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ARTICLE

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Direct measurement of DNA molecular length in solution using optical tweezers: detection of looping due to binding protein interactions

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Abstract DNA looping is caused by the interaction between DNA binding proteins located at separate positions on a DNA molecule and may play an important role in transcription regulation. We have developed a system to stretch single DNA molecules and to measure changes in molecular length. DNA molecules were prepared and 5′ end-labeled by PCR amplification. Two beads and the intervening DNA molecule were trapped and manipulated independently with dual trap optical tweezers. The trapped DNA molecule was then stretched and the extension (the distance between the two beads) was measured. The extension at the specific tension force of 30 pN was calculated and used as a molecular length. The molecular length was found to be proportional to the base pair number. The rise per residue was calculated to be 3.31±0.05 Å. The length measurement was applied to DNA fragments containing GC box sequences at two different locations separated by a distance of 2.428 kbp. The addition of GC box binding transcription factor Sp1 shortened the molecular length, suggesting DNA looping forms as a result of interaction between transcription factors.

Key words DNA looping · Optical tweezers · Transcription factor Sp1 · DNA binding protein · Rise per residue

Abbreviations *kbp* Kilo base pairs · *SV40* Simian virus 40 · *CMV* Cytomegalovirus · *PCR* Polymerase chain reaction · *TEM* Transmission electron microscopy

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1 Introduction

Protein-protein interactions on DNA, as well as protein to DNA binding, play important roles in regulating replication and transcription. These protein interactions cause structural changes in DNA molecules microscopically, as in bending near proteins, or macroscopically, as in looping. DNA looping is generated by the attachment of proteins that are bound on different sites of a DNA molecule (Schleif 1992). Cooperative interactions are important in regulating the transcription mechanism and are made easier when distant binding proteins are brought closer together by looping. Many experiments have been conducted to observe and detect such DNA looping. Biochemical approaches based on gel mobility shift assay (Krämer et al. 1988) and DNase I foot printing experiments (Hochschild and Ptashne 1986) gave indirect evidence. Observations by electron microscopy (Krämer et al. 1988; Mastrangelo et al. 1991) and atomic force microscopy (Lyubchenko et al. 1997) provided direct structural information, but looping remains ambiguous since even a random aggregation of proteins on incorrect sites could produce a looped image on a fixed sample. Hence, a new measuring technique to detect and characterize DNA looping is obviously needed. One possible method is to measure the length of a DNA-protein complex before looping, since the DNA will become shorter after looping.

To measure DNA length in solution, the DNA sould be pulled with a defined force. Recently many researchers have investigated the elastic properties of DNA molecules by stretching single DNA molecules using magnetic force and fluid flow (Smith et al. 1992), receding meniscus (Bensimon et al. 1994), optical tweezers and micropippet (Smith et al. 1996), glass needle (Cluzel et al. 1996), magnetic force (Strick et al. 1996), optical tweezers and anchored and stalled RNA polymerase complex (Wang et al. 1997).

The length of a DNA molecule of known base pairs can be calculated using a unit of base pair separation. It is widely accepted that base pair separation, or the rise per

residue, is 3.38 Å according to the X-ray analysis of aqueous B-form DNA crystal (Saenger 1988; Dickerson et al. 1982). Free DNA molecules in solution, however, are dynamic and change their structure constantly due to thermal perturbation. For example, they behave like coils and globules so that even the end-to-end distance of a molecule is not fixed. The rise per residue measured in a fixed crystal is therefore not analogous to the DNA structure in solution. Accordingly, it is necessary to define the rise per residue in solution as a standard length in our DNA length measurement method.

In this paper, we report the development of a new measurement method that stretches a single DNA molecule by using dual trap optical tweezers and attached beads. It is critical to determine the ends of molecules precisely for an accurate measurement of the absolute length of DNA because the edges of the attached beads are indistinct under the microscope. To avoid this, relative increases in DNA length for different molecules of known base pair numbers were determined. This technique is applied to measuring stretched DNA with GC box sequences to detect DNA looping caused by the addition of transcription factor Sp1.

