

Motion and Enzymatic Degradation of DNA in the Atomic Force Microscope

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ABSTRACT The dynamics and enzymatic degradation of single DNA molecules can now be observed with the atomic force microscope. A combination of two advances has made this possible. Tapping in fluid has reduced lateral forces, which permits the imaging of loosely adsorbed molecules; and the presence of nickel ions appears to form a relatively stable bridge between the negatively charged mica and the negatively charged DNA phosphate backbone. Continuous imaging shows DNA motion and the process of DNA degradation by the nuclease DNase I. It is possible to see DNase degradation of both loosely adsorbed and tightly adsorbed DNA molecules. This method gives images in aqueous buffer of bare, uncoated DNA molecules with lengths of only a few hundred base pairs, or approximately 100 nm in length.

INTRODUCTION

The first images of DNA obtained with scanning probe microscopes were encouraging but often irreproducible because of movement of the DNA molecules under the scanning tip. The ability to obtain stable and reproducible images in air (Bustamante et al., 1992; Lyubchenko et al., 1992; Thundat et al., 1992; Vesenska et al., 1992), propanol (Hansma et al., 1992), water (Lyubchenko et al., 1993a, b), and aqueous buffers (Bezanilla et al., 1994; Guthold et al., 1994; Hansma et al., 1993a) were welcome advances. However, there are many experiments that are only possible with loosely adsorbed molecules, which retain their biological function.

The invention of tapping in liquid (Hansma et al., 1994) has greatly reduced the lateral forces exerted by the AFM tip on DNA (Fig. 1), resulting in the ability to visualize DNA adsorbed loosely to mica. Even molecules attached at only one end and molecules moving along the surface can be imaged. Solutions of DNA or DNA-enzyme complexes can be directly injected into the AFM, without any rinsing of loosely bound DNA off the surface or drying of DNA onto the surface. This has made it possible to image not only DNA in motion but also real-time enzymatic degradation of DNA with the AFM.

MATERIALS AND METHODS

Preparation of nickel-treated mica

10 μ l of a solution of 1 mM NiCl₂ was placed on freshly cleaved mica for 1 min. The mica was rinsed with 2 ml of MilliQ water and then blown dry with compressed air.

Sample preparation for loosely adsorbed DNA molecules

324 bp DNA, a PCR fragment of pTZ19-T7A1 isolated using gel electrophoresis in 2% low melting agarose was prepared by E. Nudler and M. Kashlev. For the motion experiment of Fig. 2, this DNA was prepared as an elongation complex of His₆ RNA polymerase (Kashlev et al., 1993) and was injected into the fluid cell of the AFM onto mica treated with nickel. For the DNase degradation experiment of Fig. 4, the 324 bp fragments of DNA (0.5 μ g/ml) were injected into the microscope onto freshly cleaved mica in an aqueous buffer containing 5 mM HEPES, 5 mM KCl, 2 mM MgCl₂, 1 mM NiCl₂, pH 7.4.

Sample preparation for tightly adsorbed DNA molecules

ϕ X HaeIII digest DNA was obtained from Pharmacia Biotech (Piscataway, NJ). 1 μ l of the ϕ X HaeIII digest (2.5 ng/ μ l) in an aqueous buffer containing HEPES and Mg was placed on freshly split mica. The sample was subsequently rinsed with high pressure water to remove loose DNA and then dried in vacuum as described previously (Bezanilla et al., 1994) (Fig. 3).

DNase I degradation

DNase I was obtained from United States Biochemical (Cleveland, OH) and diluted with a buffer containing 20 mM HEPES, 5 mM MnCl₂, pH 7.6 just before use. After stable imaging of DNA was obtained, the DNase I was injected into the fluid cell at the dilutions noted in the figure captions. Agarose gel electrophoresis showed that DNase I was able to degrade 0.5 μ g of Bluescript plasmid DNA at room temperature in under 10 min at the dilutions and the buffer used for the AFM experiments. The buffer contained HEPES instead of Tris based on previous experiments showing that more DNA binds to untreated mica in HEPES buffers than in Tris buffers (Bezanilla et al., 1994).

AFM-imaging

All imaging was done on a Digital Instruments Nanoscope III MultiMode AFM with Nanoprobe 100 μ m, "narrow" oxide-sharpened silicon nitride cantilevers (Santa Barbara, CA) with home-built modifications for tapping in liquid. Scan rates ranged from 7 to 8 Hz, and tapping frequencies ranged from 10 to 20 kHz.

