Applications for Atomic Force Microscopy of DNA

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ABSTRACT Tapping mode atomic force microscopy (AFM) of DNA in propanol, dry helium, and aqueous buffer each have specific applications. Resolution is best in propanol, which precipitates and immobilizes the DNA and provides a fluid imaging environment where adhesive forces are minimized. Resolution on exceptional images of DNA appears to be ~2 nm, sufficient to see helix turns in detail, but the smallest substructures typically seen on DNA in propanol are ~6–10 nm in size. Tapping AFM in dry helium provides a convenient way of imaging such things as conformations of DNA molecules and positions of proteins on DNA. Images of single-stranded DNA and RecA-DNA complexes are presented. In aqueous buffer DNA molecules as small as 300 bp have been imaged even when in motion. Images are presented of the changes in shape and position of circular plasmid DNA molecules.

INTRODUCTION

Atomic force microscopy (AFM) of DNA has changed since the early images of Albrecht Weisenhorn (1961–1993). In the hope of sequencing DNA with the AFM, we started by imaging single-stranded DNAs only 20 bases long, which were not only very small but also very easily pushed by the AFM tip with the methods of sample preparation and imaging available at that time (Weisenhorn et al., 1990). Two advances created a major breakthrough: 1) imaging plasmid DNAs, which have characteristic circular shapes of known lengths, and 2) imaging in dry air, which helps to keep the DNA well bound to the substrate (Bustamante et al., 1992; Vesenka et al., 1992). Another advance, imaging in propanol (Hansma et al., 1992), was actually foreshadowed by Weisenhorn's work, since he had imaged DNA in ethanol (Hansma et al., 1991) and had shown that adhesive forces were smaller in ethanol than in water (Weisenhorn et al., 1992). Stable imaging of DNA in water (Lyubchenko et al., 1993) and aqueous buffers (Bezanilla et al., 1994a, 1994b; Hansma et al., 1993a) was hard to achieve, because DNA is soluble in these media, although Weisenhorn had also imaged small DNAs in water (Hansma et al., 1991; Weisenhorn et al., 1990, 1991). With these new methodological developments, the AFM is now capable of routinely imaging DNA curvature and DNA-protein complexes (Hansma and Hoh, 1994). Features as small as helix turns can sometimes be resolved, and DNA can even be imaged in motion in aqueous buffer (Bezanilla et al., 1994b).

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MATERIALS AND METHODS

DNA in propanol

The 2-kb bands from λ *Hin*dIII DNA were isolated from an agarose electrophoresis gel by Prep-a-Gene (Bio-Rad Laboratories, Richmond, CA) and were applied to freshly split mica and rinsed with a WaterPik (Teledyne Corp., Fort Collins, CO), as described previously (Bezanilla et al., 1994a). The sample was imaged with tapping AFM (Hansma et al., 1993b; Zhong et al., 1993), first in an aqueous buffer (data not shown), then rinsed in the AFM with water and imaged in 1-propanol (Fig. 1) at a scan frequency of 4.5 Hz and a tapping frequency of 17 kHz.

Single-stranded DNA and RecA-DNA complexes

Single-stranded φX-174 DNA, 3 ng in 0.3 μl HEPES-$MgCl_2$ buffer, was applied to freshly split mica, rinsed with water, followed by 1% uranyl acetate in water, again rinsed with water, and dried with compressed air. Complexes of RecA with single-stranded φX-174 DNA and φX-174 *Hae*III-digest double-stranded DNA were prepared (Menetski et al., 1990). One-ml aliquots were applied to mica, rinsed with water, dried, and imaged with tapping AFM in dry helium (Hansma et al., 1993b).

DNA in motion in aqueous buffer

The mica surface was prepared for imaging by treatment with $NiCl_2$. This was accomplished by placing 5 μl of 1 mM $NiCl_2$ on the mica for 1 min, then gently rinsing with 1 ml of MilliQ water. Finally, the mica was gently blown dry with compressed air and placed in a Petri dish to minimize surface contamination. The time between treatment and imaging was never more than 20 min.

