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Sample Preparation Method for Observing RNA Polymerase Activity by Atomic Force Microscopy

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1. Introduction

Transcription is a fundamental biochemical process in which an RNA molecule is synthesized according to its corresponding DNA template.

Transcription begins when an RNA polymerase (RNAP) molecule binds to a specific region of the DNA referred as the promoter. In the next step, the two strands of the DNA are separated locally to form an open promoter complex, which permits the beginning of the synthesis of the complementary RNA chain. The kinetics of this reaction have been studied in different biochemical experiments ***(1)***, which can only give population-averaged properties. Single RNAP transcription can be observed by optical microscopy through the motion of a bead tethered to the end of the DNA template, but this approach has a limited resolution ***(2)***.

Recent developments in atomic force microscopy (AFM; **refs. *3*** and ***4***) now give one the opportunity to study transcription in nearly physiological conditions with nanometer scale resolution **(Fig. 1; *5*,*6*)**.

The first step of this type of experiment consists in the formation of a stalled ternary complex ***(7)***. In a test tube, RNAP and three of the four nucleoside triphosphates (NTPs) are mixed with a specially designed DNA molecule. The protein attaches to the DNA at the promoter region to form an open promoter complex. After this, transcription starts and goes on until it stalls at a specific base along the DNA (the stall site). The DNA is specially designed to be transcribed from the promoter to the stall site by using only three different NTPs (*see* **Fig. 2**). To go further a fourth NTP is required that is not present in the test tube. These reactions allow a "sample" to be made in the test tube, the stalled ternary complex, which consists of an RNAP molecule, with a short RNA molecule already synthesized, attached to the DNA.

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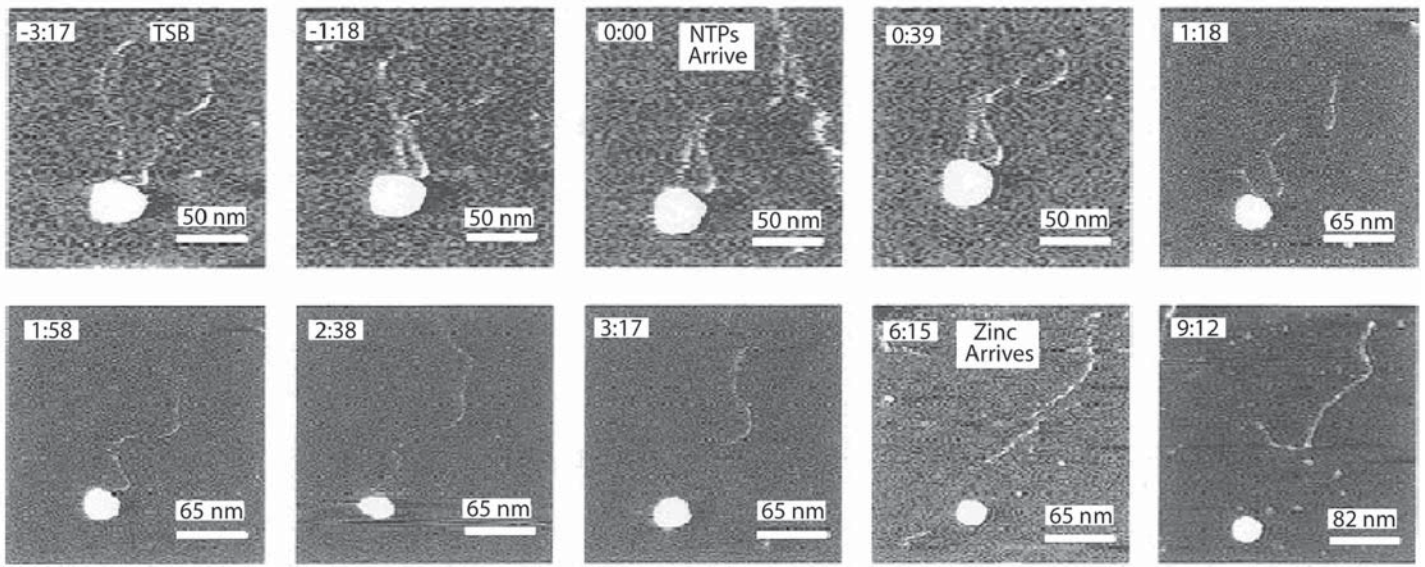

Fig. 1. Time-lapse series of images showing transcription of a 1047-bp DNA template by an RNAP molecule. On the first two images the sample is immersed in the transcription buffer (TSB). It permits a certain mobility to the DNA on the mica surface. After NTP addition (third image at time 0:00) one arm of the DNA template becomes progressively shorter until the DNA is released (2:38). The addition of zinc in the AFM chamber blocks the DNA to the mica surface and permits a better imaging. Reprinted with permission from **Ref. *5***. Copyright 1997 American Chemical Society.

