Studying RNAP–promoter interactions using atomic force microscopy

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Abstract

The most fundamental step in gene expression is transcription, during which DNA is transcribed to a corresponding RNA strand by the action of RNA polymerases (RNAPs). Over the past two decades, atomic force microscopy (AFM) has been used as one of the key tools in the investigation of transcriptional events at the single-molecule level. AFM studies have provided significant insights into the structure–function relationships of RNAP–DNA complexes in different stages of transcription. Here, we begin by illustrating the basic setup of AFM, followed by an introduction of the applications of AFM techniques, including high-speed AFM (HS-AFM) imaging, to investigate RNAP–DNA interactions with a special focus on promoter-search processes and open promoter formations. The combination of AFM with a newly developed experimental technique, DNA origami nanotechnology, will also be described.

Keywords

RNA polymerase, promoter, transcription, atomic force microscopy, DNA origami

1. Introduction

Transcription, as a first step of gene expression, is performed by RNA polymerases (RNAPs), which read a particular segment of DNA to synthesize RNA chains that are then translated into proteins. The control of transcription affects the temporal and spatial patterns of protein expression and is critical for the cellular metabolism. Therefore, the elucidation of the molecular mechanism of transcription has been one of the main focuses of molecular biology since the establishment of the ‘Central Dogma’. A large number of structural studies that used X-ray crystallography and electron microscopy led to the development of a molecular account of the interactions between RNAPs and DNA molecules [1-4]. The combination of conventional biochemistry techniques and structural studies has been a standard approach for analyzing the interactions between proteins and short DNA molecules. However, the characterization of the dynamic behavior of RNAP on a longer DNA strand comprising hundreds to thousands of base pairs (bp) cannot be achieved using only these approaches.

Single-molecule techniques are powerful options to complement this information because of their capability to detect and even manipulate transient states of protein–DNA complexes. To date, various single-molecule methods, including fluorescence-based approaches and atomic force microscopy (AFM), have been developed and used to dissect the transcription process [5-7]. Among these methods, AFM has been the standard technique used to visualize the structure of RNAP–DNA complexes [7]. A notable feature of this instrument is its capability of operating in both air and liquid environments, where the latter allows the imaging of samples in physiologically relevant conditions. In this article, we present an overview of the contributions of AFM studies to the elucidation of how RNAP locates its promoter and induces conformational changes in the duplex. We first illustrate the basics of AFM, before describing the experimental results that have been reported in the last two and a half decades. The use of a combination of high-speed AFM and DNA origami technology to study dynamic molecular events will also be described, together with future prospects.

2. Atomic force microscopy

AFM was invented by Binnig *et al.* in 1986 [8]. The central component of AFM is a cantilever with a sharp tip (probe) at its end that raster scans over the sample surface in a series of horizontal sweeps (Fig. 1). The surface topography is then reconstituted from the vertical movement of the sample stage (piezoelectric scanner), which is controlled by the feedback system. A sensitive detection of the vertical displacement of the probe during scanning is achieved by using optical lever methods, in which a small displacement (down to 0.1 nm) of the cantilever can be amplified and detected by the position-sensitive photodiode.

Immediately after its invention, AFM was introduced into the field of biological sciences by Hansma *et al.* [9]. Since then, AFM has evolved into a multifunctional tool for biological research [10-14], and has become popular in the postgenomic age. At the initial stages of the development of AFM, samples were imaged in contact mode, in which the tip remains in close contact with the sample surface during scanning. Although AFM-based studies of DNA using the contact mode have been reported [15, 16], this mode was considered inappropriate for biological samples because the interaction between the tip and the sample created a significant amount of lateral

force.

This problem was overcome by the development of the Tapping ModeTM, in which the cantilever oscillates in the **vertical direction** at close to its resonant frequency [17]. As the tip gets closer to the surface, the tip–sample interaction changes the amplitude of the cantilever oscillation. These changes are monitored by a feedback loop to maintain the tip–surface distance while the cantilever scans over the sample. In this mode, the contact between the tip and the sample surface is intermittent and, thus, the shearing interactions can be significantly reduced. As the tip-induced sample deformation is minimized, soft biological samples, such as lipid bilayers [18, 19], proteins [20–22], and nucleic acids [23, 24], can be imaged.