The Ni-treated mica disk was placed in the AFM and a dry O-ring was positioned on it. A small amount of 5 mM HEPES, 5 mM KCl, 2 mM $MgCl_2$ buffer was rinsed through the cantilever holder, and a small drop was allowed to hang from beneath it. The cantilever holder was carefully placed in the AFM and pressed onto the O-ring. When this was done correctly, there was minimal leakage and virtually no air pockets in the fluid cell. Since no DNA was present at this point, it was possible to image the Ni mica surface and verify that satisfactory imaging conditions had been obtained.

Bluescript plasmid DNA in the same buffer was injected into the fluid cell at concentrations of 0.5 and 1 ng/μl. The amount of DNA solution injected can be varied greatly, although volumes of 50–100 μl are generally adequate.

All imaging was done with a 100 μm narrow cantilever which was cleaned by placing it under UV light for 15–30 min before use in the AFM. A tapping fluid cell was used for all imaging.

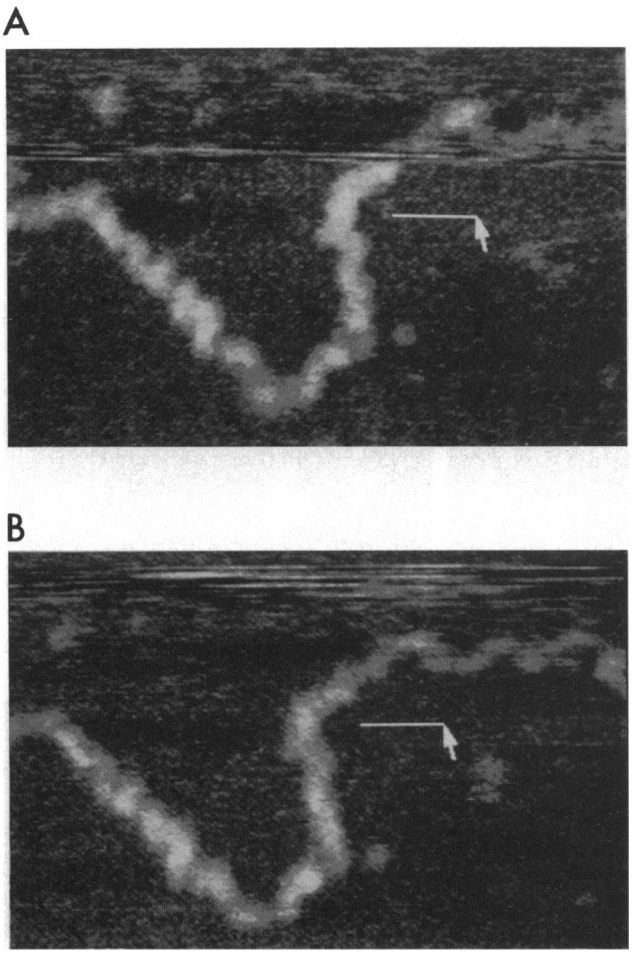

FIGURE 1 DNA in propanol showing coiling of a size comparable to the turns of the double helix. *A* and *B* are 1 min apart and are the first two in a series of five images. Bar is 10 nm.

AFM

Samples were imaged with a Nanoscope III with Multimode AFM (Digital Instruments, Santa Barbara, CA). The AFM head was leveled with the aid of a 16-piece Feeler Gauge Assortment (No. 8568A11, McMaster Carr Supply Co., Los Angeles, CA). Vibration isolation, especially the attenuation of vibrations in the frequency range 10–100 Hz, is essential for optimal AFM operation. This was accomplished by suspending the AFM from bungee cords. The AFM and the optical microscope were placed on a custom-made concrete block, 2 ft. × 2 ft. × 4 in., which was suspended with eight strands of ¼-inch diameter bungee cord (McMaster Carr Supply Co.) to each corner of the block. The tops of all the bungee cords were fastened to a loop that was raised and lowered with an electric winch that was bolted to an I-beam installed near the ceiling of the room. It is important to have the bungee cords stretched at least ½ m and preferably 1 m when the block is suspended. It is also important to be sure that the bungee cords are still elastic and not stretched to the point that the nylon covering is carrying much of the load. A correctly suspended block will have a resonant frequency <1 Hz in all directions.

