Manipulation of a Large DNA Molecule using the Phase Transition

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Abstract. A conventional method of DNA sequencing can determine up to 1000 base pairs at one time. Therefore, long DNA should be cut into many short fragments that are suitable for DNA sequencing. Those fragments, however, lose their order information. If the fragments are prepared from the terminus of the long DNA, the reorganization process can be omitted. This process consists of following unit operations; manipulation of genomic DNA, fixation with a stretched form, cutting from the terminus, recovery and amplification. In these unit operations, manipulation and cutting of DNA are focused in this report. Globular transformation suppresses break down of long genome DNA and permits manipulation of large DNA. Because globular transition is reversible, the coiled DNA can be sequentially spun from the globular DNA like a spindle. The spun DNA was successfully fixed on a glass surface in an arbitrary pattern. To prepare fragments from the stretched DNA molecule, a method to cut DNA molecule was developed. Since most restriction enzyme requires magnesium ion for their activation, the restriction enzyme was successfully activated only when magnesium ion was electrochemically supplied.

Key words: DNA, globular transformation, laser trap, local reaction, manipulation, single molecule, stretching

1. Introduction

Single molecule techniques provide a new analytical method completely different from conventional analytical methods in biology and biotechnology. The analysis based on fluorescence observation supports real time observation of biological molecules. For instance, a motion of an actin filament over heavy meromyosin was analyzed by observation of a single fluorophore bound on the actin [1]. Motion of T2 phage DNA molecule driven by pulsed field in agarose gel was visualized, and this visualization contributed investigation of mechanism of DNA separation by gel electrophoresis [2]. RNA polymerase-DNA complexes have been observed, and transcription force that a single RNA polymerase pulling DNA molecule [3] or sliding on DNA molecules array [4] were studied. Especially for analysis of DNA binding proteins, preparation and handling of DNA molecules are important. A manipulation technique for single DNA molecule contributes not only analysis of biological molecules, but also new aspects of genome analysis, such as mapping

Figure 1. Illustration of genome sequence determination method based on successive cutting and recovery of DNA fragments.

and sequencing. A typical DNA sequencing method, Sanger method, can analyze only up to 1000 base pairs from one sample at once. Then a long DNA, such as genome DNA, should be cut into many analyzable short fragments by several methods. However, these fragments lost information of their order. Therefore, reorganization process is required to determine the whole sequence after each fragment is analyzed. This process requires time and effort. If the fragments of single DNA can be prepared without loss of the order information, sequencing speed of genetic DNA will be enhanced. Figure 1 shows a concept of a novel method for preparing fragments without loss of the order information. The method consists of the following unit operations: (1) fixation of a single DNA molecule with a stretched shape, (2) cutting the DNA molecule from one terminus to obtain its fragments which are short enough to be analyzed by a conventional sequencing method, (3) successive recovery of the fragments, (4) amplification of the recovered fragment by PCR (polymerase chain reaction) method. In this report, we summarize these fundamental unit operations (1)–(3).

2. Concept

2.1. SUPPRESSION OF BREAKDOWN OF A GIANT DNA

Preparation and manipulation of giant DNA molecules have been recognized as an important technique for biochemistry and molecular biology [2–8]. Those are also important steps for genome analysis based on single molecule DNA, however, preparation of large chromosomal DNA molecules (more than several hundreds kilo basepairs) in solution is difficult because large DNA molecules are easily broken down by mechanical forces such as shear stress in flow. To suppress fragmentation of DNA due to shear stress, we have introduced globular transition. Because globular transition induces high condensation on DNA, the transition permitted manipulation of giant DNA, such as yeast chromosomal DNA. Many studies have been made on globular transition using condensing agents [9–12]. For example, PEG (polyethylene glycol) in combination with low molecular weight inorganic salt induces the DNA transformation from the coiled to the globular structure. The transformation is denoted as polymer-and-salt-induced- Ψ condensation. The globular DNA string is arranged as a hexagonal, closed packed structure [13–14]. Ψ condensation can be induced by several kinds of polymers and salts. It has been reported that the conformation of DNA can be changed from the coiled to the globular state with increasing concentrations of PEG or salt, for instance MgCl_2 or NaCl , and the DNA condensation constitutes the manner with right handed. The condensation is reversible. Removal of PEG and/or salt in solution gives coiled DNA as a result of transformation from the globular to coiled state [15].

