Studies on biomolecules using single molecule imaging and manipulation techniques

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Abstract

The best way to obtain unambiguous information about the function of biomolecules is to study their function at the single molecule level. By attaching a small tag such as a single fluorescent dye molecule to biomolecules, or a tag that is huge compared to the size of a biomolecule, we have been able to image the individual behaviours in real time under an optical microscope. Huge tags such as micrometer-sized plastic beads also allow the manipulation of individual molecules with, e.g. optical or magnetic tweezers. Using these techniques, we have observed individual behaviours of RNA polymerase, a molecular machine that copies the genetic information on DNA onto a messenger RNA. We found that some of the RNA polymerase molecules underwent linear diffusion along DNA, helping RNA polymerase to search for the promoter. We also found that a single RNA polymerase molecule rotated around the right-handed screw axis of the double helix of DNA during transcription with a rotary torque of \( \tau > 5 \, \text{pN nm} \). The present methods are potentially applicable to the examination of a wide variety of protein–nucleic acid interactions, especially those involved in the process of transcription.

Keywords: Single molecule imaging; Optical microscope; Optical tweezers; Fluorescent dye; RNA polymerase; DNA

1. Introduction

Recently, new optical methods for imaging and manipulating DNA have opened a new phase in the study of gene expression. Detailed elastic characteristics of DNA have been examined by manipulating a single molecule of DNA with an optical trap [1–3] and a magnetic trap [4]. Single-molecule process of transcription by RNA polymerase have been detected by monitoring a tag attached to DNA [5,6]. We have demonstrated that single fluorophores can be clearly observed in aqueous solution by total internal reflection microscopy, refined so that the background light is very low [7]. By combining these techniques, we have observed individual interactions of single RNA polymerase molecules with a single molecule of DNA and rotation of DNA during transcription by RNA polymerase [8].

2. Materials and methods

2.1. Single-molecule imaging of RNA polymerase–DNA interactions

2.1.1. Preparation of DNA conjugated with beads

Streptavidin-coated beads were prepared according to the method of Berliner and colleagues [9] with some modifications [8]. Biotinylated oligonucleotides complementary to both cohesive ends of the \( \lambda \)-phage DNA (Takara Biochemicals, Japan) were ligated on to the \( \lambda \)-phage DNA (Takara Biochemicals, Japan). The length of \( \lambda \)-phage DNA is about 16 \( \mu \)m. Streptavidin-coated beads were incubated with biotinylated \( \lambda \)-phage DNA. Beads conjugated with DNA were then diluted in buffer which contains an oxygen scavenger system [10]. A DNA molecule that had beads bound at both ends was selected, and suspended near the surface of a pedestal by manipulating the beads with dual optical traps under bright-field illumination [8].
2.1.2. Preparation of fluorescently-labeled RNA polymerase molecules

*Escherichia coli* core RNA polymerase was prepared and labeled with Cy3 as previously described [8]. RNA polymerase core enzyme activity was assayed under standard conditions [11]. The core enzyme activity after fluorescently-labeling was 96% of that before labeling. RNA polymerase holoenzymes were prepared by mixing the core enzymes with a 6-fold molar excess of purified σ^70, and incubating for 15 min at 30 °C in buffer [8]. Holoenzymes were stored at −80 °C.

2.1.3. Optics

A total internal reflection microscope used to visualize single fluorophores was modified to accommodate dual optical traps [8]. The optical trap mounted with a diode pumped Nd:YAG infrared laser (wavelength 1064 nm, 7910-Y4-106, Spectra-Physics Lasers, Inc., Mountain View, California) was used. Two traps were produced by passing the laser beam through a quarter-wave plate followed by a polarizing beam splitter. One of the beams was deflected with two orthogonal scanners that were operated using the mouse of a computer. Microbeads were illuminated with infrared light (700–800 nm) from a halogen lamp. The image of beads was captured by a CCD camera. Fluorophores were excited with a frequency-doubled Nd:YAG laser (wavelength 532 nm, model 140-0534-200, Light Wave Electronics, Mountain View, California). Fluorescence images were captured by a silicon-intensified target camera (C2400-08 Hamamatsu Photonics) and recorded on video tape. Bright-field images of the beads and fluorescence images of Cy3-labeled RNA polymerase molecules could be observed simultaneously. An oil-immersion objective lens (Plan NCF Fluor × 100, 1.3 NA, Nikon, Japan) was used.