RESULTS AND DISCUSSION

Imaging substructure the size of helix turns

The best resolution obtained on double-stranded DNA (dsDNA; see Fig. 1) is now considerably better than the best resolution reported previously for dsDNA (Hansma and Hansma, 1993). As before, the best images show features with a spacing similar to the helix turns of B-DNA. Now, however, the features are not simply lumps but appear as a well defined right-handed helix with deformations where the DNA strand bends. The DNA is unusually narrow: ~2 nm at the narrower regions. Imaging was fairly non-destructive, even with a scan size of only 86 nm, as can be seen from the comparison of parts *A* and *B* of Fig. 1.

As before, this helix turn resolution was obtained in alcohol: 1-propanol as shown in Fig. 1 and 1-butanol as shown previously (Hansma and Hansma, 1993). Propanol and butanol dehydrate DNA and also precipitate DNA onto the mica surface. This probably helps to immobilize the DNA, which improves the resolution. Although DNA in alcohols has an A-DNA structure, DNA that is bound to mica in an aqueous solution appears to have the slightly larger spacing of base pairs seen in B-DNA. The evidence for this comes from measurements of circular plasmids in propanol, which have the length expected for B-DNA (Hansma et al., 1993a). Thus the images of Fig. 1 show a periodicity similar to that of B-DNA, since the DNA was dried onto mica from an aqueous solution.

Much of the improved resolution seen in Fig. 1 can be attributed to tapping AFM in fluid, which has been developed recently (P. K. Hansma et al., 1994). Tapping AFM in fluid is less destructive (Bezanilla et al., 1994b) and more stable than contact AFM in fluid. Drift in imaging force was a serious problem with the previous images, which were taken with contact AFM (Hansma and Hansma, 1993). With tapping AFM, the imaging forces are controlled by the feedback loop that keeps the tapping amplitude constant, and drift to high or low force is rarely a problem. In addition to the experiment shown in Fig. 1, tapping AFM in propanol has also shown features with the spacing of helix turns on other DNA samples but with less detail.

Tips

Tip development is clearly important for reliable imaging of small substructures. The tip used for Fig. 1 imaged for only a few minutes at this level of resolution. The images immediately preceding showed evidence of a double tip, and the DNA appeared twice as wide in the images following this series. For routine imaging of small structures, one needs not only a very sharp tip but also a tip that will stay sharp and clean for the duration of the experiment.

The tip chemistry can also affect AFM imaging. For example, with contact AFM in aqueous buffer, non-destructive imaging of DNA was possible with an electron beam-deposited tip but not with an unmodified silicon nitride tip (Bezanilla et al., 1994a; Hansma et al., 1993a). In AFM of organic monolayers, hydrophobic tips adhered less to a hydrophilic surface than hydrophilic tips did (Frisbie et al., 1994). The tip used for Fig. 1 was an unmodified silicon nitride tip, which is hydrophilic (Hoh et al., 1991). The contact-AFM images showing substructure the size of helix

turns (Hansma and Hansma, 1993) were made with a tip coated with a self-assembled layer of octadecyltrichlorosilane (OTS) (Schwartz et al., 1987), which is hydrophobic. In the past, OTS tips were seldom used because the drift in imaging force was unusually rapid. Since drift in imaging force is rarely a problem in tapping AFM, OTS tips or other hydrophobic tips might be better than unmodified silicon nitride tips for imaging structures as small as helix turns on DNA with tapping AFM.

The ultimate goal, to sequence DNA with the AFM, requires a large increase in resolution, and for this resolution to be obtainable routinely. In addition, sequencing would probably require well-extended single-stranded DNA (ssDNA), which is not a natural state for ssDNA, as can be seen from Fig. 2 *A*.

Single-stranded DNA and RecA-DNA complexes

The AFM can routinely image bare, uncoated double-stranded DNA (dsDNA) (Bezanilla et al., 1995) and DNA-protein complexes (Hansma and Hoh, 1994). For this purpose, tapping AFM in dry helium is preferable. Fig. 2 and the following paragraphs describe the use of tapping AFM in dry helium for characterizing single-stranded DNA (ssDNA) and RecA-DNA complexes.