In particular, AFM's range of operation has been suitable for the analyses of various DNA and RNA structures and their complexes with proteins [24–27]. These samples are easily adsorbed onto an atomically flat mica surface using well-established methods [24, 28] and reproducibly imaged either in air or liquid environment. Unless otherwise mentioned, the works introduced in this article have been performed on mica surfaces using Tapping ModeTM AFM.

3. Promoter search

Transcription initiation requires the association of RNAP with the promoter region of a gene. The interaction between DNA and DNA-binding proteins, not only with RNAP but also with other proteins, has been thought to begin with the random binding of the protein to the DNA, followed by a translocation of the bound protein along the DNA strand until it encounters the target site [29]. Kinetic studies demonstrated that the use of this two-step process allows proteins to 'find' their recognition site with a speed that is orders of magnitude faster than what would be expected for random diffusion [30]. Subsequent studies have shown that target location by DNA-binding proteins can be facilitated by reducing the dimensionality of the search process [31, 32]. Three possible models for this site-searching mechanism have been proposed [31, 32]: sliding (one-dimensional [1D] diffusion along DNA), hopping (microscopic dissociation–reassociation events), and intersegmental transfer (direct intersegmental transfer between different sites) (Fig. 2). These different types of events have been speculated to happen simultaneously or sequentially in the initiation process of site-specific DNA reactions, and have recently been detected in DNA–restriction enzyme reactions [33–35].

The first notable AFM observation of the promoter-search process occurred in the late 1990s. Guthold *et al.* visualized the 1D sliding of the *Escherichia coli* (*E. coli*) RNAP on a promoterless DNA fragment via time-lapse AFM imaging in liquid [36]. The DNA molecule was observed to slide back and forth beneath the RNAP with an average 1D diffusion coefficient of $1.1 \times 10^1 \text{ nm}^2/\text{s}$. Intersegmental transfer and hopping were also observed in AFM imaging, with sub-minute temporal resolution [37]. In addition to the imaging of a transient structure consisting of an RNAP molecule bound to two segments of the same DNA molecule, dissociation and reassociation between RNAP and the DNA molecule were captured in successive images. However, the diffusion coefficient and lifetime of nonspecific RNAP–DNA complexes obtained from these studies suggested that the events captured on the mica surface were significantly slower than those that occur in solution. This can be explained by surface–molecule interactions, which hinder molecular diffusion. Time-lapse imaging of the

functional movement of biological molecules by AFM generally necessitates conditions that satisfy two conflicting requirements: (i) the molecules must retain enough mobility to allow their functional movement; (ii) at the same time, the molecules must be adsorbed to the mica surface and remain within the scanning area during imaging. Because of the innate slow scanning rate of the conventional instrument, researchers had to slow down the molecular diffusion and place a lot of effort into developing experimental conditions (e.g., ionic strength of buffers, mica modifications, etc.) that allow both molecular movements and AFM imaging.

Although the necessity of binding the sample to the mica surface cannot be eliminated, the requirement for sample immobilization is significantly alleviated in HS-AFM systems [38, 39], on which an entire image can be captured about 100–1000 times faster than on a conventional instrument. In the last decade, this device has been used to monitor a variety of dynamic processes of protein–DNA complexes, including DNA translocation [35, 40, 41], strand cleavage [33, 34, 42, 43], and Holliday junction resolution [44, 45], in real time at a scan rate of 0.5–3.0 frames/s. A recent HS-AFM study of RNAP revealed that RNAP searches for promoter regions using all of the above-mentioned mechanisms, i.e., 1D sliding, hopping, and intersegmental transfer [46] (Fig. 3). Consistent with the work of Bustamante *et al.* [37], HS-AFM movies have shown that hopping and intersegmental transfer events are preceded and followed by sliding, which supports the possibility that a combination of the three mechanisms facilitates the targeting of RNAP to the promoter.

4. RNAP–promoter complexes

After RNAP finds the promoter, the RNAP–DNA complex undergoes a series of conformational changes that result in local unwinding of the DNA double helix to expose the template strand [1, 47–49]. The resulting complex is called open promoter complex (PR_O). In prokaryotes, the formation of PR_O and the subsequent initiation of transcription cannot be achieved by the core RNAP itself. The core RNAP has to be bound to other protein factors, the so-called σ -factors, which recognize specific sequences in promoters [50]. The primary σ factor in *E. coli* is σ^{70} . It should be noted that σ^{70} alone can form a holoenzyme by binding to core RNAP without the need of any additional protein factors. Therefore, the *E. coli* σ^{70} -RNAP holoenzyme system has been one of the best model systems for studying PR_O s.

