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Visualization of a Specific Sequence on a Single Large DNA Molecule Using Fluorescence Microscopy Based on a New DNA-Stretching Method

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A method for analyzing large DNA which makes it possible to obtain spatial information on the positions of specific sequences along a DNA molecule has been developed. Making use of the fact that large DNA molecules are stably elongated under an alternating-current field in a concentrated linear polymer solution, the direct observation of elongated individual λ DNA molecules with fluorescence probes was carried out using fluorescence microscopy. The spatial positions of the fluorescent spots of the probe (fluorescence-labeled restriction endonuclease *Eco*RI) on DNA molecules were determined by image analysis. As expected, fluorescent spots of *Eco*RI were observed at certain positions on λ DNA, where sequences to which *Eco*RI binds are located. Finally, the potential application of single large DNA molecule analysis using this DNA-stretching method is discussed.

The combination of gel electrophoresis and hybridization is widely used to determine the positions of specific sequences on large DNA molecules (1). Fluorescence in situ hybridization (2) (FISH) is another powerful technique for analyzing chromosomal DNA. On the whole, however, current widely used techniques for analyzing large DNA molecules show poor spatial resolution. Therefore, a more useful method is needed. Schwartz *et al.* demonstrated the imaging of restriction endonuclease digestion of elongated individual DNA molecules by fluorescence microscopy after fixation in agarose gel (3). Cai *et al.* improved this technique and showed the rapid construction of excellent ordered restriction endonuclease maps of yeast artificial chromosomes (YAC) (4). Michalet *et al.* carried out the high resolution mapping of cosmid contigs on a YAC and precise measurements of gaps between cosmids by dynamic molecular combing (5). Allison *et al.* demonstrated higher resolution mapping of *Eco*RI sites of λ DNA and 35 kb cosmid using atomic force microscopy (AFM) (6). These reports indicate that the analyses of individual DNA molecules using microscopy enable us to obtain detailed spatial information in DNA analysis. However, since large DNA molecules assume a random coiled conformation in an aqueous solution, if a large DNA molecule is to be analyzed, it becomes difficult to accurately measure the position of the probe along the DNA in its natural state. Thus, when the analysis of single large DNA is to be carried out, the DNA should be taken straightened conformation by some method such as dynamic molecular combing. Although this single DNA molecule analysis using microscopy seems to have great potential, there have been a few studies on DNA elongation with regard to large DNA molecule analysis.

Recently, we found that large DNA molecules are elongated in concentrated neutral linear polymer solutions under an alternating-current electric field (7, 8). This DNA elongation occurs due to the entanglement of DNA and the surrounding linear polymer chains. DNA that can be elongated varies greatly in size (from ca. 50 kb to ca. 1 Mb), and this DNA-stretching phenomenon is the basis for a new method for DNA analysis, i.e., if DNA is straighten under the microscope, it should be possible to obtain accurate information on the spatial positions of the probes on the DNA. Based on these results, we examined the effectiveness of single DNA molecule analysis using this new DNA stretching and straightening technique with fluorescent probes. In this study, fluorescence-labeled restriction endonuclease *Eco*RI was used as a fluorescent probe. *Eco*RI can recognize and bind to a specific sequence of DNA, GAATTC. Therefore, fluorescence-labeled *Eco*RI is appropriate for use as a fluorescent probe in a preliminary demonstration of single DNA molecule analysis.

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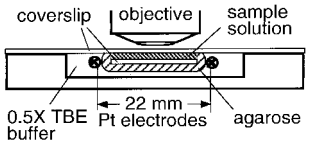

FIG. 1. Schematic cross-sectional view of the cell specially designed for direct observation of individual large DNA molecules.

MATERIALS AND METHODS

Restriction endonuclease *Eco*RI (New England Biolabs, Inc.) was labeled by rhodamin as follows: First, *Eco*RI was biotinylated by Sulfo-NHS-LC-Biotin (purchased from PIERCE) through its amino groups. Rhodamine-avidine complexes were then attached to *Eco*RI through the biotins (9). During this preparation, *Eco*RI was bound to heparin (HiTrap Heparin, Amersham Pharmacia) to protect the binding site of the enzyme. Finally, the fluorescence-labeled *Eco*RI was washed and eluted with a buffer solution containing 742 μM NaCl. The activity of the fluorescence-labeled *Eco*RI was checked by digestion of λ DNA.