2 Materials and methods

2.1 DNA preparation

DNA molecules were prepared by PCR amplification. Both ends of the DNA were labeled using 5′ end-labeled primers simultaneously. Each end was labeled differently to prevent the two ends of a single DNA from attaching to the same bead. The reaction was performed using DNA polymerase LA-Taq (TaKaRa, Japan) and λ-phage DNA as a template for DNA molecules to evaluate the rise per residue. One primer of each pair was 5′ end-labeled with biotin (TaKaRa, Japan) and the other was 5′ end-labeled with digoxigenin (TaKaRa, Japan). The ZAP Express vector (Stratagene, USA) was used as a template for DNA molecules with GC box sequences.

Agarose gel electrophoresis was used on DNA fragments to remove the unreacted primer oligonucleotides. A 1% agarose gel with Howry buffer (80 mM tris, 4 mM Na_2EDTA, 25 mM sodium acetate and 50 mM acetic acid) was used.

2.2 Protein-coated beads

Streptavidin (Boehringer Mannheim, Germany) was coupled to 2.73-μm diameter carboxylated polystyrene beads (Polyscience, USA) using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimid according to the manufacturer's protocol. Polyclonal antidigoxigenin (Boehringer Mannheim, Germany) was bound to 1.909-μm diameter carboxylated polystyrene beads by the same method.

2.3 Bead-DNA-bead assembly

DNA fragments were mixed with streptavidin-coated beads in a buffer solution of 10 mM tris-HCl, pH 7.5, containing 1 mM EDTA, and 150 mM NaCl and introduced into a micro chamber made of two glass cover slips and a spacer. The antidigoxigenin-coated bead suspension was added, and the micro chamber was filled with the buffer solution. The bead-DNA-bead assemblies were formed after an incubation of one hour at room temperature. The concentration of DNA and the two kinds of beads were kept at the same molecular ratio in order to efficiently obtain a single DNA molecule between the two beads.

2.4 Sp1 binding reaction to GC box sequences

Sp1 (Promega, USA) and the DNA with GC box sequences were mixed in a buffer solution of 12 mM HEPES-KOH, pH 7.5, containing 50 mM KCl, 5 μM $ZnSO_4$, and 0.5 mM dithiothreitol and incubated in ice for one hour. A molar ratio of seven Sp1 proteins per GC box was used to reduce binding at non-specific sites of DNA (Mastrangelo et al. 1991).

2.5 Gel mobility shift assay

The sequences of the probes used in the gel mobility shift assay were as follows:

(1) the GC box sequence in SV40 promoter (SV40 probe)

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5'-GATCCGGAACTGGGCGGAGTTAGG
       GCCTTGACCCGCCTCAATCCCTAG-5'
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(2) the GC box sequence in CMV promoter (CMV probe)

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5'-GATCCGTCAATGGGCGGGGGTCGTTGGGCGGTCAGCCA
       GCAGTTACCCGCCCCCAGCAACCCGCCAGTCGGTCTAG-5'
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Sp1 was mixed with double-stranded poly (dI-dC) (4 mg for SV40 and 40 ng for CMV probe) in 10 mM HEPES buffer, pH 7.9, containing 1 mM $MgCl_2$, and 1 mM dithiothreitol. After incubation on ice for 15 minutes the ^{32}P-end-labeled probe (2×10^4 cpm) was added to the mixture and incubated again at 24 °C for 25 minutes. Unlabeled DNA probes were used 300 times in the competition experiments. The reaction mixture was electrophoresed in a 4.5% native polyacrylamide gel to separate protein-bound probes from free probes.