Molecules of native single-stranded ϕX-174 DNA, in the absence of a denaturing agent, have compact structures (Fig. 2 *A*) held together by short regions of intramolecular base pairing. The diameters of the ϕX-174 DNA molecules in this image are 90–120 nm. These circular molecules are 5386 bases long, which corresponds to a length of 1700 nm if extended at the base spacing of double-stranded B-DNA. Deflection-mode AFM imaging (Fig. 2 *A*, *inset*,) shows in more detail the structure of the top surface of the molecule, whereas height-mode imaging gives height information about the molecules.

AFM of RecA-DNA complexes can be seen in Fig. 2 *B*. RecA protein binds to ssDNA at a ratio of one 38-kDa RecA monomer per four nucleotides and, in the process, extends the ssDNA. The resulting RecA-ssDNA filament binds to dsDNA, forming a RecA-coated filament, ~10 nm in diameter, with both the ssDNA and dsDNA in the center. SsDNA in this complex slides along the dsDNA in search of the sequence complementary to the ssDNA, partially unwinding the dsDNA in the process. At the complementary sequence, the strand exchange reaction occurs, in which the ssDNA displaces its like sequence in the dsDNA. In the presence of ATP, strand exchange is followed by ATP hydrolysis and dissociation of RecA from the DNA (West, 1992). In this system (Fig. 2 *B*), ATP has been replaced with a non-hydrolyzable analog, ATP[γS], which traps the RecA on the DNA (Menetski et al., 1990). The dsDNA in the experiment of Fig. 2 *B* is ϕX-174 *Hae*III digest, containing 11 DNA fragments ranging in size from 72 to 1358 base pairs. The ssDNA is circular ϕX-174, as described above. On the right side of Fig. 2 *B*, parts of two extended circles of ϕX-174 can be seen, fused over a distance of ~350 nm, comparable in length to the 1078-bp fragment of ϕX-174 *Hae*III DNA. These fused circles might simply be circles in such close physical proximity to one another that the AFM tip cannot distinguish between them or they might result from a strand exchange reaction between the double-stranded fragment and two circular molecules of ϕX-174 DNA. Junctions between other molecules corresponded in length to other fragments of ϕX-174 *Hae*III DNA.