2.2. Direct observation of DNA rotation during transcription by RNA polymerase

2.2.1. Preparation of stalled transcription complex

*E. coli* RNA polymerase holoenzyme was purified and supplemented with excess ε subunit [12]. DNA template for transcription was prepared from T7 D111 DNA [13] by polymerase chain reaction. The downstream primer had been biotinylated at nine sites at ~10 base intervals. Stalled transcription complex was prepared as described before [12].

2.2.2. Preparation of beads

Fluorescent, carboxylated microbeads (20 nm, excitation 580 nm, emission 605 nm, Molecular Probes) were amino-derivatized with ethylene diamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The microbeads and biotin-x-cadaverine were conjugated to 850-nm carboxylated magnetic beads (Seradyn) with EDC, and streptavidin was bound to the biotinylated magnetic beads [12].

2.2.3. Transcription on the glass surface

A flow chamber was made of two coverslips, which had been sonicated in water, stored in methanol, and dried by burning methanol with a lighter. Stalled transcription complex in buffer infused into the flow chamber, and attached to the coverslip surface [12]. After that streptavidin-coated magnetic beads were infused and attached to the downstream end of the DNA where nine nucleotide residues were biotinylated. Then, all four NTPs (nucleoside triphosphates) were added to allow further transcription [12].

2.2.4. Microscopy

Samples were observed at 23 ± 2 °C on an Olympus IX70 inverted microscope with a 100× oil-immersion objective. Magnetic beads were illuminated with a halogen lamp obliquely from above through a ring-shaped optical-fiber assembly. Fluorescent daughter beads were imaged with standard epi-fluorescence optics. Superimposed bright-field and fluorescence images were projected on a silicon-intensified target camera (C2400-08 Hamamatsu Photonics) and recorded on a video tape. A disk-shaped neodymium magnet, to which a conical iron piece was attached to enhance the magnetic force, was placed above the sample. The magnetic bead was pulled upward by a magnet. Vertical pulling force was calibrated by tethering the magnetic beads with 16-μm-long λ-DNA and measuring the amplitude of Brownian motion [14]. The force varied among beads and was 0.05–0.2 pN.

3. Results and discussion

3.1. Single-molecule imaging of RNA polymerase–DNA interactions

3.1.1. Observations

Fig. 1a shows a schematic drawing of the experimental arrangement. An unstained λ-phage DNA molecule with both ends attached to beads was captured and stretched to full extension by dual optical traps under bright field illumination. The DNA was brought near the surface of a rectangular pedestal 8 μm wide and 2 μm deep, which had been made on a glass surface by chemical etching. Individual interactions of single fluorescently-labeled RNA polymerases with the DNA were directly observed by the evanescent field produced when a laser was totally reflected in the interface between the glass and the solution [7]. Fig. 1b shows the bright field image of the beads in the optical traps, between which a single DNA molecule had been suspended. An oblique band between the beads indicates a part of the rectangular pedestal. Fig. 1c shows the fluorescence image of the same field as in Fig. 1b.
illuminated by the evanescent field. The concentration of RNA polymerases was 9 nM.

3.1.2. Linear diffusion

Some of the RNA polymerases that were nonspecifically bound moved along the DNA (Fig. 2). The direction of movement along the DNA was random, indicating that the movement was due to thermal diffusion. This observation provides direct evidence of the linear diffusion of single molecules of RNA polymerase, bound to the nonspecific binding sites, along a single DNA molecule. The fraction of RNA polymerases that diffused further than the space resolution of the optical microscope (~0.2 μm) was 10 out of 381 molecules analyzed by the image processor. If other RNA polymerases underwent thermal diffusion but their diffusion could not be observed because of the small diffusion range (<0.2 μm), then the diffusion constant $D$ can be estimated to be in the order of $10^{-10}$ cm$^2$ s$^{-1}$ by inserting the average lifetime of nonspecific binding $t_0 = 0.2$ s ($1/4.6$ s$^{-1}$) into the equation [15]. This diffusion constant is one order of magnitude smaller than that predicted by a kinetic study [16,17] and three orders of magnitude smaller than that in solution. The average range of linear diffusion for the lifetime of binding (~0.2 s) has been calculated to be ~90 nm, i.e. 300 bps. The probability of polymerase binding to the promoters would be greatly increased by diffusion along DNA, compared to RNA polymerase undergoing random association/dissociation (hopping) along the DNA [17–20]. The present method could be extended to examine the effects of regulatory proteins and different σ factors on the interactions of DNA–RNA polymerase, and the elementary process of transcription directly at the single molecule level. Furthermore, this method could be applied to the study of other
DNA binding proteins such as helicase and restriction endonuclease.