2.6 Dual trap optical tweezers

Beads were observed under an inverted microscope (Nikon, TMD 300, Japan) equipped with a Plan Apochromat 100× oil-immersion objective (numerical aperture = 1.4), an oil immersion condenser lens for high-magnification objectives, and a 100-W halogen lamp. Images were detected with a Newvicon camera (Hamamatsu Photonics, C2400-07, Japan), enhanced with an image proces-

sor (Hamamatsu Photonics, DVS-3000, Japan) and recorded with a video cassette recorder (SONY, EVO-9650, Japan). A linear polarized TEMoo mode laser beam from a Nd-YAG laser (Spectron Laser Systems, SL902T, UK) emitting at 1064 nm was divided in two by a polarizing beam splitter cube and sent to a galvano mirror scanner system. They were then recombined at a second polarizing cube and went into the microscope through the epifluorescence port. The two trapped laser beams were independently manipulated over the field of microscopic view (about 30 μm×20 μm) using a galvano scanner controller (General Scanning Inc., CX-660, USA) by changing the incident angle of the laser beams into the back aperture of the objective. The laser power incident to the microscope was measured by a thermal detector (Newport, Model 835, USA). The optical tweezers' trapping force was calibrated by applying the Stokes drag law and by holding a single polystyrene bead steady against relative constant flow with a liner translator (Sigma Koki, HPA-30, Japan). The trap stiffness was 0.057 pN nm^{-1} (for 1.909-μm diameter bead at a laser power of 140 mW without the objective).

2.7 Fluorescence microscopy

DNA fluorescently labeled with YOYO-1 (Molecular Probes, USA) was excited by an argon ion laser (Coherent, INNOVA 300, USA) at 488 nm, and the resulting fluorescent images were detected with a silicon-intensified target video camera (Hamamatsu Photonics, C2400, Japan), processed with an image processor (Hamamatsu Photonics, ARGUS-10, Japan) and recorded with a video cassette recorder (Sony, CVD-1000, Japan). To reduce photo bleaching of dyes and the resulting DNA cleavage, oxygen was enzymatically scavenged with glucose oxidase (216 μg/ml), glucose (4.5 mg/ml), catalase (36 μg/ml), and 5 μM dithiothreitol (Kishino and Yanagida 1988).

2.8 Measurement of DNA length

DNA molecules were invisible but detectable because one bead followed when the other was trapped and moved. A DNA molecule was stretched when one bead was held stationary and the other was moved away. The first bead shifted slightly from its position in accordance with the tension force applied to the DNA molecule and was pulled continuously until the held bead or the moved bead tore away abruptly from the trap. Bead images during this stretching procedure were captured and analyzed on a Power Macintosh 8500 computer using public domain NIH image software (developed by the US National Institutes of Health and available on the Internet by anonymous FTP from Zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI). After setting the threshold of the captured bead image, the x- and y-coordinates of the bead's center were determined. The diameter of a bead was tentatively defined as the distance between