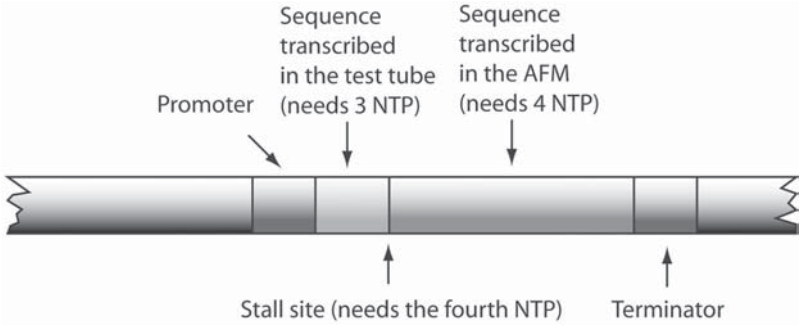

Fig 2. Schematic representation of the DNA template showing the positions of the promoter, stall site, and the terminator.

The stalled ternary complex is then deposited onto a mica surface and introduced into the AFM observation chamber. Unfortunately, the AFM tip interacts relatively strongly with the sample it is imaging. This interaction requires a relatively strong anchoring of the specimen to the support (mica in our case). Because transcription is a dynamical process, we are facing a paradox; the sample has to be fixed strongly enough to the support to permit AFM imaging and at the same time, it has to be free enough to permit its movements to occur. In the case of the transcription, this problem particularly concerns the binding of DNA to surfaces. A DNA molecule has to be attached strongly enough to the mica to permit its imaging and it has to be free enough to allow RNAP to translocate it.

This problem can partially be solved by modulating DNA adhesion to mica by the use of two different buffers: an "imaging" and a "transcription" buffer. The imaging buffer promotes DNA adhesion whereas the transcription buffer releases DNA from the surface and permits to the RNAP to pull on the DNA and to transcribe it. These buffers differ in their zinc concentrations: this ion has been demonstrated to promote DNA adhesion to the mica ***(8,9)***.

The RNAP molecule is not concerned by the adhesion/activity problem; it sticks to the mica in both transcription and imaging buffers and adhesion to mica does not inactive all the RNAPs bound to it.

An additional problem in imaging dynamical processes by AFM is the maximal scanning frequency of the microscope. The majority of biochemical reactions occur at speeds that are for the moment inaccessible to AFMs (RNAP translocates along DNA at a maximal speed between 12 and 19 bases per second; **ref. *10***). This problem can be solved by slowing the natural transcription speed. The easiest way to achieve this is by reducing the quantity of NTPs available to the RNAP.

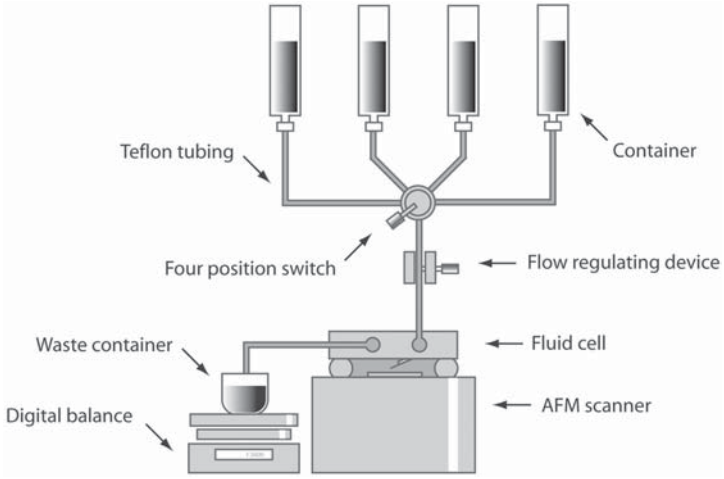

Fig. 3. Schematic diagram depicting the flow-through system. The four position switch permits to select one of the four different solutions to flow through the AFM fluid cell. The digital balance permits to determine when a new solution comes in contact with the sample.

To switch from the imaging to the transcription buffer or vice versa , one needs an exchange device that does not perturb AFM imaging. The best results are obtained by a gravity-driven flow-through system consisting of four containers suspended above the AFM and that are connected to the fluid cell through a four-position switch. A buffer is continuously flowing through the fluid cell during the experiment and the switch allows the buffer to be changed without changing the flow-rate (i.e., with a minimum perturbation). The flow rate can be controlled by a micrometer screw on the tube leading from the switch to the fluid cell. (*See* **Fig. 3**.)

A balance is used to measure the effluent to determine the flow rate. By calibrating the weight of the liquid between the switch and the fluid cell and by knowing the flow rate, one can determine when a new solution reaches the sample.

Before starting the experiment, it is advisable to check if the RNAP has conserved its activity and if it stalls at the right site when fueled by three NTPs. This test is accomplished by running the reaction in a test tube once with three and once with four NTPs. Both samples are eventually deposited onto a mica surface and imaged in air with the AFM. In the first case, one should see isolated RNAP and DNA molecules. In the second case, the RNAP molecule should be bound to the DNA at the stall site.

