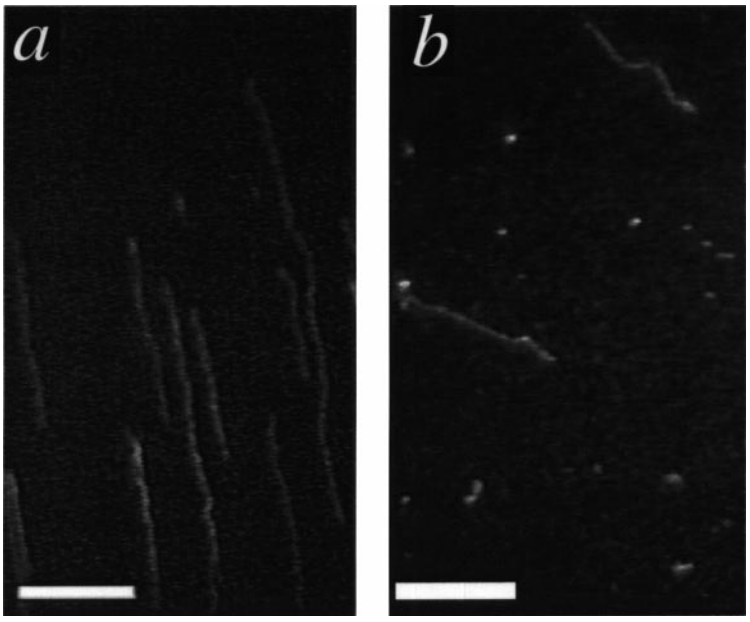

FIGURE 4 Fluorescent micrographs of a 0.01 mg/ml solution of λ-phage DNA in TE buffer deposited on a TESPA (3-aminopropyltriethoxysilane; Pierce)-coated glass microslide; molecular combing is used to prepare DNA for light microscopy. The molecules are stained with an oxazole yellow homodimer, YOYO-1. (*a*) The DNA solution has a low ionic strength and does not contain any divalent cations; the majority of the polymers appear as separate monomers. (*b*) The DNA solution contains 2 mM $MgCl_2$; numerous dimers and multimers appear in the micrograph. Bars = 10 μm.

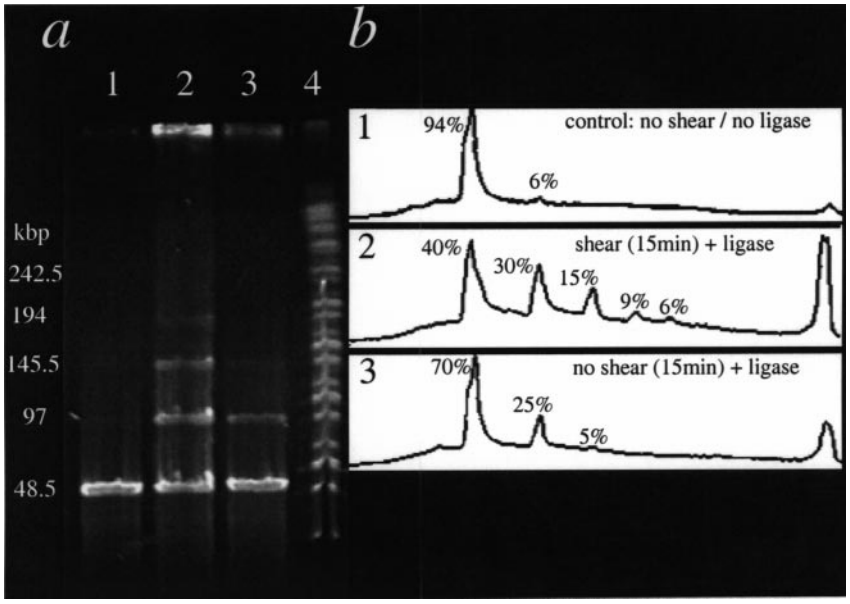

FIGURE 5 Gel electrophoretic separation of the products of ligation of λ-phage DNA. *Lane 1*: Control sample. *Lane 2*: DNA molecules that have been sheared for 15 min at a rate of 10 s^{-1} in the presence of ligase. *Lane 3*: Unsheared DNA that has been incubated with ligase for 15 min. The same concentrations of DNA and ligase were used for lanes 2 and 3. A midrange marker (New England Biolabs) is loaded onto *lane 4*. DNA is resolved in 1% agarose gel at 15°C that is subjected to a voltage gradient of 6 V/cm and a switch time ramped from 1 to 25 s. (*a*) Electrophoretic gel. (*b*) Densitometric analysis of the gel.