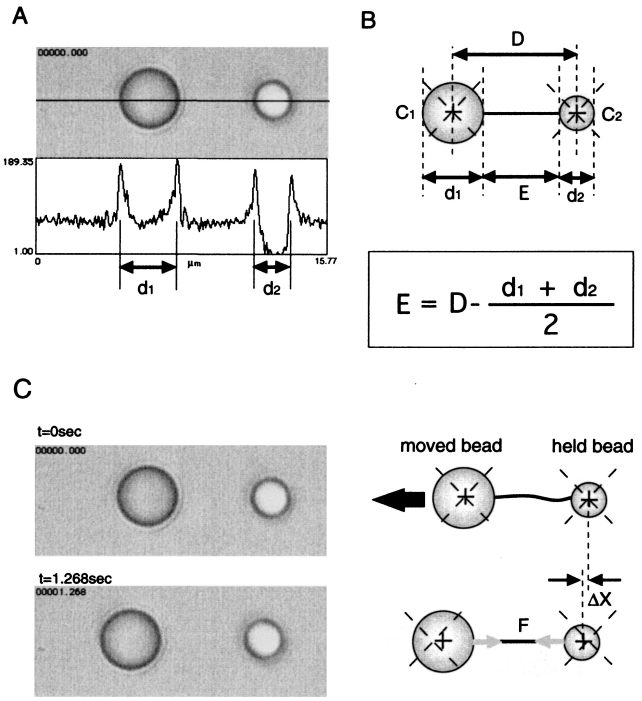

Fig. 1 A–C Evaluation of DNA extension and tension force. **A** Bead images and density profile plot. d_1, d_2: bead diameter. **B** DNA extension (*E*) was obtained by subtracting bead radii from the distance between two beads (*D*). C_1, C_2: bead center. **C** The tension force (*F*) was calibrated from the displacement (ΔX) of the right bead from the trapping spot using the trap stiffness

the sharp peaks in a density profile plot along a straight line passing through the centers of the beads, although it is not sure that this peak corresponds to the surface boundary of a bead (Fig. 1 A). The stretched length of DNA was calculated from the distance between bead centers by subtracting the two bead radii (Fig. 1 B). The tension force F applied to the DNA molecule was calculated from the displacement of the trapped bead based on trap stiffness (Fig. 1 C).

3 Results

3.1 DNA-bead system assembly

The purity of different base pair DNA molecules obtained by the PCR method was first checked by agarose gel electrophoresis. Each fragment resulted in a single band, indicating that there was no significant contamination by different length DNA fragments (Fig. 2 A). These DNA molecules were incubated to add beads to the ends, according to the procedure described above. After one hour of incubation, one out of about 100 beads was followed by another bead when manipulated, and a DNA molecule was assumed to be connecting them. This assembly formation was confirmed by fluorescence visualization. A fluores-

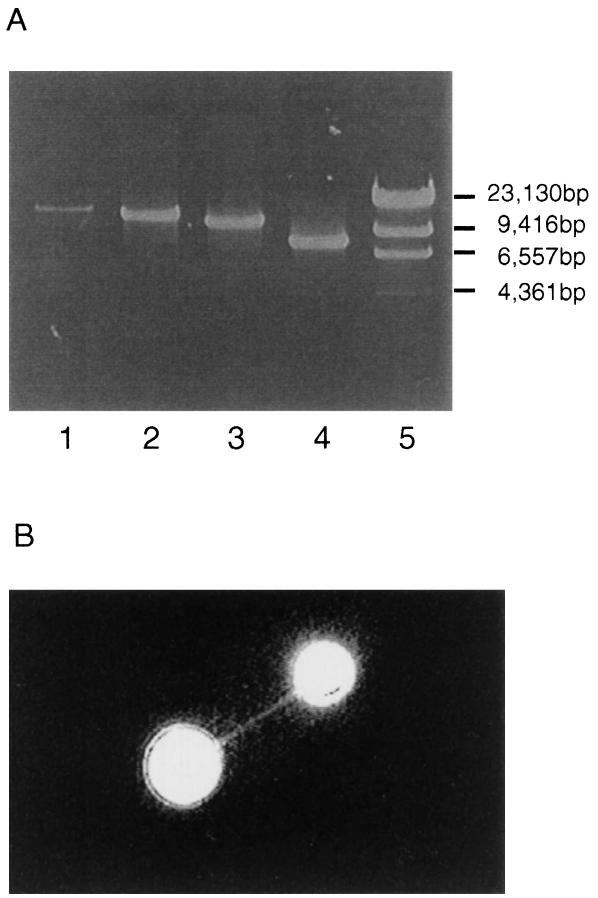

Fig. 2 **A** Agarose gel electrophoresis of DNA fragments for molecular length measurement. Lane 1, 20.707 kbp: 2, 14.991 kbp: 3, 12.039 kbp: 4, 7.960 kbp: 5, size marker, *λ-Hin*dIII digest. A 1% agarose gel with Howry buffer (80 mM tris, 4 mM Na_2EDTA, 25 mM sodium acetate and 50 mM acetic acid) was used. DNA fragments were visualized with ethydium bromide. **B** A fluorescence image of a single DNA molecule. Each end of a 14.991 kbp DNA molecule was attached to micro polystyrene beads (1.909 μm and 2.73 μm in diameter) and stretched by dual trap optical tweezers. DNA molecule was fluorescently labeled with YOYO-1 and excited by an argon-ion laser operating at 488 nm

cence image of the bead-DNA-bead assembly is shown in Fig. 2 B. For length measurement experiments, the DNA was not stained because the fluorescent staining could affect its mechanical properties. The molecular extensions were inferred from the position of the beads following the procedure described in 2.8.

