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Biochemistry®

Reprinted from
Volume 36, Number 3, Pages 461–468

Biochemistry

 Volume 36, Number 3 January 21, 1997

Accelerated Publications

Escherichia coli RNA Polymerase Activity Observed Using Atomic Force Microscopy†

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Received September 27, 1996; Revised Manuscript Received November 22, 1996⊗

ABSTRACT: Fluid tapping-mode atomic force microscopy (AFM) was used to observe *Escherichia coli* RNA polymerase (RNAP) transcribing two different linear double-stranded (ds) DNA templates. The transcription process was detected by observing the translocation of the DNA template by RNAP on addition of ribonucleoside 5′-triphosphates (NTPs) in sequential AFM images. Stalled ternary complexes of RNAP, dsDNA and nascent RNA were adsorbed onto a mica surface and imaged under continuously flowing buffer. On introduction of all four NTPs, we observed some DNA molecules being pulled through the RNAP, some dissociating from the RNAP and others which did not move relative to the RNAP. The transcription rates were observed to be approximately 0.5−2 bases/s at our NTP concentrations, approximately 5 μM. The RNA transcripts were not unambiguously imaged in fluid. However, in experiments using a small single-stranded (ss) circular DNA template, known as a rolling circle, transcripts up to 1 or 2 microns long could be observed with tapping mode AFM once the samples were dried and imaged in air. This confirmed our observations of the transcriptional activity of RNAP adsorbed onto mica. This work illustrates that the development of tapping-mode in fluid has made it possible to use AFM to follow biological processes at the molecular level and get new insights about the variability of activity of individual molecules bound to a surface.

Transcription is a central biochemical process in gene expression that is still not fully understood. The kinetics of the reaction have been studied under a variety of conditions in biochemical experiments (Sen & Dasgupta, 1994, 1996). These experiments are restricted to observing the population-averaged properties of proteins. Single RNA polymerase (RNAP)[1] transcription has been observed with optical microscopy (Schafer et al., 1991). In that experiment, stalled

† S.K. was supported by the Department of Histology of the University of Fribourg, Switzerland, N.H.T. was supported partially by a NATO fellowship and NSF Grant 9622169, and B.L.S. by the MRL Program of the NSF Grant (Award No. DMR-9123048). This work was also supported by NSF Grants MBC 9118482, MCB 9317466, BIR 9318945, NIH Grant GM-32543, and a grant from the Lucille P. Markey Foundation to the Institute of Molecular Biology, University of Oregon.

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1997.

[1] Abbreviations: AFM, atomic force microsopy; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; RNAP, RNA polymerase; NTPs, ribonucleoside 5′-triphosphates; bp, base-pair; nt, nucleotides; TSB, transcription buffer.

S0006-2960(96)02440-3 CCC: $14.00

ternary transcription complexes (Levin et al., 1987) were adsorbed on a glass surface with a 40 nm diameter gold particle fixed at the end of the DNA template. By analyzing the spatial extent of the Brownian motion of the gold particles by differential interference contrast light microscopy, Schafer et al. (1991) detected translocation of DNA templates with single RNAP molecules. More recently, Yin et al. measured the force produced by a single RNA polymerase during transcription using optical tweezers (Yin et al., 1995).

The continuing development of atomic force microscopy (Binnig et al., 1986) now offers the opportunity to study transcription in an even more direct way. The ability of the AFM to operate in liquids (Drake et al., 1989) has long made the instrument attractive for the study of the dynamics of biological systems. The invention of tapping-mode in fluid (Hansma et al., 1994) has reduced damaging shear forces present in contact mode and enabled reproducible imaging of proteins physisorbed to mica surfaces (Fritz et al., 1995; Thomson et al., 1996a). DNA has been imaged with improving success and reproducibility in recent years [for a review see Hansma and Hoh (1994)]. Fluid tapping-mode was essential for obtaining sequential images showing DNA motion on mica surfaces and degradation of DNA by DNase I (Bezanilla et al., 1994). Previously, transcription complexes of *Escherichia coli* RNAP had been imaged in air (Rees et al., 1993); imaging these complexes in fluid (Guthold et al., 1994) and monitoring the transcription process was the next step. To ensure that transcription proceeded at a slow enough rate to be observable by AFM the NTP concentration was kept below 5 μM.

In observing the transcription process a paradoxical problem had to be overcome. To be able to image DNA with the AFM under fluid, not only must the DNA molecule be bound sufficiently stongly not to be disturbed by the tip, the DNA must also be bound loosely enough for the RNAP to be able to translocate it. This compromise was made by adjusting the concentration of Zn^{2+} ions in the reaction chamber following the observation that certain divalent cations promote the adhesion of DNA onto mica (Hansma, 1996). Practically this was achieved by imaging the complexes in a zinc buffer intially, then rinsing the zinc away using a buffer suitable for maintaining RNAP activity and then introducing an NTP-containing buffer. The window of opportunity for observing transcription as the zinc concentration decreased and the DNA detached from the mica depended mainly upon the flow rate. Sequential AFM images were acquired to observe the RNAP in action under the various buffer conditions.