Bacteriophage λ DNA (48.5 kb; contour length, 16.5 μm) was purchased from TaKaRa. Polyacrylamide (PA; 10 wt%, average molecular weight: 700,000–1,000,000) was purchased from Tokyo Chemical Industry Co., LTD. The polyacrylamide was mixed with TBE buffer and stirred for more than 24 h. The solution was then mixed with 2-mercaptoethanol (2-ME; as an antioxidant), catalase, glucose oxidase, glucose, dithiothreitol (DTT), YO-PRO-1 (intercalating fluorescent dye, Molecular Probes, Inc.), and λ DNA. The enzymes were added in order to reduce the concentration of dissolved oxygen (10), which causes photobleaching of the dye and scission of DNA. DNA/PA solution was then mixed with fluorescence-labeled *Eco*RI solution to form complexes of DNA and *Eco*RI. The solution contained EDTA, which removes free Mg^{2+} ions. In the absence of free Mg^{2+} ion, *Eco*RI can not digest DNA, and merely binds to the specific sequence of DNA (11, 12). The final concentrations of the materials in the sample solution were as follows: DNA, 0.1 μM (in nucleotide); YO-PRO-1, 0.8 μM; PA, 5.2 wt%; Tris, 38 mM; Borate, 36 mM; EDTA, 1 mM; NaCl, 148 mM; glycerol, 1% (v/v); 2-ME, 4% (v/v); catalase, 0.016 mg/ml; glucose oxidase, 0.08 mg/ml; glucose, 1.8 mg/ml; DTT, 1 mM; fluorescence-labeled *Eco*RI, 0.1 μg/ml. The sample solution was incubated for at least 20 minutes at 37°C just prior to the direct observation.

DNA molecules were observed using a specially designed electrophoresis cell (see Fig. 1) prepared as follows: DNA/*Eco*RI solution (30 μl) was placed on a 76 mm × 24 mm cover slip and covered by a 18 mm × 18 mm cover slip. This sample sandwich was covered with melted 1 wt% agarose solution containing 0.5 × TBE and cooled to form a gel. The sandwich was placed on the cell and connected to a pair of 24 mm-long Pt electrodes through 0.5 × TBE buffer solution. The AC electric field (53 V/cm in amplitude, 30 Hz) was generated by a combination of a function generator and an amplifier. Individual DNA molecules were observed by a microscope (OLYMPUS BX-50) equipped with a ×100 oil-immersed objective. To observe the green fluorescence of YO-PRO-1 and the red fluorescence of rhodamine at the same time, a dual-band pass filter (model U-DM-FI/TR, OLYMPUS) was used. The images were recorded on videotape using a high-sensitivity SIT camera (Hamamatsu Photonics) or a color chilled 3CCD camera (C5810-01, Hamamatsu Photonics), and analyzed with a personal computer using image-analysis software (NIH image).

RESULTS AND DISCUSSION

Figure 2 shows images of elongated λ DNA and the time course of the apparent contour length, $R_l(t)$, and the x component of the position of the center of mass, $g_x(t)$, of DNA in a concentrated linear PA solution under an AC field. The x-axis is in the direction of the field. As shown here, λ DNA is loosely elongated in the same direction as the field due to the support of surrounding polymer chains. The fluorescence intensity of the DNA is distributed uniformly, except at both ends. This indicates that the segments of elongated DNA are distributed almost uniformly. Notice that the fluctuation of $R_l(t)$ is small. The fluctuation of $g_x(t)$ is also small, since the entangling PA solution is highly viscous, and Brownian motion is reduced. These results indicate that the elongated DNA is stable enough for the color CCD camera to capture clear images.

Next, we elongated a complex of λ DNA and fluorescence-labeled *Eco*RI under the microscope. Figure 3 shows an example of a complex of λ DNA and fluorescence-labeled *Eco*RI in the PA solution. A red fluorescent spot of the probe can be seen on the DNA, which is visualized as a green fluorescent string. When there is no external field, the DNA molecule with a fluorescent probe assumes a random coiled conformation (see Fig. 3A). On the other hand, when the AC field is applied, the DNA assumes a stable loosely elongated and straight conformation (see Fig. 3B). Thus, it is possible to determine the spatial position of the fluorescent spot of the probe along the DNA molecule. In Fig. 3B, a schematic DNA molecule (green bar) and the positions of GAATTC sequences to which *Eco*RI will bind (red bands) are illustrated as a reference. The position of the red fluorescent spot corresponds to the rightmost of the five red bands, i.e., *Eco*RI is binding to the GAATTC sequence. In this experiment, however, five fluorescent spots of *Eco*RI were not observed at the same time on one λ DNA molecule. This is because we could not obtain a concentrated fluorescence-labeled *Eco*RI solution due to our limited ability to purify the obtained sample solution.

Next, using captured images similar to Fig. 3B, we determined the spatial positions of the fluorescent

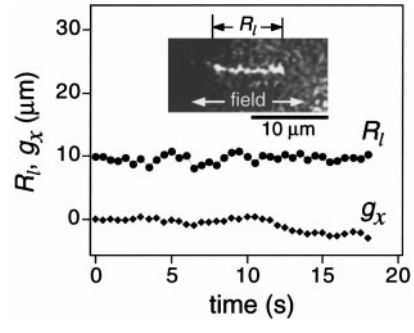

FIG. 2. Time course of $R_l(t)$ and $g_x(t)$ in a 5.2 wt% PA solution under an AC field (53 V/cm in amplitude, 30 Hz). Sampling interval is 0.5 s. Inset: Elongated and straightened λ DNA captured by the SIT camera. In this image, no *Eco*RI is binding to the DNA.