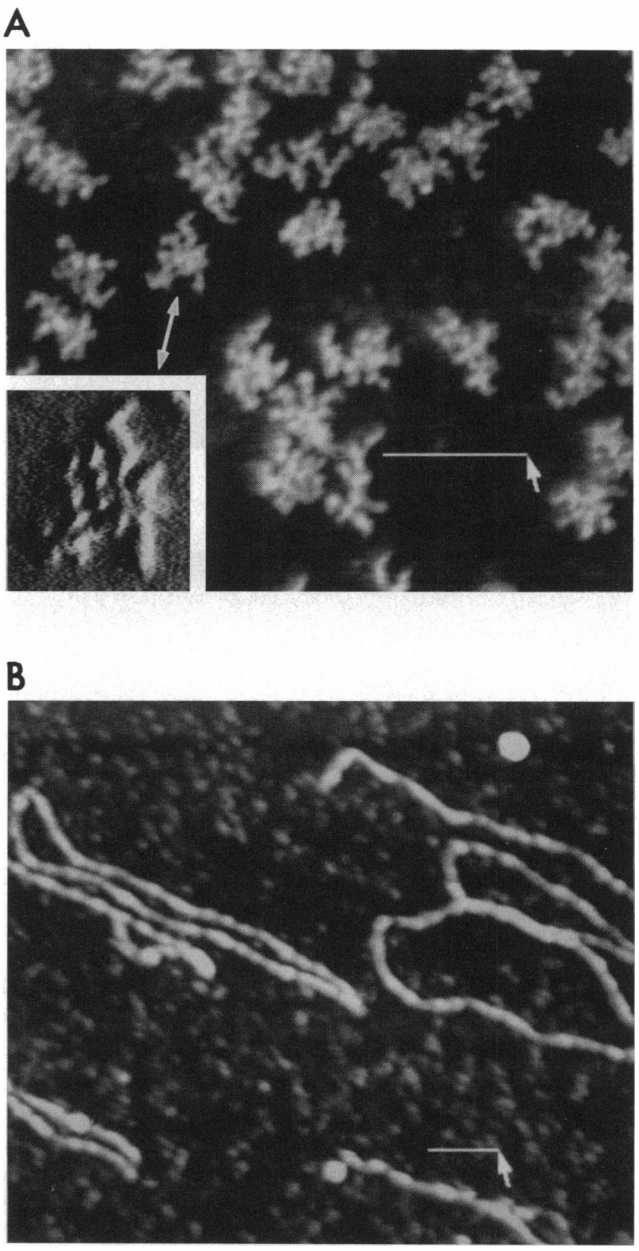

FIGURE 2 (*A*) Single-stranded ϕX-174 DNA molecules fold into compact shapes. Height mode image. (*Inset*) Deflection mode image of the molecule indicated by the double arrow; length and width of the molecule in both images is ~140 × 90 nm. This sample was rinsed with uranyl acetate. Similar images are obtained with untreated DNA. (*B*) After incubation with RecA and single-stranded binding protein, the molecules are extended to a length of ~2 μm, and junctions are seen between some molecules. Bars are 200 nm.

DNA in motion

Bluescript plasmids absorbed to Ni mica often show movement from image to image (Fig. 3). During an imaging session new plasmids may suddenly appear as they fall out of solution and are absorbed to the surface. Conversely, plasmids that are absorbed can sometimes lift off the surface and return into solution or move to a completely different area. This is probably due to the action of the tip loosening the plasmids as the area is scanned repeatedly. An example of this is the plasmid indicated in Fig. 3 *B*, which appears to have shifted completely to the left in Fig. 3 *D*.

Although the plasmids are stable enough to image, they usually do not stay completely absorbed to the surface. In Fig. 3 *D* several of the plasmids have missing sections. These reappear or shift along the plasmid in later images. These discontinuities arise when part of a plasmid is no longer anchored to the Ni mica surface (Bezanilla et al., 1994b), and the AFM cannot image it. Plasmids may also undergo conformation changes as evidenced by the plasmid in the upper left corners of the images in Fig. 3.

Time resolution

The maximum scan speed of an AFM that will give quality images depends on the cantilever spring constant, its effective mass, and the damping constant of the cantilever. It has been shown that in fluid the effective mass of the cantilever is increased and therefore the scan speed is reduced (Butt et al., 1993). Furthermore, in the tapping mode of operation the resonant frequency of the cantilever is much lower in liquid than in air (mostly due to this increased effective mass), and this places an upper limit on scan speed. Thus, the sample surface is not tracked as accurately when the scan speed increases beyond this theoretical limit.

The above limitations mean that, in general, the better the time resolution of the images the lower the spatial resolution. These effects can be reduced by adjusting the gains, but they cannot be completely negated. Bluescript on Ni-treated mica in buffer solution has been reliably imaged in tapping mode at a scan rate of 12 Hz with proportional gain set at 10 or more. At 256 samples per line and 256 lines it took roughly 20 s to capture each image. The plasmids appeared wider at this scan speed than at lower speeds, while the measured heights were comparable. After 45 min of imaging at this rate, damage to some DNA molecules was evident.

CONCLUSION

Advances in AFM of DNA have been made in three areas. 1) There is an improvement in the best resolution obtained (Fig. 1). 2) AFM can be used more easily now for routine imaging of DNA and DNA-protein complexes at a resolution comparable to that typically seen by electron microscopy. 3) The AFM can now image DNA in motion (Fig. 3) and some processes involving DNA such as RNA polymerase binding to DNA and DNase I digesting DNA (Bezanilla et al., 1994b; Guthold et al., 1994).