3.2 Molecular length measurement

DNA molecules of four different sizes (7.960 kbp, 12.039 kbp, 14.991 kbp, and 20.707 kbp) were examined. Two beads of the assembly were trapped and manipulated independently. Usually the smaller bead was held at a fixed position and the other was moved away at a constant velocity of about 1 μm/sec. The bead images in the DNA stretching procedure were analyzed and the tension force

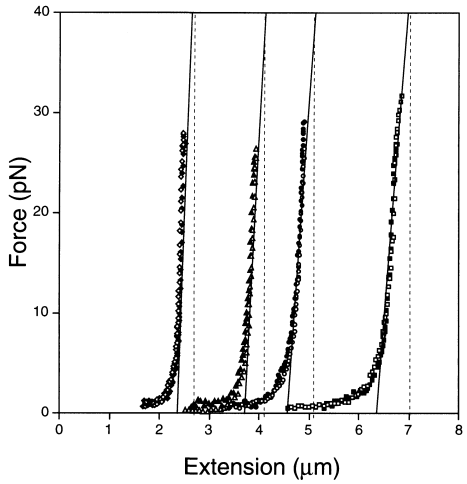

Fig. 3 Force versus extension curves of DNA molecules. DNA molecules were stretched in the buffer of 10 mM tris, pH 7.5, containing 150 mM NaCl and 1 mM EDTA. The base pair numbers of DNA are 7.960 kbp (◆, ◇), 12.039 kbp (▲, △), 14.991 kbp (●, ○), and 20.707 kbp (■, □), Contour length of each DNA, assuming 3.38 Å/bp is shown using the dotted line. The solid lines show linear approximations of elastic modulus

Table 1 The results of length measurement of different size DNA

Base pair number		DNA length (μm)	
(kbp)	G+C content (%)	at 30 pN	at 0 pN
7.960	57.56	2.55±0.12 (16)	2.34±0.09 (16)
12.039	57.30	3.98±0.04 (9)	3.68±0.05 (9)
14.991	56.78	4.95±0.15 (12)	4.53±0.12 (12)
20.707	46.09	6.78±0.11 (15)	6.32±0.11 (15)

The results are given as mean±standard deviation (number of the molecules examined)

F and the DNA extension were evaluated. Typical force versus extension curves and the contour length of each DNA molecule, calculated with a value of 3.38 Å/bp from B-form crystal, are shown in Fig. 3.

With a small stretching force (<10 pN), the DNA molecule was extended up to 90% of the contour length reflecting the entropic elasticity of DNA. Tension force rose rapidly when contour length approached, and 100% contour length was not reached, since one of the beads escaped from the trap when the tension force exceeded the maximum trapping power of about 30 pN. In this linear stretched region, the extension can be approximated as the elastic modulus. The averaged data were analyzed for each molecule size, and the molecular length at specific tension forces of 30 pN and 0 pN was calculated and is shown in Table 1.