3.2. Direct observation of DNA rotation during transcription by RNA polymerase

3.2.1. Observations

To observe relative rotation between RNA polymerase and DNA, we employed an optical microscopy technique based on the tethered-particle method [5] (Fig. 3a). A DNA template, 4971 base pairs (1.7 \( \mu \)m) long and containing strong promoter, was constructed (Fig. 3b). Daughter fluorescent beads attached to the end magnetic bead served as markers of rotation. We pulled the magnetic bead upward at \( \sim 0.1 \) pN with a magnet for two reasons: to confine the rotation in a horizontal plane, and in the hope of restraining the DNA from supercoiling, which would interfere with torque transmission to the end bead. Up to a few percent of beads in an observation chamber rotated continuously (Fig. 4), invariably clockwise when viewed from top in Fig. 3a. Threading a right-handed double helix of DNA through RNA polymerase will, in a simple mechanism, produce clockwise rotation. Not all beads rotated, some being stuck on the glass surface; others fluctuated in both directions, presumably being attached to nicked DNA or tethered to an inactive RNA polymerase. Generally, the rotation was slower at lower NTP concentration, (NTP). When NTPs were absent, unidirectional rotation was not observed. For our DNA template with the full transcript length of \( \sim 4500 \) bases, the expected number of revolutions is \( 4500/10.4 \) (base pairs per turn of DNA [21]) \( \sim 430 \). At least \( \sim 180 \) consecutive revolutions have been observed, suggesting that thousands of base pairs can be transcribed without extensive rotational slippage.

3.2.2. Comparison of rotation and transcription rates

To compare with the rate of rotation, we measured the rate of RNA elongation in solution. The elongation rate
divided by 10.4 will be the rotation rate if the polymerase faithfully tracks the DNA helix. Indeed, this seemed to be the case at (NTP) below \( \sim 20 \mu M \). Compared to the elongation rate, the rotation rate apparently saturated at a lower (NTP), around \( \sim 20 \mu M \). The apparent saturation of rotation rate is explained if the maximal torque of RNA polymerase, \( T_{\text{max}} \) is already reached at the observed maximal rotation rate of \( \sim 0.2 \) revolutions s\(^{-1}\) (rps) or flexible DNA cannot sustain this much of torque and collapses by supercoiling. To rotate a bead of diameter \( D = 850 \text{ nm} \) in bulk water at this speed, the required torque will be \( > 5 \text{ pN nm} \) [12]. This value is close to the critical torque at which DNA supercoiling begins.

In summary, our results indicate that RNA polymerase rotates DNA by tracking its right-handed helix, that RNA polymerase does so over thousands of base pairs, and that RNA polymerase can produce \( > 5 \text{ pN nm} \) of torque. Whether RNA polymerase rotates around DNA or DNA around RNA polymerase is an issue in vivo [22], but our experiment with fixed RNA polymerase cannot answer this problem. Some uncertainty remains in the degree of tracking fidelity, but several beads made many revolutions at the rate commensurate with precise helical tracking. Although the mechanism of tracking is yet unknown, genuine rotary motors may also employ tracking as the rotary mechanism. In the bacterial flagellar motor [23,24], the rotor consists of circularly arranged identical subunits. Driving units are also circularly arranged, but one unit suffices to produce efficient rotation [24]. A single unit may thus track along the rotor subunits thereby causing rotation. In the \( F_1 \)-ATPase in which an asymmetric rotor rotates in 120° steps [25] tracking mechanism is unlikely.

4. Perspective

The methods described in this article could be extended to examining the effects of regulatory proteins on the interactions DNA–RNA polymerase and the elementary process of transcription directly at the single molecule level.

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