The activity of RNAP on mica, which has not been demonstrated previously, was confirmed using a rolling circle template. This is a small circular ssDNA template that is thought to mimic the transcription bubble. In general, it was very difficult to observe the small RNA transcripts produced by the linear templates. However, the rolling circle complexes produce huge transcripts (up to 9000 bases) that were visible using tapping mode in air once the samples were rinsed and dried.

EXPERIMENTAL PROCEDURES

Preparation of the Stalled Ternary Complexes

DNA and RNAP were mixed in a 1:1 molar ratio in a transcription buffer (TSB: 20 mM Tris pH 7.9, 5 mM $MgCl_2$, 50 mM KCl, 1 mM β-mercaptoethanol) and incubated for 10 min at 37 °C to allow the RNAP to associate with the promoter and form an open promoter complex. To make a stalled ternary complex, three appropriate NTPs were added and the reaction incubated at ambient temperature for 5 min.

Reaction Conditions for the 373-Complex. In this case we used a 373 bp DNA fragment with a T7A1 promoter (see Figure 1) (Severinov et al., 1995). DNA and RNAP (0.12 mM, each) were incubated as described above, and then ApU [adenyl (3′−5′) uridine; Sigma, USA] and an NTP mixture (ATP, CTP, GTP; Pharmacia, USA) were added to final concentrations of ~0.5 mM for ApU and 20 μM for each NTP, respectively. This allowed formation of the stalled ternary complex, producing a nascent RNA fragment 20 bases in length. The ApU dinucleotide was necessary for chain initiation, but the RNAP cannot incorporate a dinucleotide into a growing RNA chain (Nudler et al., 1994). After incubation of the reaction mixture at room temperature for 5 min, the complex was prepared for imaging.

Reaction Conditions for the 1047-Complex. A 1047 PCR-derived fragment (containing a λ_{pr} promoter, see Figure 1) was allowed to associate with RNAP (~0.1 mM, each) as discussed above. To form a stalled complex producing an RNA fragment of 70 bases, a 100 μM NTP mixture (ATP, GTP, UTP, Pharmacia, USA) was added at 37 °C and the complex incubated at room temperature.

Reaction Conditions for the Circular 42 Base ssDNA (Rubin et al., 1995). The sequence of the rolling circle was GATTCCTTTCTTCTTTCCTTC repeated twice. 1 mM circular DNA was incubated with 0.4 mM RNAP at 37 °C for 10 min. An NTP mixture (ATP, CTP, GTP, UTP) was added to the reaction at a final concentration of 2.5 μM for each NTP. The reaction mixture was incubated at room temperature, and transcription was allowed to proceed for a few minutes before imaging, to ensure the formation of short transcripts. Note that with the rolling circle, it is not necessary to add just three NTPs to facilitate stalling, the RNAP will act continuously on the DNA template until all NTPs are depleted. Initially, we controlled the amount of NTPs added such that, on average there were about six of each NTP available to each polymerase. In this low concentration, the RNAP cannot make a transcript greater than 10−20 bases long.

Flow-Through System

The flow-through system we used to exchange buffers while imaging has been described previously (Thomson et al., 1996b). It consists of four 20 mL containers suspended above the AFM, connected by Teflon tubes to the fluid cell through a four-position switch. The switch made it possible to select a different buffer without changing the flow rate. The height of the containers was adjusted to keep the fluid levels roughly the same, thus giving a constant liquid flow driven by gravity. Additionally, the flow rate was controlled by a micrometer screw on the tube leading from the switch to the cell. A balance measured the weight of the effluent in order to determine the flow rate. The set-up was calibrated by measuring the weight of the liquid that sits between the fluid cell and the switch. Thus, by reading the weight of the liquid coming out the fluid cell we could determine when a new solution reached the sample.