Molecular lengths measured were plotted as a function of base pair number in Fig. 4. Error bars representing standard deviation were too small to be shown. The molecular lengths increased linearly from 7 kbp to 20 kbp. The best fits for the base pair number and molecular length at 30 pN and 0 pN were calculated using a least squares regression

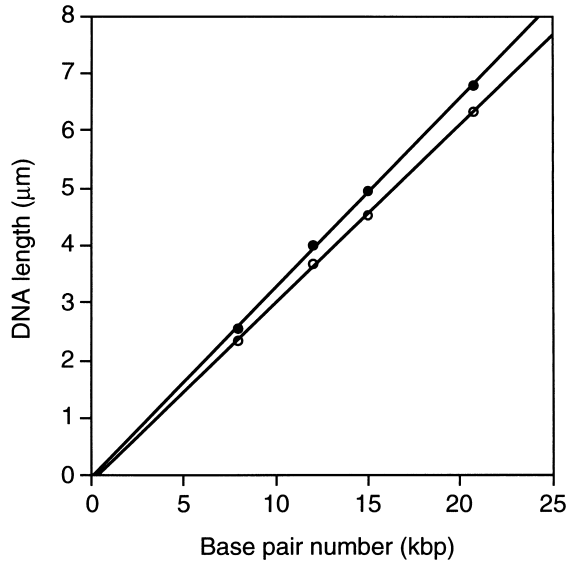

Fig. 4 Base pair numbers versus DNA length at 30 pN (●) and 0 pN (○). The best fit equations for the linear approximation are y = 0.331x–0.047 (30 pN) and y = 0.311x–0.109 (0 pN)

analysis and are also shown in Fig. 4. The rise per residue was evaluated to be 3.31±0.05 Å (at 30 pN) and 3.11±0.04 Å (at 0 pN) from the slopes of the best fit. The intercepts at the y axis were close to zero but not exactly zero.

3.3 Sp1 binding ability of GC box sequences

To examine Sp1-dependent DNA looping, we used DNA with GC box sequences. The structure of the DNA molecule is shown in Fig. 5 A. This DNA was synthesized using the PCR method from the ZAP Express vector, which was derived from λ-phage DNA. The vector had a 4518 bp pBK-CMV phagemid region containing GC box sequences derived from SV40 and CMV promoters. We designed the primer sequences from J and gam regions of the ZAP Express phage DNA. The PCR gave a single DNA product of 15 kbp as analyzed by agarose gel electrophoresis (data not shown). The Sp1 binding ability to both GC box sequences was checked by a gel mobility shift assay. Since the SV40 promoter contains five GC box sequences, we chose the third GC box as the probe (Mitchell and Tjian 1989). As shown in Fig. 5 B, Sp1 bound to the SV40 and CMV probes. The amount of poly (dI-dC) had to be decreased to 40 ng for the CMV GC box sequence, suggesting relatively weak binding affinity. The addition of excess unlabeled probes completely prevented Sp1 binding to the labeled DNA probes, indicating that the Sp1 binding was specific.

3.4 Molecular length of the DNA with GC box sequences

In order to investigate the effects of Sp1 on molecular length, the length of DNA synthesized by PCR was meas-