2. Materials

2.1. AFM (Nanoscope III)

1. Tapping mode in liquids option.
2. Tapping mode in air option.
3. Scanner with a maximal lateral scan size of about 13 μm.
4. 100-μm Long silicon nitride triangular cantilevers with a spring. constant of about 0.1 N/m. Sharpen tip with a radius of curvature of about 5–15 nm for tapping in liquids.
5. Silicon cantilevers with a nominal spring constant of 30 N/m for tapping in air.
6. Fluid cell.
7. O ring.

2.2. The Flow-Through System

1. Four containers of about 20 mL.
2. Four-arm container holder.
3. About 10 m of Teflon tubing.
4. A four-position switch.
5. A flow-regulating device (micrometer screw squeezing the tube leading from the switch to the fluid cell).
6. Digital balance.
7. A 200-mL waste container.

2.3. Buffers and Solutions

1. Transcription buffer: 20 m*M* Tris, pH 7.9, 5 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* β-mercaptoethanol.
2. Transcription buffer with NTPs: 20 m*M* Tris, pH 7.9, 5 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* β-mercaptoethanol, NTP mixture (ATP, CTP, GTP, UTP); 2.5 μ*M* for each NTPs.
3. Imaging buffer 20 m*M* Tris, 5 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* β-mercaptoethanol, 1.5 m*M* $ZnCl_2$, pH 7.5.
4. Stalled ternary complex NTP solution: 100 μ*M* NTP mixture containing 3 NTP in the transcription buffer.
5. Stalled ternary complex DNA–RNAP solution: 100 μ*M* specially designed DNA (*see* **Notes 1–3; Fig. 2**); and 100 μ*M* RNAP.

3. Method

3.1. Observation of the Stalled Ternary Complexes in Air

1. Mix DNA and RNAP in a 1:1 molecular ratio in transcription buffer and incubate it for 10 min at 37°C to allow the RNAP to associate with the promoter.
2. Add the three appropriate NTPs to the solution and incubate for 5 min at room temperature to make the stalled ternary complex.
3. Deposit 5–10 μL of the solution onto freshly cleaved mica.
4. Wait a couple of minutes.

5. Rinse in excess deionized water and dry in a stream of compressed air.
6. Observe the sample using tapping-mode AFM in air.

3.2. Preparation of the Sample for AFM Imaging in Liquids

1. Prepare the flow-through system with the following buffers: transcription buffer with no NTPs, transcription buffer with the four NTPs, and imaging buffer.
2. Prepare the AFM for tapping mode imaging in liquids (*see* **Note 4**).
3. Mix DNA and RNAP in a 1:1 molecular ratio in transcription buffer and incubate it for 10 min at 37°C to allow to the RNAP to associate with the promoter.
4. Add the three appropriate NTPs to the solution and incubate for 5 min at room temperature to make the stalled ternary complex.
5. Dilute the solution 1:10 in the imaging buffer.
6. Deposit 5–10 µL of the solution onto freshly cleaved mica and introduce the sample into the AFM before any drying occurs.
7. Connect the fluid cell to the flow-through tubing and rinse the sample with the imaging buffer to wash away any loosely bound complexes and to ensure that the DNA is easily visualized.
8. Image the sample and search a field containing several RNAP molecules attached to their DNA.
9. Switch the transcription buffer with no NTPs on and let it flow through the fluid cell until the DNA shows diffusive motion on the mica.
10. Switch the transcription buffer with NTP on to reinitiate transcription and capture every frame for 5–10 min (*see* **Notes 5** and **6**).
11. Switch to the imaging buffer to re-attach DNA to the mica and capture several frames.
12. Stop the experiment and analyze the images by searching the RNAPs, which translocated their DNA. The individual images recorded by the AFM can be assembled in a movie using NIH image or Adobe Premiere (*see* **Note 7**).

4. Notes

1. The DNA should have no more than 2000–3000 base pairs. Longer molecules makes it difficult to follow the progression of the transcription.
2. The stall site should be located towards the middle of the DNA, but asymmetrically to give two different DNA arm lengths to observe directionality of movement of the RNAP in the AFM movie.
3. Among other companies Microsynth, MWG Biotech, and Eurogentech can manufacture the DNA required for this experiment.
4. The cantilever oscillation frequency for tapping mode imaging in liquids should be about 10 kHz for the cantilevers suggested above.
5. By selecting the movie option in the AFM capture menu, the microscope automatically records every frame, allowing the experimenter to concentrate to other tasks.
6. A compromise has to be found between the temporal and the spatial resolution of the AFM images. Scanning at low speed, large areas allow several RNAP–DNA complexes to be followed but with a limited temporal resolution. Inversely scan-

ning at high speed a small area diminishes the chances to capture one of the 20% active RNAP molecules. A scanning speed of 5 to 10 Hz with a resolution of 256 × 256 over a 500-nm large scan size is a good value with which to begin.

7. Because of the thermal drift, individual images taken by the AFM will be shifted. Before making an animation, the frames have to be aligned first. This can be achieved with the freeware image processing software NIH image.

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