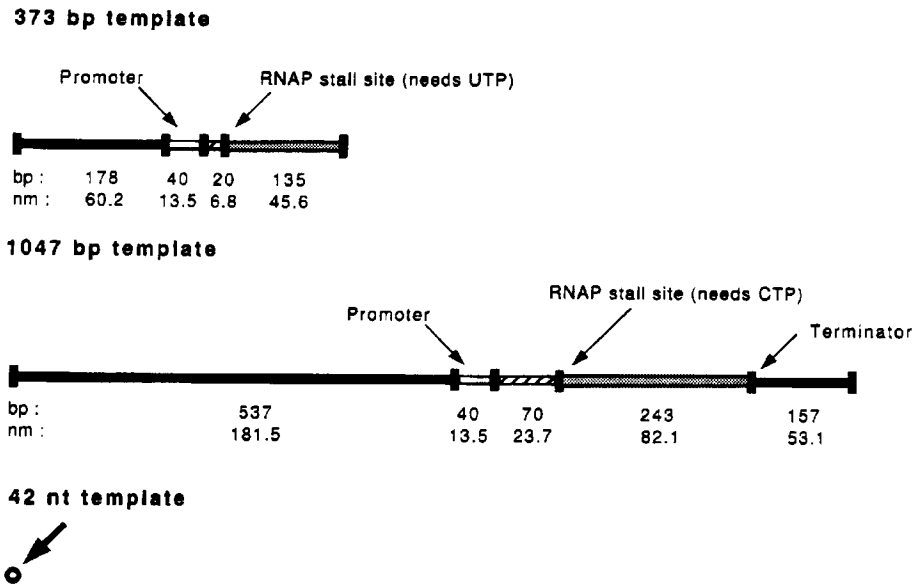

FIGURE 1: Schematic representations of the 373 and 1047 linear dsDNA templates showing positions of the promoters, stall sites, and the terminator. The 42 nt ssDNA circular template is represented at the same scale at the bottom of the figure. The 373 template contained a T7A1 promoter and the transcription start site was positioned approximately in the middle of the template. The 373 dsDNA had no termination sequence and so was expected to be released after completion of the reaction. The stall site occurred 20 bp downstream of the start site. The 1047 bp dsDNA had a λ_{pr} promoter, again positioned close to the middle of the template. The stall site was 70 bp downstream and a terminator sequence was located a further 243 bp downstream. The lengths of the DNA regions in nm are based on B-DNA conformation with a repeat of 0.338 nm/bp.

AFM Sample Preparation and Imaging

For imaging under fluid, the solutions of ternary complexes were diluted 1 to 100 in a low salt buffer containing zinc and 7 μL of the diluted solution was deposited on a freshly cleaved mica surface and introduced into the microscope fluid cell immediately before any drying of the sample could occur. The low salt zinc buffer was composed of 20 mM Tris, 5 mM KCl, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol, and contained between 1 and 2 mM $ZnCl_2$ at pH 7.5. Higher salt buffers discourage adsorption of proteins to mica, presumably through competition for surface binding sites. Once in the microscope the sample was first rinsed with flowing zinc buffer to wash away any loosely bound complexes and to ensure the DNA present was easily visualized. Next, transcription buffer was selected to rinse away the zinc and detach the DNA from the surface. When the DNA showed diffusive motion on the mica the transcription buffer with NTPs was selected to reinitiate transcription. Finally, the zinc buffer was reintroduced to re-attach the DNA strongly to the mica.

Samples for imaging in air were prepared by depositing the RNAP–DNA complex solution on mica, waiting a couple of minutes to allow protein adsorption, and then rinsing with excess deionized water and drying in a stream of compressed air. The experiment with the rolling circle was carried out as the solution experiments above, but after the second introduction of zinc buffer the sample was rinsed in excess water and dried for imaging in air. The buffer used to show the RNA on the mica in air contained 3 mM $ZnCl_2$ rather than 1 or 2 mM.

We used a Nanoscope III AFM (Digital Instruments, Santa Barbara, CA) with a "D" scanner having a maximal lateral scan size of about 13 μm. A Plexiglas tapping-mode fluid cell similar in design to a commercial glass one was used for imaging in liquids. For fluid imaging, we used 100 μm long silicon nitride triangular cantilevers with nominal spring constants of about 0.1 N/m. Electron beam deposition tips were grown in a JEOL JSM-5300 LV SEM on their oriented twin tips (Mark Wendman, Digital Instruments) and had estimated radii of curvature between 5 and 15 nm. For imaging in air, 125 μm long silicon diving board-shaped levers with spring constants of about 30 N/m were used. The AFM was operated at line acquisiton frequencies between 3 and 7 Hz, both in liquids and in air. The cantilever oscillation frequency was tuned to 12–15 kHz for tapping mode in liquids and to 280–320 kHz for tapping in air. Post data acquisition processing was carried out using NIH Image.

RESULTS

Activity of the RNAP in Vitro

Stalled ternary complexes were formed using the method of Levin et al. (1987). The RNAP was incubated with the DNA template in a transcription buffer (TSB) (see Experimental Procedures) that maintained activity of the complexes and inhibited unspecific binding of the RNAP to the DNA. Three NTPs were then added to the RNAP/DNA open promoter complexes in solution to initiate transcription up to a stall site (the position on the DNA where the RNAP pauses because it lacks the fourth NTP to continue). The resulting stalled elongation complex is composed of RNAP, DNA, and a growing RNA transcript. In these experiments we used two different linear double-stranded (ds) DNA templates with different promoters: one of 373 base pairs (bp) and one of 1047 bp (Figure 1). The 1047 bp dsDNA had a termination sequence, while the 373 bp template had no termination sequence.