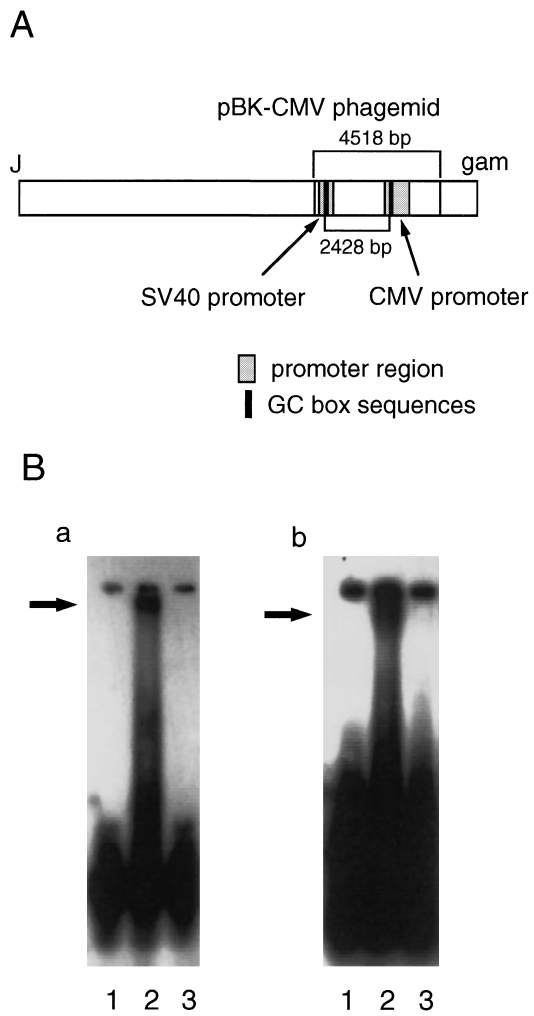

Fig. 5 **A** The structure of the DNA with GC box sequences. The DNA has two GC box sequences derived from SV 40 and CMV promoter. GC box sequences of both promoter regions are shown. **B** Gel mobility shift assay. DNA probes were ^{32}P-end-labeled and detected by auto radiography. (a) SV 40 probe at the poly (dI-dC) concentration of 4 mg. (b) CMV probe at the poly (dI-dC) concentration of 40 ng. Lane 1, DNA probe: lane 2, DNA probe with the addition of Sp1: lane 3, competition experiment with adding unlabeled DNA probe. The arrow shows the retarded band

ured in the presence or the absence of transcription factor Sp1, which binds to the GC box with high affinity. The results of the measurement with a pulling force of 30 pN are shown in Table 2. The original length of the DNA when Sp1 was not added was measured to be 4.76 μm. The base pair number was determined to be 14.52 kbp using the relation between the DNA length and the base pair number obtained above, and roughly agreed with the results from agarose gel electrophoresis. When Sp1 was added, the molecular length data can be classified into two groups. Table 2 shows that the mean length of 20% of the DNA molecules examined became 1.03 μm shorter than the length of the other DNA, corresponding to the base pair numbers of 3.12 kbp. The rest of the DNA molecules became almost as long as when Sp1 was not added. The force versus ex-

Table 2 Effect of Sp1 addition on the length of DNA with GC box sequences

	DNA length (μm)	Calculated base pair number (kbp)
without Sp1	4.76±0.14 (15)	14.52±0.43 (15)
with Sp1* (group 1)	4.69±0.15 (33)	14.31±0.44 (33)
with Sp1* (group 2)	3.66±0.13 (8)	11.19±0.41 (8)

* With the addition of Sp1 the DNA lengths distributed into two groups. The DNA length of group 1 kept almost the same as when Sp1 was not added. The DNA length of group 2 became shorter than that of group 1. The results are given as mean±standard deviation (number of the molecules examined)

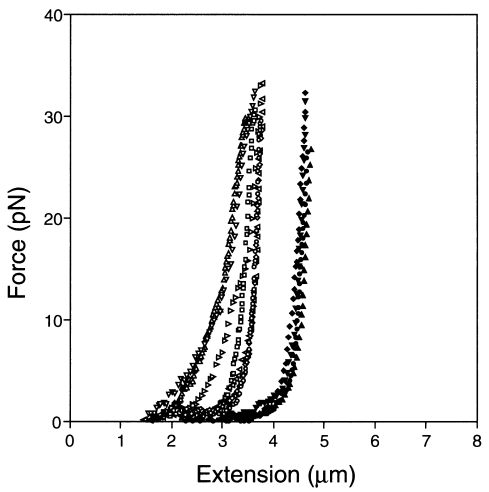

Fig. 6 Force versus extension curves of the DNA with GC box sequences. Transcription factor Sp1 was added to the DNA and the solution was incubated before forming the bead-DNA-bead assembly. Filled signs correspond to the DNA with original length. Open signs show when the DNA shortening occurred

tension curves of the DNA with Sp1 were plotted in Fig. 6. The curves of DNA molecules whose length stayed constant show the typical patterns of DNA stretching. In contrast, the shortened DNA molecules gave more complex curves. In some curves, the force started to rise at the shorter extension of DNA and rose gently.