Most AFM-based studies of PR_O s have been performed in the dry condition. However, despite the simplicity of the method, relevant information about the properties of RNAP–DNA complexes can be recovered, and deeper insights into the structure, topology, and molecular interactions can be gained from static AFM images.

Several observations based on AFM have shown that the template DNA appears to be bent by the binding of the RNAP to promoter regions (Fig. 4). Rees *et al.* first found RNAP- σ^{70} at the λP_L promoter and showed a mean bend angle of 54° [51]. In a similar work, Revetti *et al.* reported that the DNA bend angle at the λP_R promoter varied from 55° to 88° [52]. Furthermore, a detailed analysis of the AFM images revealed that the formation of the RNAP- σ^{70} -DNA complex at the λP_R promoter caused a reduction of the contour length of ~ 30 nm (~ 90 bp) relative to the free DNA molecule. Taken together with the bend angle measurements, these results led Revetti *et al.* to propose that the reduction of the DNA contour length can be attributed to the wrapping of the DNA molecule around the RNAP by $\sim 300^\circ$. Those authors provided further insights on this wrapping: assuming a persistence

length of 53 nm for duplex DNA [53], the formation of a $\sim 300^\circ$ bend of the 90 bp segment of DNA requires 60 kJ/mol. This energy must be compensated by the energy stemming from the interaction between the RNAP and the promoter. It has been estimated that the free energy of formation of the PR_O at the λP_R promoter is -60 kJ/mol [54]. Thus, without the energy cost of DNA bending, the free energy required for PR_O formation would be around -120 kJ/mol, which would probably render the complex too stable to move on to the next phase of transcription. The energy required for DNA bending may have the benefit of facilitating promoter clearance.

Subsequent studies showed that the extent of DNA wrapping in RP_O depends on the sequence of promoters, particularly on sequence determinants of the upstream region of the promoter (UP-elements) [55]. Importantly, it was found that the sequence of the upstream region of the promoter can affect DNA wrapping, even in the absence of the RNAP α -subunit carboxy-terminal domain (α -CTD), which is a determinant for protein–DNA interactions with UP-element subsites; this implies that other effects, such as the intrinsic DNA curvature in upstream DNA, may also determine the structural features of RP_O .

5. Methods to identify polarity of DNA strands

As discussed in the previous section, specific interactions between proteins and their target DNA sites are generally investigated in dry conditions by measuring contour lengths from the ends of the template DNA to the position of the protein. Therefore, a DNA fragment carrying the required binding site (e.g., promoter) flanked by two DNA arms of different lengths has been used commonly in AFM-based studies of DNA–protein complexes. However, the interpretation of systems in which proteins that move along the template dynamically is complicated and difficult, because it is impossible to determine the polarity of the naked DNA strand in AFM.

To date, several methods have been devised to determine the polarity of the DNA strand in AFM images. A popular method is end modification with a biotin moiety [56–59]. This is easily achieved by a PCR-based preparation of the DNA strand using a biotinylated primer. The biotinylated end can be decorated with streptavidin [56–59] or streptavidin-conjugated gold particles [59], which results in a small blob at the end of the DNA in topographic AFM images.

The strategy developed by Billingsley *et al.* does not rely on labeling with additional proteins [60]. In their approach, a single-stranded oligonucleotide primer is designed to allow the formation of a loop and stem while retaining 3' extensions, to facilitate the annealing of the primer to the template. This enables the incorporation of a DNA stem loop into the linear DNA at one end after a single-cycle DNA polymerase reaction (see also [61] for the improved protocol). Importantly, the presence of the loop has no effect on the activity of RNAPs regarding their location on promoters.

DNA origami nanotechnology [62] provides an alternative approach for the identification of strand polarity. DNA origami is a technique in which a long, single-stranded DNA ('scaffold strand') is folded into a predefined shape with the aid of many short oligonucleotides that are complementary to different parts of the scaffold strand ('staple strands'), thus giving stability to the structure [62]. Endo *et al.* used DNA origami nanostructures in HS-AFM-based single-molecule observation systems [63, 64]. For the analysis of transcription, a rectangular DNA origami nanoplateform with external dimensions of approximately 400×20 nm was designed to

attach a template dsDNA with a prescribed orientation [65] (Fig. 5). In this design, two connectors that were used to anchor the template DNA were located at different distances from the nearest ends of the platform, thus enabling the determination of the polarity of the template. A 1000-bp-long template dsDNA containing the T7 promoter region was incorporated between the predesigned sites on the origami structure, to study the transcriptional events performed by T7 RNAP. In a fashion similar to that of *E. coli* RNAP, T7 RNAP exhibited a sliding motion along the template DNA in the absence of NTPs (Fig. 6). Furthermore, the elongation process, during which the RNAP initially binds to the promoter region, moves downstream on the template, synthesizes the nascent RNA, and finally dissociates from the DNA, was successfully monitored with a temporal resolution of 0.5 frames/s under the condition each NTP is supplied. This method may be applied to the study of other types of RNAPs and their regulatory factors.