Tapping-mode AFM imaging in air of the stalled ternary complexes was used to determine if nonspecific binding of the RNAP to DNA occurred. We looked for complexes in which the RNAP was bound to the DNA at locations other than at the RNAP stall site, such as at the end. Only samples where the binding of RNAP was consistent with the expected

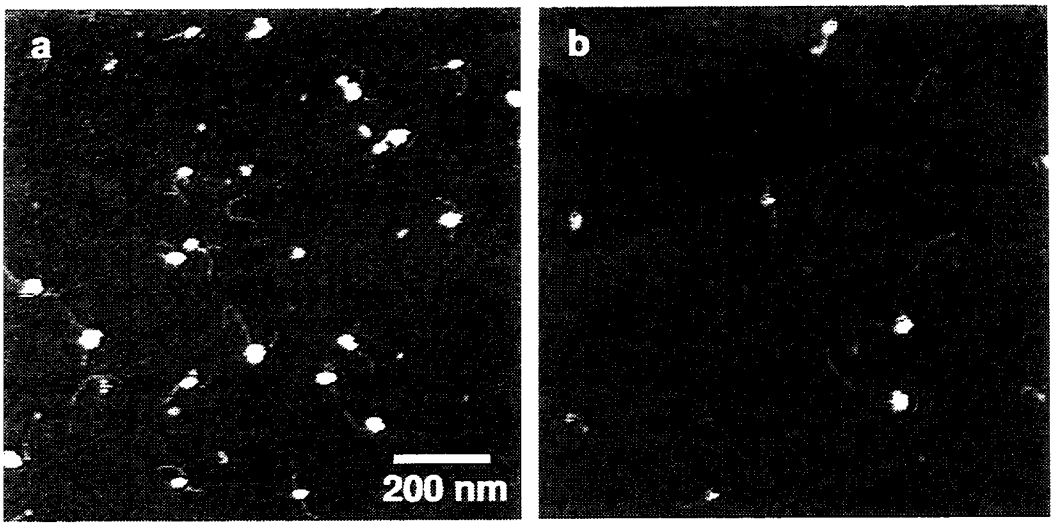

FIGURE 2: Transcription complexes produced in vitro and imaged in air. Stalled ternary complexes (a) before and (b) after the addition of the missing NTP in the test tube. The complex is composed of an RNAP molecule, the 373 bp long dsDNA template and nascent RNA (indistinguishable in these images). In a one can see the RNAP (bright globular feature) located around the middle of the DNA template, consistent with the expected stall site. In b many of the RNAPs have released their templates. One or two are inactive and did not release their DNA. The lower number of proteins and DNA fragments visible in this frame compared to a is probably, on the basis of other tests, due to the lowering of the affinity of RNAP and DNA to mica in the presence of a higher concentration NTPs. We found that the presence of 1 mM NTPs decreases the number of RNAP on the mica surface by more than 50%. It is thought that the binding of NTPs to mica also competes with DNA binding to mica. Subsequent washing of the mica surface with water does not appear to remove the NTPs, since the DNA still does not bind in greater quantities to the mica.

stall site position were used for further experiments. The activity of the complex in solution was tested by looking at samples of the complex with tapping AFM in air before and after the addition of the missing NTP to the test tube (Figure 2). Figure 2a shows a stalled ternary complex obtained with the 373 bp dsDNA before the addition of the fourth NTP. One can distinguish the RNAP (the bright globular features) on the DNA template at a location compatible with the position of the stall site. Figure 2b depicts the complex after transcription was allowed to proceed in the test tube for at least 20 min. Many of the RNAPs present have released their templates and several isolated DNA fragments are visible on the field. This suggested that the stalled ternary complexes were active in solution.

Demonstration of RNAP Activity on Mica Surfaces

Schafer et al. (1991) showed that RNAP is active even when bound to a glass surface. We discovered that in the buffers we used to keep the complexes active DNA did not adhere strongly enough to glass to be imaged by the AFM. Additionally, the glass was not flat enough to visualize the DNA as clearly as we would like. We therefore chose mica as our support for imaging the complexes. Mica can be cleaved to reveal an atomically flat surface and also, the adhesion of DNA to mica can be controlled by adjusting parameters such as the pH and the monovalent and divalent cation concentrations. Since, however, the surface chemistry of glass and mica differ, one cannot be certain that the RNAP will conserve its activity on the mica.

To test RNAP activity on mica, we used ternary complexes with a 42 nucleotide (nt) circular ssDNA template (Rubin et al., 1995). A short circular ssDNA with nonspecific initiation of transcription is thought to mimic the transcription bubble that forms when RNAP melts dsDNA templates; this ssDNA was termed a "rolling circle" (Daubendiek et al., 1995). The rolling circle template has several advantages over a linear one: it is short and circular, which means that it is held very close to the RNAP, and is therefore clearly distinguishable in AFM images from the long pieces of RNA that are produced during transcription. Another advantage is that, under our conditions, transcription continues for tens of minutes, as long as NTPs are available in the solution.

Previously it has been demonstrated that rolling circle complexes produce transcripts between 400 and 9000 nt long (Daubendiek et al., 1995). Therefore, we thought it should be possible to image these large transcripts produced by RNAP adsorbed to mica. A 9000 nt transcript would be 2.7 μm long in the absence of intrastrand base pairing, which is a reasonable assumption for the template sequence (see Experimental Procedures). In contrast, our linear templates produce smaller transcripts: 52.4 nm (155 nt) and 105.8 nm (313 nt) for the 373 and 1047 bp templates, respectively. These would be released from the protein as soon as transcription terminated and could be flushed away by the flowing buffer. We discovered that RNA synthesized on mica is best visualized in the AFM if rinsed with a Zn^{2+} buffer and imaged in air.