4 Discussion

DNA molecules were stretched and DNA length was measured as extension at a specific tension force of 30 pN. The rise per residue we measured was 3.31±0.05 Å at a stretching force of 30 pN and 3.11±0.04 Å at 0 pN under linear extrapolation. These values roughly agree with the rise per residue of 3.38 Å in B-form DNA seen in X-ray crystallography. The discrepancy, however, indicates that the base pairs under no stretching force favor more compact base pair shapes. In other words, the base pair distance in crystals is expanded by a hydrate force corresponding to nearly 40 pN, judging from the curves in Fig. 3.

We examined the molecular length of four different base pair numbers, and compared the G+C content (%) of each DNA. As shown in Table 1, the G+C contents of 7.960 kbp, 12.039 kbp and 14.991 kbp are almost the same. On the other hand that of 20.707 kbp DNA is 10% less than the others. Our experiments could not precisely determine whether DNA length depended on G+C content. Therefore, it appears that the G+C content of the DNA doesn't influence the length measurement result.

In the best fit for the relation between the base pair number and the molecular length, the intercept on the y axis was not zero but −47 nm, probably a result of experimental errors in measuring the DNA extension. Because the accuracy of bead center measurement was within 3 nm, the experimental errors are not in image capturing or in bead center calculation. The diameter of beads measured by the method described above is 6% larger than the diameter that was measured by TEM or by using the principle of centrifugal photo sedimentation (manufacture's information). These facts suggest that bead diameter was overestimated due to ambiguity in determining bead edges. In addition, the accuracy of our bead boundary evaluation was within 30 nm. Accordingly, bead image calculation errors could be the most plausible reason for the experimental error. Consequently, four different sizes of DNA molecules must be compared for more accurate results.

As shown in a fluorescence image of bead-DNA-bead assembly, we can consider that the assembly used in the length measurement method has a single DNA molecule between two beads. Because multiple DNA molecules will affect the DNA length distribution, we controlled the concentration of DNA and beads to the same molar ratio and kept their concentrations in the incubation low to have a single DNA molecule in the assembly.

We applied our technique to the detection of DNA looping caused by transcription factor Sp1, which is known for GC box sequence binding (Kadonaga et al. 1987) and for protein-protein interactions (Pascal and Tjian 1991). Although electron microscopy and scanning transmission electron microscopy confirmed both DNA looping and the nexus structure composed by the Sp1 multimer at the specific DNA sites (Mastrangelo et al. 1991), the measured loop contour lengths were imprecise. By stretching the DNA, we could evaluate loop contour shortening. As shown in Table 2, the average shortening of the stretched DNA was estimated to be 1.03 μm, which corresponded to the 3.12 kbp measured with the rise per residue at the same 30 pN stretching force. This shortening can be interpreted as the result of Sp1 binding on the two GC box sites on DNA and looping out the intervening base pairs. The DNA examined here has two GC box sequences 2.428 kbp apart, 0.69 kbp shorter than that observed above. The different patterns of the shortened DNA curves in Fig. 6 suggest that this discrepancy could be attributed to a non-specific interaction between Sp1 proteins and DNA. Figure 6 also shows that the effects of this interaction remain even when the DNA molecule is pulled by a 30 pN force. Stronger ten-

sion forces will counteract this weaker non-specific interaction.

As shown in the gel mobility shift assay, the Sp1 binding capability of a CMV GC box was found to be weak. This weak binding ability may explain why only 20% of the DNA with GC box sequences becomes shorter with the addition of Sp1.

Here we used PCR amplification to label both ends of the DNA molecule. Recent developments in PCR amplification enabled us to obtain over 35 kbp of DNA molecules (Barnes 1994), making it possible to prepare any kind of DNA molecule containing interesting sequences by using specific primers and template DNA. We have shown here that our length measurement method is successful in detecting DNA shortening due to looping even in the presence of non-specific interactions. This method could also be used to investigate DNA looping in which other transcription factors are at work.

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