entropy loss associated with the oligomerization process and favors two-body interactions between the ends of different DNAs as opposed to the ends of the same DNA molecule. By analogy with classical models of polymers under shear (de Gennes, 1991; Doi and Edwards, 1989), we propose that enhanced intermolecular interactions are due to an increased probability of collisions among DNA molecules and by the enhanced deformation of the DNA molecules in flow (Leduc et al., 1999; Smith et al., 1999; Simonson and Kubista, 1993; Odegaard-Jensen et al., 1996; Doyle et al., 1997). Because the relaxation time of a single λ-phage DNA molecule is equal to $\tau \approx 0.42$ s (Sobel and Harpst, 1991), the Weissenberg number $\dot{\gamma}\tau$, which compares the flow time scale, $1/\dot{\gamma}$, with the macromolecular time scale, τ (Batchelor, 1967), is higher than unity as soon as the shear rate reaches $\sim 2.5\ s^{-1}$. When $\dot{\gamma}\tau > 1$, classical polymer physics models (de Gennes, 1991; Doi and Edwards, 1989) predict that a shear flow deforms and stretches DNA molecules. This leads to a statistical bias against intramolecular pairing and in favor of intermolecular interactions by exposing the ends outside the otherwise undeformed molecule (Cates and Witten, 1986).

A dilute DNA solution can also be effectively modeled as a suspension of Brownian colloidal particles. Here, $\dot{\gamma}\tau > 1$ signifies that Brownian diffusion is overcome by the rate of collisions between individual molecules, which is enhanced by the flow. The fluid "layers" between the fixed upper cone and the moving lower plate of the rheometer move at different speeds (Fig. 1 *b*). Hence DNA molecules that belong to neighboring fluid layers may collide, which increases the probability of contact between DNA molecules ends (Fig. 6). Therefore, we propose that shear enhances DNA association both by deforming the molecules and by increasing their frequency of contacts.

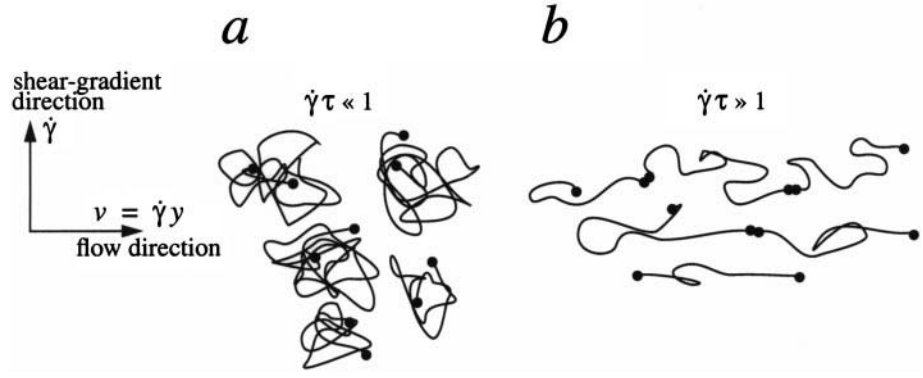

FIGURE 6 Potential mechanism of shear-induced λ-phage DNA assembly. Shear promotes unwinding of the molecules and enhances the probability of contact of cohesive ends, which are represented by small black points. (*a*) Conformation of DNA molecules at shear rates, $\dot{\gamma}$, that are small compared to the inverse of the relaxation time of the molecule, $1/\tau$. (*b*) DNA conformation at high shear rates.