The rolling circle complexes on mica were inserted in the AFM fluid cell and flushed continuously with transcription buffer containing 2.5 μM NTPs at a flow rate of 0.5−1 mL/min. Initially, unattached or loosely bound complexes on the surface were flushed away with the flowing transcription buffer, leaving only those which were firmly anchored to the surface. After the NTPs had flowed through for about 20 min, the chamber was flushed with a buffer containing 3 mM Zn^{2+} for several minutes to encourage attachment of the RNA to the surface. The sample was then removed from the microscope, rinsed with excess deionized water, dried with compressed air, and imaged in air using tapping-mode AFM. The presence of long RNA strands were observed on the surface, indicating that these complexes were active on mica (Figure 3). Some RNAPs still have the RNA transcript associated with them (see arrow, Figure 3). These elongated strands have only been observed in air samples

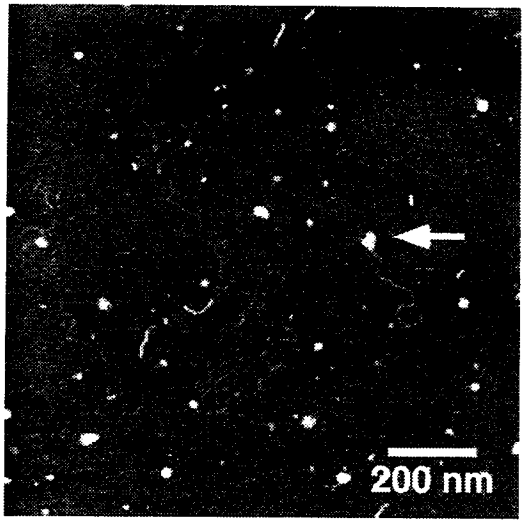

FIGURE 3: Demonstration of the activity of the RNAP on the mica surface. Tapping-mode AFM images taken in air after the mica surface has been exposed to 2.5 μM NTPs, then a 3 mM Zn^{2+} solution, and then rinsed with water and dried. The RNAPs with the rolling circle templates produce huge RNA transcripts (long filamentous structures), many over 1 or 2 microns long, showing that RNAP on mica can maintain activity. The arrow indicates an RNAP molecule that is still attached to its transcript.

because RNA is difficult to image under fluids because it has a low affinity for mica. Again, assuming no intramolecular base pairing, a transcription rate can be estimated by measuring the length of the RNA produced while the NTPs flowed through the chamber. The rates of transcription for the RNAPs estimated in this way from Figure 3 and other images varied between 0.4 and 1.0 base/s. As a control, samples of the initial complex were diluted 1 to 100 and imaged in air. The initial transcripts were so short that they were not visible with the AFM.

Transcription occurring on the mica surface was visualized in our experiments with linear DNA templates by following the movements of the DNA templates relative to the RNAP. However, DNA does not adsorb strongly enough to mica in our transcription buffers to be easily imaged by the AFM. In order for it to be imaged easily, we chose to promote its adhesion to the mica. We achieved this by adjusting the Zn^{2+} concentration in the flowing buffer. It has, however, been reported that this cation can have an inhibitory effect on the RNAP (Niyogi & Feldman, 1981). In a similar experiment with the rolling circles, we found that RNAP on mica was active even after exposure to a zinc buffer, as shown by the following experiment: A 3 mM Zn^{2+} buffer was initially flowed over the sample for about 5 min. Transcription buffer was then flowed for about 5 min before the NTPs were added, to remove excess Zn^{2+}. In this case, we could again observe RNA fragments synthesized by RNAP bound to mica (data not shown). The RNA concentration was lower than that in Figure 3. However, the presence of RNA on the mica demonstrates clearly that this inhibition is reversible, at least for some of the proteins.

Time Lapse Imaging of Transcription

RNAP is a processive enzyme which translocates along natural dsDNA templates during transcription at a maximal speed of between 12 and 19 bases per second in vivo (Kornberg & Baker, 1991). In order to observe this phenomenon using the AFM we prepared stalled ternary complexes with the 373 and 1047 bp dsDNA templates. The resulting stalled complex was then diluted in a low salt zinc buffer and deposited on freshly cleaved mica. In the buffers we used, the RNAP adhered strongly enough to the surface to be consistently and reproducibly imaged. To visualize the DNA during the reaction we adopted a flow-through system driven by gravity (see Experimental Procedures). Buffer was constantly flowing through the fluid cell during the imaging at a rate of 0.2−0.5 mL/min. This system allowed the exchange of buffers without disturbing imaging by switching a valve on the input tubing of the fluid cell from one buffer container to another. By varying the Zn^{2+} concentration "on line" using the flow-through system we could adjust the concentration of the divalent cation in such a way that the DNA was bound loosely enough to be translocated by the RNAP but still with sufficient strength to be imaged by the AFM. Practically, this was achieved by depositing the ternary complexes in a Zn^{2+} containing buffer and imaging under this buffer. Then the transcription buffer, without Zn^{2+} was selected to flow over the sample until the DNA appeared to have some mobility on the surface. Then transcription buffer containing between 0.5 and 5 μM NTPs and no Zn^{2+} was selected. This solution contained all four NTPs needed to complete transcription. After the NTPs entered the fluid cell, we observed dramatic changes occurring in the images. Finally, a zinc buffer was introduced to encourage adhesion of the DNA to the mica for clear visualization in the AFM.