2.2. DIRECT LASER TRAPPING OF SINGLE DNA [16]

The transformation into globular state has an advantage not only toughness against shear stress but also manipulation with laser trapping. Laser trapping has been applied for biological research [17–19], because the laser beam can be focused to about the size of its wavelength, and suspended particles can be trapped without physical contact [20–22]. The trapping force is originated from the difference in refractive index of the particles and that of the surrounding media. It has been difficult to trap biological macromolecules such as DNA molecules, because these molecules give low difference in refractive index and the trapping force cannot overcome the Brownian motion. This globular transformation results in a higher refractive index of DNA because the globular state is highly condensed and increases the density. We have demonstrated that a single DNA molecule can be trapped optically using a Nd:YAG laser (1064 nm in wavelength) upon transformation from the coiled state to the globular state.

2.3. STRETCHING OF GIANT DNA MOLECULE

Several stretching methods have been proposed for application of optical mapping. Bensimon et al. developed a process called 'molecular combing.' They anchored one terminus of DNA on surface, and extended DNA molecules by a moving interface which was generated from evaporation of DNA solution [23–24]. Yokota et al. also developed a method for straightening DNA molecules for optical restriction mapping. By their method, DNA molecules in solution at the intersection of a straight-edged coverslip and a slide were also stretched by a moving interface, however, the interface was driven by a moving coverslip [25]. Even those developments, straightening of large DNA molecules is not so easy because large DNA such as chromosomal DNA is extremely fragile and easily broken down by shear stress of accompanying flow in the solution.

As described previously, the globular transition permits manipulation of long DNA molecules, therefore we tried to apply the globular transition to stretching of DNA. First of all, we induced the globular transition to a single yeast chromosomal DNA molecule, and attracted to the tip of needle electrode of tungsten by dielectrophoresis, a kind of electrostatic force. The attracted globular DNA was adhered at the tip. When the globular DNA was reverted to the coiled state by reducing concentrations of the condensing reagents, the coiled DNA was sequentially spun from the globular DNA like a spindle. By manipulating the tip of the needle electrode, chromosomal DNA was spun and fixed on a glass surface successfully in arbitrary pattern.

2.4. CUTTING OF DNA BY LOCALLY ACTIVATED RESTRICTION ENZYME

Development of cutting method is also important to achieve a sequential fragment preparation of the new analysis method of genome. Several methods to cut DNA molecules have been proposed already. Focusing of ultra-violet (UV) laser could cut DNA molecules [8, 26–27], however irradiation of UV causes serious damage on DNA molecules, such as formation of pyrimidine dimer. Cutting methods based on local activation of restriction enzyme are more suitable for this purpose, because restriction enzyme produces no damages and keeps biologically active terminus at the cutting site. Washizu et al. cut DNA molecules to touch a latex bead coated by restriction enzyme [28]. The latex bead can be easily manipulated by a laser trapping technique. We proposed different methods to localize restriction enzyme activity. Suitable temperature and divalent cation, such as magnesium ion, are required to activate most restriction enzymes. We have already reported a cutting method based on control of local temperature [29]. Although this method can be easily applied to ordinary restriction enzymes, it is difficult to activate restriction enzyme only in narrow area. Hoyer et al., reported a cutting method based on control of local magnesium ion [30]. Irradiation of ultraviolet ray (UV) induced UV photolysis of a caged compound of magnesium ion. The locally elevated magnesium ion activated a restriction enzyme *Apa* I and cut DNA molecules. Because

irradiation of UV sometimes injures DNA molecules, a cutting method free from UV irradiation has been required.

We have developed a new method based on control of local concentration of magnesium ion. Because DNA molecules were suspended in a buffer containing a chelater, ethylenediaminetetraacetic acid (EDTA), the concentration of magnesium ion in the suspension was extremely low, and an activity of restriction enzyme was completely suppressed. The restriction enzyme can be locally activated by raising concentration of magnesium ion locally. We have developed a electrochemical method to control local concentration of magnesium ion. We fabricated a needle electrode made of magnesium metal. The release of magnesium ion from the needle was controlled by applying dc voltage to the needle electrode. The balance between release and diffusion of magnesium ion determined the distribution of magnesium ion. We demonstrate in this report that local concentration of magnesium ion can be controlled electrochemically and also single DNA molecule can be cut by a locally activated restriction enzyme.