6. Conclusions

In this article, we gave an overview of the manner in which AFM techniques have been used to study RNAP–promoter interactions, with a view to obtaining a better understanding of the mechanisms via which the RNAP locates promoters on the template DNA, and of the subsequent conformational changes induced by RNAP binding. In addition to conventional dry or wet imaging, advances in HS-AFM have promoted these studies. We also illustrated methods used to determine the polarity of the template DNA strand in single-molecule AFM imaging experiments. Precise identification of the promoter position in the template strand is of great help, especially for dynamic time-lapse AFM imaging, and enables the recovery of more accurate information from the images obtained. By extending and combining the various approaches described in sections included above, studies of more complex systems, e.g., events that occur on templates with tandemly or convergently aligned promoters [60, 66], can be performed in greater detail. The process of transcription elongation can also be monitored and analyzed using time-lapse AFM imaging [46, 65, 67], although we did not focus on this aspect in depth here. It should also be noted that recent advances in tip manufacturing and improvements in feedback systems enable the possibility of reaching a nano-range resolution in solution, as exemplified by the imaging of the double-helical structure of a DNA molecule [68-71]. We anticipate that the entire process of transcription will be monitored at the single-molecule level with unprecedented spatial and temporal resolution in the near future.

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Figure legends

Figure 1. Basic principles of AFM operation. The standard AFM setup includes a cantilever with a nanoscale tip positioned over the AFM sample surface. The tip's position is monitored by a laser which reflects off of the cantilever onto a photodiode. The positional information is then processed and displayed on the PC monitor. The information gathered can be displayed as a topographical image in which the tip is scanned in a raster pattern over or across the sample. From ref [23].

Figure 2. Schematic of various promoter-search mechanisms by RNAP. In sliding, the RNAP diffuses one dimensionally along the DNA molecule. Hopping is transient association and dissociation of the RNAP repeatedly occur along the DNA. In intersegment transfer the RNAP translocate from its initial position on the DNA to a distant site via the formation of an intermediate loop complex.

Figure 3. HS-AFM images of promoter-search processes of *E. coli* RNAP obtained at 1 frame per second (fps). Scale bar, 100 nm. In addition to hopping (frame 4–8), successive images show dissociation and random walk of RNAP, followed by reassociation to the same DNA (frame 9–28). Images were reproduced from [46] with modification.

Figure 4. AFM analysis of the open promoter complex (PR_O). (A) Measurement of DNA contour lengths in the absence (top) and presence of *E.coli* RNAP- σ^{70} . Reproduced with permission from [55]. (B) A schematic of showing how the RNAP wraps DNA around itself, and how the bend angle θ is defined. The schematic is inspired by original work by Rivetti *et al* [52].

Figure 5. DNA origami nanoplatform for the single-molecule analysis of transcription. (A) Design of the DNA origami nanostructure. The template DNA can be anchored between sites a and b. (B) Graphical explanation of the incorporation of the template DNA onto the origami platform. (C) Design of the PCR-amplified template DNA containing a T7 promoter and two ssDNA linkers for attachment to origami platform between sites a and b. (D) Successive HS-AFM images of the purified DNA origami nanoplatform carrying the template DNA. Images were acquired at scan rate of 1 fps. The elapsed time is shown in each image. Scale bar, 100 nm. (E) Overlay of the DNA strands from 6 successive images. An illustrated DNA origami platform and the connection sites a and b are also shown. From ref [65].

Figure 6. (A) HS-AFM images of T7 RNAP sliding on the template DNA attached to the platform. Arrows indicate position of RNAP. Images were acquired at scan rate of 0.5 fps. Scale bar, 100 nm. (B) Shapes of the dsDNA chain and the positions of RNAP (red circle). Rectangles represent the position of sites a (red) and b (purple) on the DNA nanoplatform. From ref [65].

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6