Figure 4 shows six images selected from a "movie" (sequential images) taken with the 373 bp complex. As one can see, in the first image, there are five RNAPs, four of which are associated with DNA (labeled a−d). After addition of the NTPs, in the final image, it can be seen that three of the RNAPs (b−d) have released their DNA. Protein a does not appear to move relative to its DNA template and is assumed to be inactive. Proteins c and d both dissociate from their DNA templates but there is no clear indication that transcription has occurred. Protein b appears to transcribe its template: the lower arm of the template lengthens between the image at time 00:00 (minutes:seconds) when the 5 μm NTPs arrive and the image at time 01:13 (the second image after the NTPs arrive). The image acquistion time was 37 s. In the image at time 06:43, the arm has lengthened still further and the template appears to be in the process of being released. Finally, it is released and the zinc sticks the DNA down around the RNAP in the last image. From these images, it was estimated that this RNAP transcribed its DNA with a rate of approximately 0.8 bases/s, by measuring the extension in length of the upstream arm. It should be emphasized that the temporal resolution of the AFM is still relatively poor at present, since an image takes between 30 s and a min to acquire. It is possible that some of the proteins observed releasing their DNA were in fact transcribing it, but finished the template within the image acquisition time. It also appears that on some proteins transcription occurred, but since the DNA was not clearly imaged during transcription, we could not follow the process (e.g., Figure 5). One can easily imagine that this DNA was pulled through the RNAP since the DNA has moved relative to the RNAP and was subsequently released. These images were taken about 6 min apart, but none of the five intervening

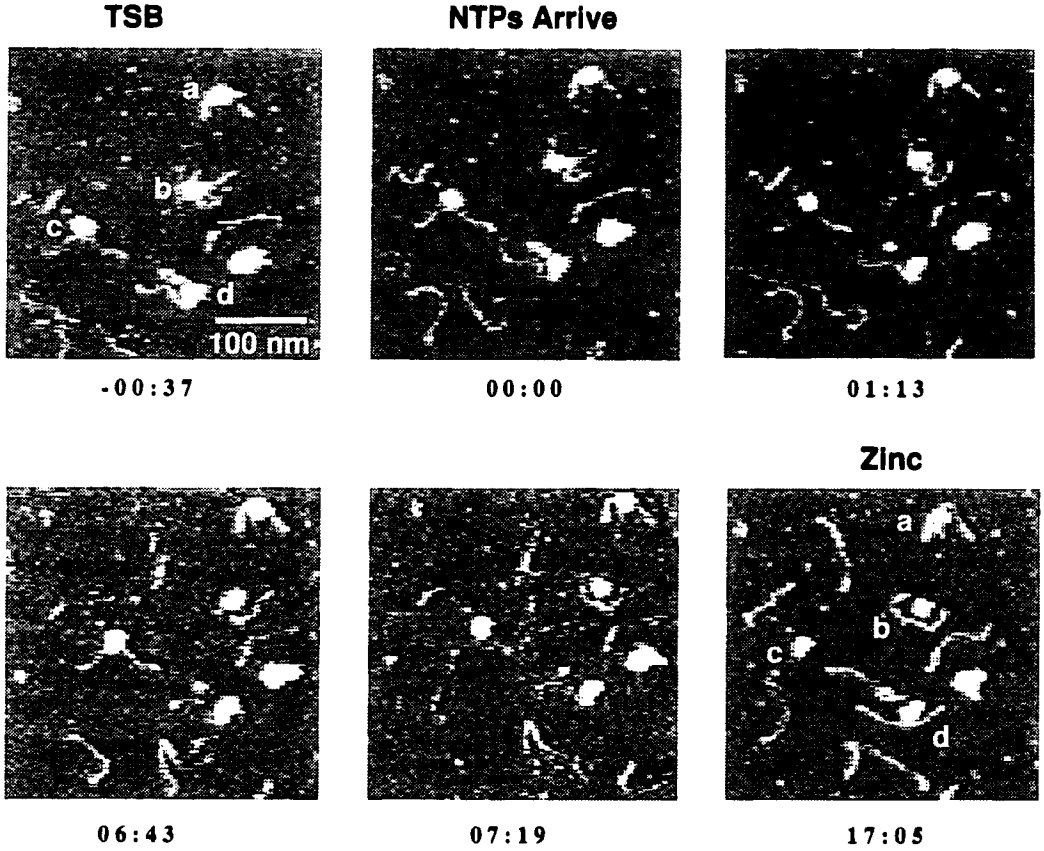

FIGURE 4: Selected time-lapse images of a field of five RNAP molecules, four of which are associated with a 373 bp dsDNA template. When a buffer containing 5 μM NTPs enters the microscope fluid cell dramatic changes are observed. The RNAP labeled "a" appeared inactive, "b" appeared to transcribe its template and then release it, "c" released its DNA template, and "d" may have released its template. For the RNAP marked b there are intermediate images showing the DNA being pulled through the RNAP during transcription. Addition of a zinc buffer at the end of the experiment sticks the DNA down firmly to the mica for clearer imaging.