3. Experimental Methods

3.1. SUPPRESSION OF FRAGMENTATION BY GLOBULAR TRANSITION [31]

Toughness of globular DNA against shear stress was examined with yeast chromosomal DNA as prepared by the following procedure. Yeast (*Saccharomyces cerevisiae*) cells (S288C strain) were cultured in YPD solution (5 g/l yeast extract (nacalai tesque), 10 g/l polypeptone (Wako), 2% glucose (nacalai tesque)), at 30 °C for 3 days. The cultured yeast cells were centrifuged at 3000 rpm for 5 minutes and the precipitates were washed once by 50 mM EDTA solution and twice by CES solution (0.5 M sorbitol (nacalai tesque), 0.1 M sodium citrate (nacalai tesque), 50 mM EDTA). The precipitates were mixed with a melted low melting point agarose (Gibco BRL), and the mixtures were solidified in 50 μ l of agarose plug. The plug was treated with 0.14% Zymolyase (Seikagaku) at 37 °C for 3 hours. The following treatment was deproteinization with 0.5 mg/ml ProteinaseK (Promega) at 50 °C for overnight. For transition to globular structure, the gel plugs containing the chromosomal DNA were soaked in a solution (60 mg/ml PEG, 0.6 M NaCl) for overnight. The extracted DNA was stained with a fluorescence dye YOYO-1 iodide (Molecular Probes) for visualization.

First of all, toughness of globular DNA was examined by a method based on observation of shape of DNA before and after subjection of shear stress. A gel plug containing the globular DNA on a cover slip was heated to about 70 °C (melting point of the agarose gel). The melted gel plug was observed by an inverted fluorescence microscope. Effect of shear stress on globular chromosomal DNA was further investigated by using a pulsed field gel electrophoresis. The gel plug containing globular DNA or coiled DNA (non-globular DNA) were prepared. Both samples were melted in a micro tube, and treated with a vortex mixer (Vortex Genie 2, Scientific Industries) for 10 sec, with different strength, weck stress conespon-

Figure 2. Optical system for laser manipulation of globular DNA.

ded to 3 on a vortex mixer dial and strong stress was 6, by a vortex mixer. These chromosomal DNAs were analyzed by pulsed field gel electrophoresis system.

3.2. LASER TRAPPING OF GLOBULAR DNA

Figure 2 depicts the experimental apparatus for DNA manipulation. Fluorescent stained DNA molecules were observed using a Carl Zeiss inverted microscope, Axiovert 135 TV, equipped with a 100 $\times$, 1.3NA oil-immersion objective lens and with a high sensitivity Hamamatsu SIT camera. Images were recorded on video tape. The 1064 nm Nd:YAG laser beam, expanded to a diameter of 7.5 mm, was introduced into the microscope through a dichroic mirror. The beam was focused in the object plane of the fluorescence image by the objective lens to trap DNA molecules optically.

Figure 3. Illustration of the method to stretch chromosomal DNA.

Globular DNA induced by PEG-MgCl₂ was optically trapped using 180 mW of 1064 nm light from a Nd:YAG laser. The infrared laser induces less damage to biological molecules such as DNA compared with visible light from krypton ion or argon ion lasers [26]. Before trapping of globular DNA, transition was confirmed by the fluorescence microscope. At the condition of [PEG] = 60 mg/ml and [MgCl₂] = 50 mM, all of the DNA molecules existed in the globular state; no coiled DNA was observed.

3.3. STRETCHING OF GIANT DNA MOLECULE

Figure 3 illustrates the procedure of stretching of DNA molecules schematically. As described previously, yeast chromosomal DNA was prepared in agarose gel, and transformed to globular structure by soaking in PEG/NaCl solution (Figure 3A). The globular DNA molecules were extracted from the gel by diluting agarose gel (Figure 3B). One of the globular DNA molecules was anchored at the tip of a needle electrode (Figure 3C), and transformed to coiled state (Figure 3D). The

coiled DNA molecule was spun from the chromosomal DNA in globular state and straightened on the glass surface (Figure 3E).

To stretch the DNA molecule according to Figure 3, condensing reagent was diluted with distilled water. During dilution, DNA molecule should be anchored to avoid drift of DNA molecules due to flow of solution accompanied with buffer exchange. To anchor, the globular DNA molecules were manipulated by gradient force, because these cannot be driven by a Coulomb force. Application of $10V_{\text{eff}}$, 1 MHz of AC voltage attracted and anchored the globular DNA molecules to a tip of a sharp needle electrode.