Based on this physical insight, we have used our new approach to oligomerize DNA fragments that are smaller than λ-phage DNA. For shorter DNA molecules, the relaxation time τ is decreased, and therefore, at a given shear rate, the Weissenberg number is decreased. Hence we find that to pass the threshold $\dot{\gamma}\tau \approx 1$ and induce assembly, higher shear rates are required, as expected for shorter DNA molecules. In dilute conditions, the relaxation time of pBR322 DNA is ~45 times smaller than that of λ-phage DNA. Accordingly, the onset of assembly of the (short) pBR322 DNA fragment occurs at a shear rate that is ~45 times larger than for the (long) λ-phage DNA molecule, which was verified experimentally (data not shown). On the other hand, our model and experimental results predict that larger DNA fragments, including genomic DNA, should easily be polymerized under shear, a possibility that we shall test in the near future. Note, however, that according to our data (Figs. 4 and 5), shear-mediated exposure of the ends of DNA molecules is not sufficient to promote DNA assembly, which suggests that a purely entropic argument cannot be used and enthalpic interactions between of the molecules remain essential.

Antagonistic effects of ions

The presence of ions in solutions may a priori have two opposite effects on the extent of DNA assembly under shear. On one hand, for divalent-ion concentration between 0 and 2 mM (Mg^{2+} or Ca^{2+}), the persistence length of a DNA molecule, which is proportional to the bending constant of DNA, decreases from ~350 nm to ~50 nm (Hagerman, 1988). At equilibrium, decreased polymer flexibility increases the polymer's rotationally averaged radius of gyration and the instantaneous aspect ratio. Shear can couple to a rigid molecule more effectively and, therefore, further enhances polymer fluctuations (Leduc et al., 1999; Mason et al., 1998). Hence increased ionic strength may decrease the propensity of a polymer to unwind and to expose its ends to allow DNA polymerization under shear. On the other hand, increased ionic strength may promote the interaction of cohesive ends by screening the electrostatic repulsion and/or by bridging two phosphate groups of the DNA molecule (Baumann et al., 1997). Because high concentrations of Mg^{2+} or Ca^{2+} trigger the formation DNA multimers (see Fig. 4), potential ion-induced reduction of polymer interactions via increased DNA flexibility is clearly overcome by enhanced interactions between DNA cohesive ends.

Implications

Much of biological and medical scientific effort is and will be based on improved cloning devices. Here we have presented direct experimental evidence that, contrary to conventional wisdom, a uniform shear flow can promote and control the formation of multimers from quiescent DNA monomers. We observe DNA assembly at low shear rates and disassembly a higher shear rates. The findings reported in this paper could have great repercussions for molecular biology and biotechnology. Our novel, fast, and efficient method for DNA assembly could directly improve conventional techniques of chromosome mapping and synthesis of novel genes (Petka et al., 1998). Our shear-based method for promoting DNA-DNA interactions could also be used to enhance the rate of association between DNA (or RNA) molecules obtained from cell extracts and the (short) primers immobilized on substrates such as gene chips in genomic applications. In current protocols, the reaction between complementary DNA molecules and primers is extremely slow because it is mostly diffusion-limited. Our results could form the basis for a new method by which DNA fragments are continuously subjected to a shear flow to expose their ends, which would greatly enhance their probability of contact and interaction with the primers immobilized on the gene chip (Christens, private communication).

The findings reported here, as well as the tunability of DNA properties (Pecora, 1991; Wirtz, 1995), may offer a new framework in which to understand the physics of end-associating polymers under shear, including telechelic polymers, which are commonly used as thickeners and in "smart" materials (Broze et al., 1981; Witten, 1988). According to our results, shear would induce and control the transient assembly of telechelic polymers, which indeed display shear-thickening at low shear rates and subsequent shear-thinning at high shear rates (Broze et al., 1981; Witten, 1988). Low temperatures are used here to "freeze" the DNA macrostructures (i.e., the newly formed multimers) generated by the shear flow from quiescent DNA monomers; high temperatures render DNA concatenation-reversible and short-lived. λ-phage DNA has the advantage over classical telechelic polymers of being readily amenable to light-microscopy observations and not requiring an extremely long time to equilibrate.

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