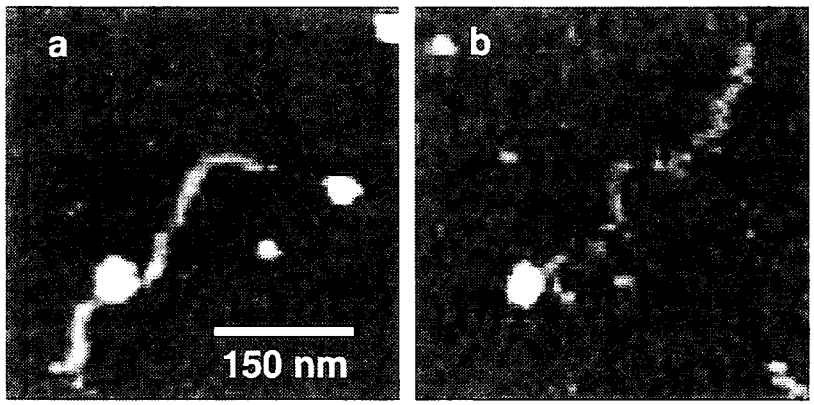

FIGURE 5: Complex of RNAP with the 1047 bp dsDNA before (a) and after (b) the addition of 0.5 μM NTPs. The DNA appears to have been pulled through the RNAP and may well have been transcribed. However, these two images were acquired about 6 min apart but none of the intervening frames showed intermediate stages in transcription because the DNA was too mobile to be imaged clearly.

images showed intermediate stages in transcription because the DNA became too mobile to be imaged clearly.

With the longer 1047 template it was also possible to obtain a sequence of images with intermediate stages in the transcription. Figure 6 shows a sequence, with an image acquisition time of 39 s, of a 1047 template being pulled through the enzyme. One can see by comparing the images at −3:17 and −1:18 (i.e., before the NTPs arrived) that the DNA is mobile enough to move on the mica. As the NTPs flowed into the fluid cell (image 0:00), the arm on the left becomes progressively shorter until eventually the DNA is released in the image at 2:38. After release, a 2 mM Zn^{2+} buffer solution was added for better imaging of the DNA. In this case the RNAP obviously read through the terminator. The rate of transcription was estimated from the decrease in length of the downstream DNA arm to be 1.9 bases/s.

In general, RNA is more difficult to image than DNA because it base pairs with itself to form compact structures that are hard to distinguish from small particles of debris. In contrast, we were able to image the RNA produced in the rolling circle experiments since the sequence of the DNA template was designed so that the RNA would not have much base pairing (the template was 86% C and T) and the RNA could be microns long.

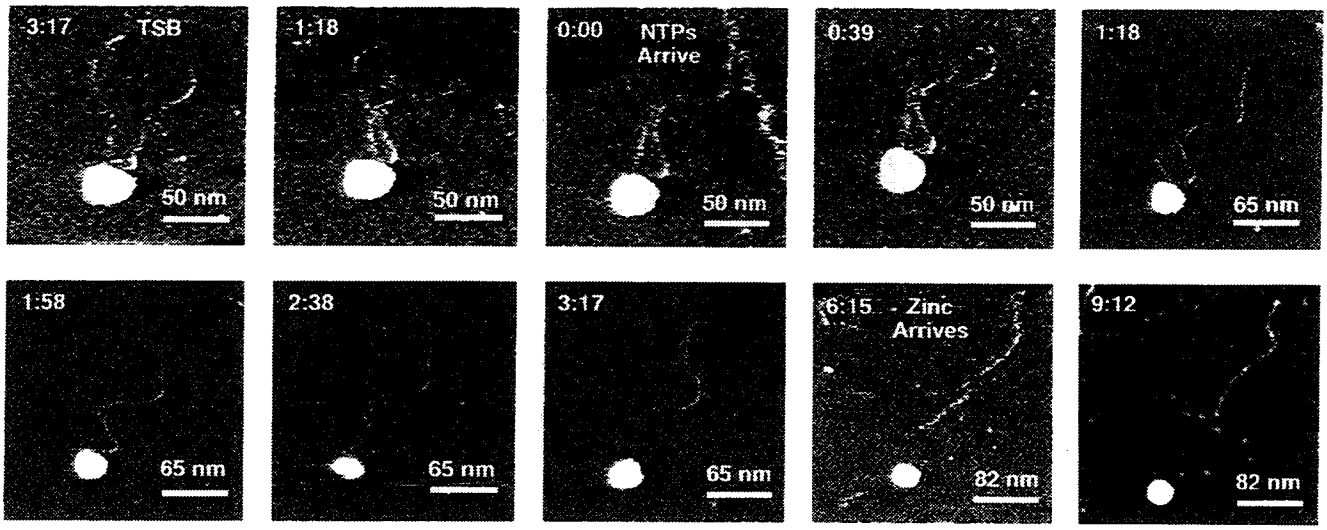

FIGURE 6: Time-lapse series of images showing transcription of the 1047 dsDNA template by an RNAP molecule. The first two images show that before the NTPs enter the DNA has mobility on the mica. The six images after NTP addition, from time 0:00 onwards are sequential and show that one arm of the DNA template becomes progressively shorter until it is released (02:38). The 1047 bp template has a terminating sequence, but it appears that the RNAP read through the terminator to the end of the DNA.