After anchoring the DNA, the condensing reagent was diluted with distilled water, and then the globular DNA unfolded to coiled state. Because the phase transition to coiled state progressed from the terminal of the globular, the coiled DNA was sequentially spun from the globular DNA like a spindle. Accompanied with sequential extraction of DNA, the extracted DNA can be fixed on a surface of glass coverslip through crosslinked magnesium ion.

3.4. CUTTING OF DNA MOLECULES

To demonstrate control of local concentration of magnesium ion, distribution of magnesium ion was observed in the buffer containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and $0.1 \mu\text{M}$ magnesium green that specifically bound to magnesium ion. During application of dc voltage to the needle electrode, the fluorescence due to magnesium ion was observed by the microscope. Magnesium ion to activate the restriction enzyme *EcoRI* was supplied by applying dc voltage on the needle electrode of magnesium metal. The needle electrode was fabricated by electropolishing. Dc voltage (+10V) was applied between a magnesium wire (diameter: 1 mm) and stainless steel electrode in 0.5 M EDTA solution (pH 8.0) for 60 seconds. During the dc voltage application, the magnesium wire was vibrated up and down with about 1 Hz to sharpen its tip. The sharpen needle was covered with nail manicure except its tip. The shape of the tip was confirmed by a microscope observation. The needle electrode was mounted on the stage of the microscope.

4. Results and Discussions

4.1. SUPPRESSION OF FRAGMENTATION BY GLOBULAR TRANSITION [31]

Figure 4 (a) and (b) show microscope images of coiled and globular chromosomal DNA molecules respectively. As shown in Figure 4 (b), DNA molecules were condensed by transition to globular structure. Those molecules were treated with shear stress generated by melting of agarose gel. DNA images after the treatment are shown in Figure 4 (c) and (d). In the melted gel plug, the coiled DNA molecules were significantly stretched by the shear stress (in Figure 4 (b)). On the other hand, globular DNA molecules kept the same structure (in Figure 4 (d)). This result suggests that globular DNA is tough against a shearing stress, and fragmentations

Figure 4. Photographs of coiled and globular DNA before and after exposure to shear stress.

are suppressed. This suggestion was confirmed by an analysis of pulsed field gel electrophoresis. Figure 5 shows analysis of pulsed field gel electrophoresis both for a coiled (lane 1–3) and globular yeast chromosomal DNA molecules (lane 4–6) treated with the Vortex Genie 2 (vortex mixer). Lane 1 and 6 denote chromosomal DNA without any treatments. Lane 2 and 5 denote the DNA molecules treated for 10 sec with the Vortex Genie 2 of dial 3. Lane 3 and 6 also denote treatment of 10 sec with the Vortex Genie 2 of dial 6, which caused much higher shear stress than dial 3. Shorter DNA fragments observed in lane 3 and 4 demonstrated that the coiled DNAs were easily fragmented by shearing stress. Although coiled DNAs were fragmented (lane 3 and 4), transition to globular structure suppressed the fragmentation due to shear stress (lane 4 and 5).

These results demonstrate that globular transition suppresses fragmentation of giant DNA. This means globular DNA can be manipulated without fragmentation caused by shear stress. We tried to apply the transition for a recovery of a single DNA molecule into the glass capillary. Figure 6 shows sequential photographs that a single globular DNA molecule was recovered into the capillary in solution. The recovered single DNA molecule firmly kept its structure in fluid during the recovering process.

Figure 5. Pulsed field gel electrophoresis to analyze effects of shear stress.

Figure 6. Manipulation of a globular DNA molecule using a capillary.

Figure 7. Sequential photographs of trapped and free T4 phage DNA molecules in the globular state at $t = 0$ (a), 0.24 sec (b), 0.64 sec (c), 0.87 sec (d) and 1.14 sec (e): scale bar, $5 \mu\text{m}$. The position of the optical trap is indicated by white arrows.

Figure 8. Trapping force for globular T4 phage DNA with respect to that of $0.5 \mu\text{m}$ latex bead. Trapping force of the bead was around 1 pN when laser power was 200 mW.