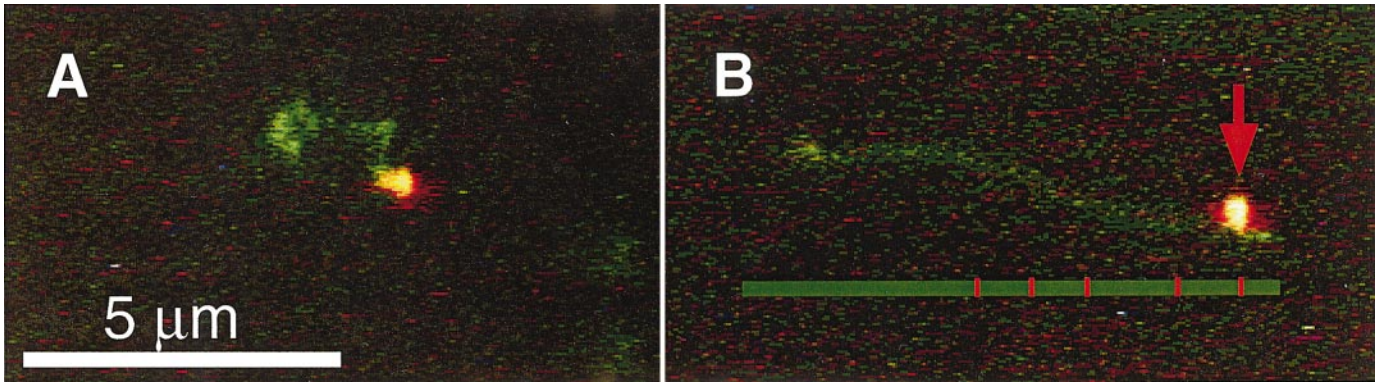

FIG. 3. A complex of λ DNA and fluorescence-labeled *Eco*RI in 5.2 wt% PA solution captured by the color CCD camera. (A) Under no external field. (B) Under an AC field (53 V/cm in amplitude, 30 Hz). Red bands indicate positions of the specific sequence (GAATTC) to which *Eco*RI binds. The arrow indicates the position where *Eco*RI is actually binding.

spots on the DNA. Figure 4 shows plots of the distance from the 5′ end to the fluorescent spot along λ DNA in base pairs. The distances were obtained as follows: First, the apparent contour length of λ DNA is measured. Next, the distance from one end of the DNA to the fluorescent spot is measured. The percentage of the interior covered by the fluorescent spot is then obtained. Since the DNA is elongated almost uniformly and the total number of base pairs of λ DNA is already known (48502 bp), the distance from one end of the DNA molecule to the fluorescent spot in bp is easily obtained. Finally, by comparing the results to a restriction map of the complete sequence of λ DNA (13), the distance from the 5′ end of the DNA molecule to the fluorescent spot is determined. As shown in Fig. 4, the positions of the fluorescent spots are close to known positions of the specific sequence of λ DNA to which *Eco*RI binds. Thus, the analysis of single DNA molecules using this new DNA-stretching technique makes it possible to obtain information on the spatial positions of specific sequences in a large DNA molecule.

Recently, techniques of individual DNA molecules mapping, using fluorescence microscopy or AFM, have been developed and reported (3, 4, 5, 6). These reports show that the analysis of single large DNA molecules, which makes it possible to determine the spatial positions of specific sequences is feasible. The method described here should contribute to this new approach in DNA analysis. One of the benefits of our technique is that individual DNA molecules over the size range of 50 kb–1 Mb can be elongated stably in the polymer solution without anchors. Therefore, after the analysis, DNA fragments that have a specific sequence of interest can be recovered for further analyses, for instance, using a silica capillary (14). This method could be useful especially for analyzing DNA that is precious and a very small amount. In addition, multiple fluorescent probes with different colors may make it possible to identify the positions of different specific sequences on a single large DNA molecule in one experiment. This technique may be a powerful tool for identifying the positions of specific sequences, such as the sequences of newly-found markers and mismatch positions. More practical analyses of individual large DNA molecules using DNA-stretching in concentrated polymer solutions under an AC field will be reported elsewhere.

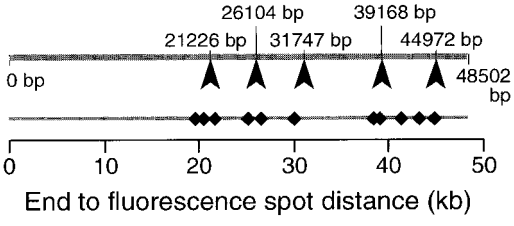

FIG. 4. Plots of the positions of fluorescent spots along λ DNA based on the distance from the 5′ end of DNA to the fluorescent spot. *Eco*RI restriction map of λ DNA is shown for reference. Arrows indicate the site of the specific sequence, GAATTC, of λ DNA.

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