DISCUSSION

In these experiments we have demonstrated the activity of RNAP on mica both by observing RNA transcripts after the reaction occurred and observing the translocation of dsDNA by RNAP on addition of NTPs. It should be noted that the translocation of DNA by RNAP under our conditions was unidirectional and only observed on addition of NTPs. Activity of RNAP on mica was confirmed by incorporation of radioactive NTPs and subsequent analysis by gel electrophoresis (Guthold et al., unpublished results).

Active RNAPs on the mica surface synthesized RNA molecules of different lengths from the rolling circle DNA template. There were long RNA molecules associated with RNAP that could not be measured because of aggregation as well as shorter RNA molecules not attached to RNAP. These results suggest that there is a large variation in both the transcription rate and the extent of transcription for different RNAP molecules. The extent of transcription is known to be highly variable for rolling circle complexes (Daubendiek et al., 1995) and complexes with dsDNA templates in the test tube. It is apparent from our studies that there is a decrease in activity and transcription rates on mica. This is probably a consequence of the surface interactions between the RNAP and the mica. It is possible that the different orientations of the RNAP have an additional detrimental effect on the activity and transcription rate. The influence of a surface on the activity and transcription rate could be investigated by controlling the orientation of RNAPs.

A major problem one encounters when imaging transcription is that if one wants to estimate percentage activity of the RNAP one must image a field of many complexes. This type of experiment is shown in Figure 4 (which is actually a software zoom of a field of view four times as large, therefore the pixels are noticeable). However, in order see the DNA clearly one must image only one complex (such as that shown in Figure 6). The image acquisition time is limited by the speed of the tip across the surface (Butt et al., 1993). This means that one can acquire scans faster if a smaller area is imaged. Since the RNAP only takes a few minutes to transcribe, one must compromise on scan rate versus scan area, depending on whether one is interested in observing the percentage activity of the RNAP or obtaining the best sequence of intermediates during transcription. For these reasons it is difficult to assess exact percentage activities of RNAP using AFM, but our observations suggest that it could be as low as 10% or 20% on mica.

The observation of the transcription of DNA on mica surfaces opens a new field of applications of the AFM in the study of complex biochemical processes. In this study we obtained a few intermediate images of the transcription process, where the DNA templates appeared to be pulled through and released by the RNAP. We also noticed that the transcription rate of the RNAPs on mica was relatively slow, in the range of 0.5−2 bases/s. It is not surprising that the rate is slow since the RNAP is bound to a surface, had been exposed to Zn^{2+}, and the NTP concentration was low: typically 5 μM. Pausing is also evident in these experiments. For example there was an apparent pause of several minutes in the experiment shown in Figure 6 starting at ca. 1 min. Sequence dependent pauses in transcription have been shown by Levin and Chamberlin (1987) and Nudler et al. (1994).

If the DNA adheres too strongly to the mica it may increase the incidence of pausing. Using optical tweezers Yin et al. (1995) have shown that RNAP can exert a force of up to 14 pN on the DNA. In light of this result, it is perhaps suprising that the transcription process can be observed by AFM because adhesion forces in contact AFM between tip and sample often exceed 14 pN for biological samples in fluid (Radmacher et al., 1995) although less is known about imaging forces with tapping-mode AFM. However, motion of the arms of the DNA or free DNA across the mica surface was always observed prior to transcription occurring: if the DNA is free enough to move by itself, it is reasonable to suppose that the RNAP can pull it through for transcription. In the sequences showing transcription, the DNA sometimes follows the same contour across the mica as it is pulled through the RNAP. This resembles the tube-like motion of fluorescently labeled DNA pulled through an entangled solution of DNA (Perkins et al., 1994).

The use of electron beam deposited (EBD) tips may be necessary for obtaining movies of transcription. Previously, it has been noted that EBD tips gave substantial improvement in the imaging of DNA under aqueous buffers because tip-induced damage and motion of DNA were reduced (Hansma et al., 1993). It is not clear yet whether the gentle imaging arises from the surface chemistry of EBD tips or is a consequence of lower adhesion between the DNA and the tips due to their sharpness.

In this study, we have demonstrated the ability of the AFM to monitor complex biochemical processes at molecular resolution. Improvements in AFM scanning speeds should eventually enable such processes to be monitored with much higher temporal resolution giving insights into biological mechanisms and kinetics.

ACKNOWLEDGMENT

We thank Digital Instruments for AFM support and David Vie for growing the EBD tips.

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BI9624402