4.2. LASER TRAPPING OF GLOBULAR DNA

Figure 7 shows laser trapping of a globular T4 phage DNA molecule. The free DNA molecules were translated by moving the microscope stage. The globular DNA molecule indicated with white arrows was trapped at the laser focal point while a free DNA molecule moved leftward (Figure 7 a–c) or upward (Figure 7

Figure 9. Stretching a yeast chromosomal DNA molecule.

c–e). At this laser power, it was possible to trap the globular DNA, however, the coiled DNA could not be trapped. When a coiled DNA molecule arrived at the laser focal point by moving the stage, it was pushed downward by the optical pressure of the laser beam and escaped from the trap (data not shown). Figure 8 shows the trapping force of a globular T4 phage DNA condensed by several reagents. In this figure, the trapping force of the globular DNA was represented with respect to that of a $0.5\ \mu\text{m}$ bead to compensate error in focusing of the laser. These results demonstrated that the trapping force increased with laser power, and all the condensing reagents that we examined permitted laser trapping. These results strongly suggest that the DNA transformation into the globular structure, induces a higher refractive index and lower viscosity drag force due to its decreased size. Laser trapping of a single supercoiled λ -DNA using krypton ion laser operating at 647 nm was also reported by Chiu et al [32]. In their experiment, λ -DNA molecules were stained with YOYO-1 dye. It can be observed that YOYO-1 dye induces DNA condensation within about 30 minutes in the absence of any condensing agents. This suggests that the mechanism of the trapping observed by Chiu et al. might be attributed to the YOYO-1 induced condensation, but the condensed structure is still unknown. On the other hand, the globular transformation induced by condensing agents has been extensively studied by means of spectroscopy and microscopy so that the morphology and experimental conditions of the globular DNA have been already opened up [9–11]. On the other hand, it might be difficult to transform DNA condensed by YOYO-1 reversibly, because YOYO-1 dye binds to DNA molecule with high affinity.

4.3. SEQUENTIAL STRETCHING OF GIANT DNA

Figure 9 shows the stretching of a yeast chromosomal DNA molecule. By manipulating the tip of the needle electrode, chromosomal DNA was spun and fixed on a glass surface successfully in arbitrary pattern.

Figure 10. (A) Experimental setup for the visualization. (B) Magnesium ion distribution visualized by a fluorescence, magnesium green.

It demonstrates that our method has an advantage to the previous methods, because DNA molecules can be stretched to form a pattern such as squares, which permit to observe much longer DNA molecules in a limited microscope field. From the fluorescence image, no kink was observed during the stretching. This result suggests that globular DNA is packed orderly without an entanglement, even DNA is very long such as chromosomal DNA. After stretching the chromosomal DNA, we found that only a very small fluorescence attached at the tip of the electrode. It indicates almost the entire chromosomal DNA in the globular form was spun and fixed on the glass surface.

4.4. CUTTING OF DNA MOLECULES

Figure 10 shows the image of magnesium ion distribution visualized by the fluorescence of magnesium green. When +5V dc was applied to the needle electrode of magnesium metal, magnesium ion was electrochemically released from the tip of the needle electrode. Although Figure 10 has demonstrated that magnesium

Figure 11. Sequential photographs of DNA cutting. Time elapsed is shown in lower-right corner of each photograph.

ion can be supplied electrochemically, it was not demonstrated yet whether this reaction also released inhibitors of restriction enzyme. We confirmed that released magnesium ion could activate restriction enzyme (data not shown).

Figure 11 shows sequential photographs of DNA digestion by local activated restriction enzyme *EcoRI*. During the application of $-2V$ dc voltage, no cutting reaction was observed (data not shown). However, after the application of $+2V$ dc voltage, the cutting reaction was initiated. The cutting of DNA molecules was observed only the restricted area close to the tip, and no cutting was observed in the far area from the tip. The restriction enzyme was activated according to the diffusion of magnesium ion from the tip of the needle electrode. The position of cutting sites almost agreed to a restriction map of *EcoRI*, which was obtained by DNA sequence of λ -DNA (data not shown). These results indicate that the local restriction enzyme activity can be controlled by applying the dc voltage to the needle electrode of magnesium metal, and this method can be used for cutting individual DNA molecules.

5. Conclusions

Micro-manipulation techniques for a single DNA molecules will possibly provide novel analytical methods in biotechnology. Laser trapping and electrostatic force are useful tools for manipulation of single DNA molecules. Following techniques have been developed: (1) manipulation of large chromosomal DNA using the globular transformation, (2) stretching of single DNA molecule based on reversed reaction of globular transition, (3) cutting of stretched single DNA by activation of enzyme reaction locally.

Acknowledgement

We wish to thank Prof K. Yoshikawa of Kyoto University and Dr M. Ishikawa of JRCAT for valuable suggestions and discussions.

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