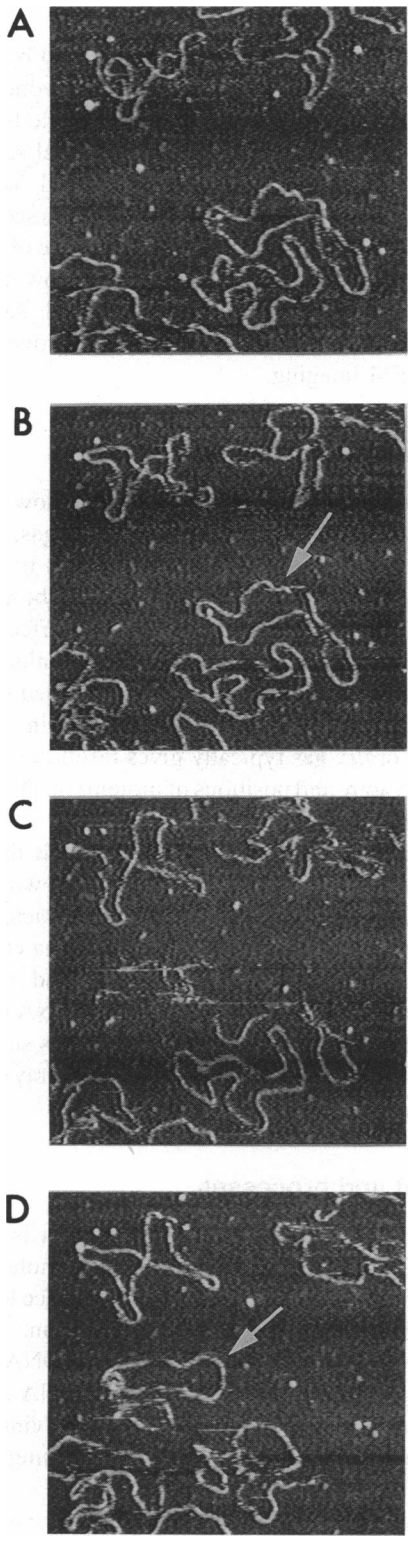

FIGURE 3 Time-lapse images of plasmid DNA in motion in the AFM. Plasmid DNA in a buffer containing 5 mM HEPES, 5 mM KCl, and 2 mM $MgCl_2$ was injected into the fluid cell onto fresh Ni-treated mica. These images were captured ~4 min apart; the time required to capture each image was 1.25 min. Discontinuities in some plasmids indicate that sections of the DNA are not anchored to the surface. The plasmid indicated in *B* has moved to the left in *D*. The plasmid in the upper left of the images undergoes various conformal changes. All images were captured in the "up" direction. Image size, 725 × 725 nm; tapping frequency, 12 kHz; scan speed 6.1 Hz.

High resolution imaging

The best resolution on DNA now appears to be ~2 nm, if helix turns can be seen in detail. It would be wonderful to get this resolution routinely and to see the double helix as it is bent and straightened by ligands and unusual sequences of bases, but this goal has not yet been reached. The smallest features typically seen on DNA are 6–10 nm in size, and there is as yet no real way to know how much of the observed fine structure is due to the shape of the tip and how much is due to the shape of the molecules being imaged. As discussed above, tip development is a key area for improving the resolution of AFM imaging.

Routine imaging

Good methods of sample preparation now allow routine imaging of DNA and its complexes in air or dry gas. The sample preparation needs are for DNA to bind well to the surface under conditions where the buffer salts can be washed off. One question is whether the sample surface affects the DNA conformation. This question, which is not unique to AFM, has been addressed recently for DNA adsorbed to mica and silylated mica (Bezanilla et al., 1995). Routine imaging of DNA in air or dry gas typically gives resolution at the level of DNA curvature and positions of proteins on DNA in DNA-protein complexes.

Results on routine imaging of DNA include the measurements of bend angles in complexes of DNA with RNA polymerase (Rees et al., 1993) and the characterization of ligand-induced DNA bending (H. G. Hansma et al., 1994), polysome spacing (Allen et al., 1993), and supercoiling (Samori et al., 1993). Unusually stretched DNA (Thundat et al., 1994) and other DNA-protein complexes such as biotinylated DNA complexed to streptavidin (Murray et al., 1993; Shaiu et al., 1993) have also been imaged.

Movement and processes

Now that DNA in motion can be imaged, it is possible to investigate the flexibility of short DNA molecules, with lengths of only two to four times the persistence length of the double helix, which is ~140 bp (Hagerman, 1988). One question in interpreting AFM "movies" of DNA is whether bending is occurring at points where the DNA is nicked or melted. The ability to image processes involving DNA and enzymes is on the frontier of a new field of single molecule enzymology of DNA.

In the last 5 years, AFM of DNA has progressed from the level of asking, "are we sure this is really DNA?" to the problem of developing methods for reliably imaging DNA to the point where we can use the AFM to answer scientific questions about DNA.

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