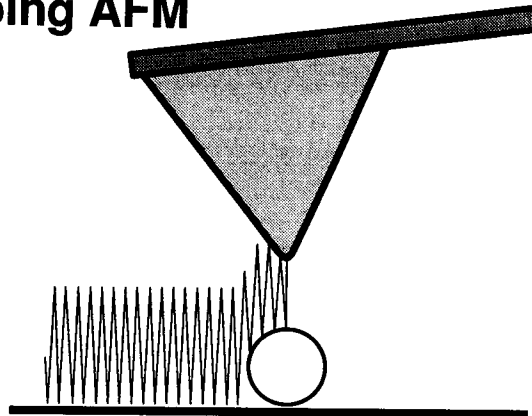
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Tapping AFM



Contact AFM

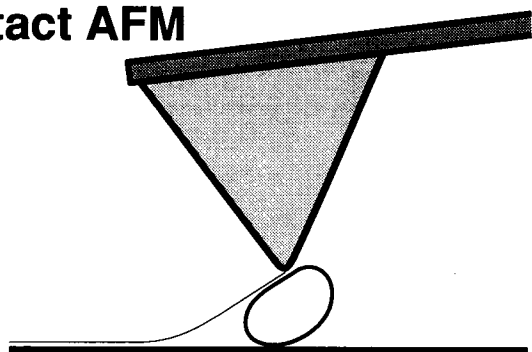


FIGURE 1 Diagram of tapping AFM and contact AFM. AFM tips on cantilevers raster scan across sample surfaces, following the paths indicated by the thin lines. Surface features are detected by changes in the cantilever deflection (Binnig et al., 1986; Rugar and Hansma, 1990). (*top*) In tapping mode, the AFM tip oscillates over the sample surface at high frequencies, touching the sample only at the bottom of the oscillation. This reduces lateral forces on the molecules. For tapping in air (Hansma et al., 1993b; Zhong et al., 1993), the cantilever oscillates at its resonant frequency. Because the resonant frequency of the cantilever is too low in liquid, tapping in liquid can be achieved instead by oscillating the sample under the tip (Hansma et al., 1994). (*bottom*) In contact mode, molecules must be well adsorbed to the substrate to avoid being moved and/or damaged by the tip. Because the images reflect the convolution of the tip shape with the molecule, the measured dimensions of molecules are larger than the actual dimensions.

RESULTS

DNA in motion

A DNA solution was injected into the AFM onto mica rinsed with 1 mM NiCl_2 (Ill et al., 1993), giving reasonably stable images of loosely adsorbed DNA molecules that were observed to change positions from one image to the next (Fig. 2). Animation of consecutive images gave dramatic time-lapse movies of these DNA molecules in motion. Some molecules showed motion in only one end of the molecule, whereas the other end remained firmly tethered to the mica (e.g., the molecule indicated with an *arrow* in Fig. 2 A). This partial binding may arise from an uneven distribution of nickel ions on the mica or from how the DNA lands on the mica initially. Some molecules were observed to move

across the field of view, such as the molecule indicated with the *arrow* in Fig. 2 C, which changes conformation in Fig. 2 D and moves to the left in Fig. 2 E.

The motion of the tip may contribute to the movement of the molecules. But the ability to image these molecules without apparent damage, even when a molecule has been displaced, is in contrast to contact mode AFM where molecules must be tightly adsorbed to the substrate. With contact AFM, the lateral forces induced by the tip were sometimes sufficient to scrape even tightly adsorbed DNA off the substrate. All images were taken with unmodified silicon nitride cantilevers, unlike contact mode AFM of DNA adsorbed to unmodified mica, which required electron beam deposited tips to image in aqueous solutions (Bezanilla et al., 1994; Hansma et al., 1993a). Using a silylated mica substrate, Lyubchenko et al. (1993a, b) have imaged long molecules of lambda DNA in water with unmodified silicon nitride tips.

DNA adsorption was too weak for stable images on fresh-split, untreated mica or on mica rinsed with MgCl_2 . The importance of Ni^{2+} may be because Ni^{2+} ions form coordination complexes with N and O atoms, many of which are octahedral or tetragonal (Phillips and Williams, 1966; Snow et al., 1993). In contrast, Mg^{2+} ions have 2S electrons in the outer shell producing a more uniform, spherically symmetric surface charge. Furthermore, Ni^{2+} has a similar ionic radius to Mg^{2+} , but it has a higher ionization potential and is more polarizable, which may allow it to form more stable coordination complexes between the negatively charged mica surface and the negatively charged phosphate backbone of the DNA.

Enzymatic degradation

The nuclease DNase I was used to cleave double-stranded DNA adsorbed to mica in the AFM. DNase I was chosen because it is a very active, relatively nonspecific endonuclease that cleaves double-stranded DNA in the presence of manganese. The experiments were performed on DNA that was dried onto mica (Fig. 3) or DNA that was injected in solution into the AFM (Fig. 4). The DNA in Fig. 3 was tightly adsorbed to the mica by rinsing the sample with high pressure water to remove loose DNA and then drying in vacuum (Bezanilla et al., 1994). Fig. 3 A is a field of DNA molecules before introducing DNase I. The DNA did not move on the mica, indicating that it was very well adsorbed. DNase I was introduced 1 min before the image in Fig. 3 B. Note that the piece of DNA indicated with an *arrow* in Fig. 3 A is cleaved in Fig. 3 B. Part of this piece then disappears (Fig. 3 C), and the last piece moves to the right (Fig. 3 D, *arrow*). The degradation continues (Fig. 3 E) until, 14 min after putting in DNase I, there is very little intact DNA remaining (Fig. 3 F). There is however, more debris in the background as compared with earlier images, which may be an accumulation of DNase I and degraded DNA on the surface.

Another DNase experiment used a solution containing 0.5 $\mu\text{g/ml}$ DNA injected directly into the microscope onto freshly cleaved mica in an aqueous buffer containing 1 mM

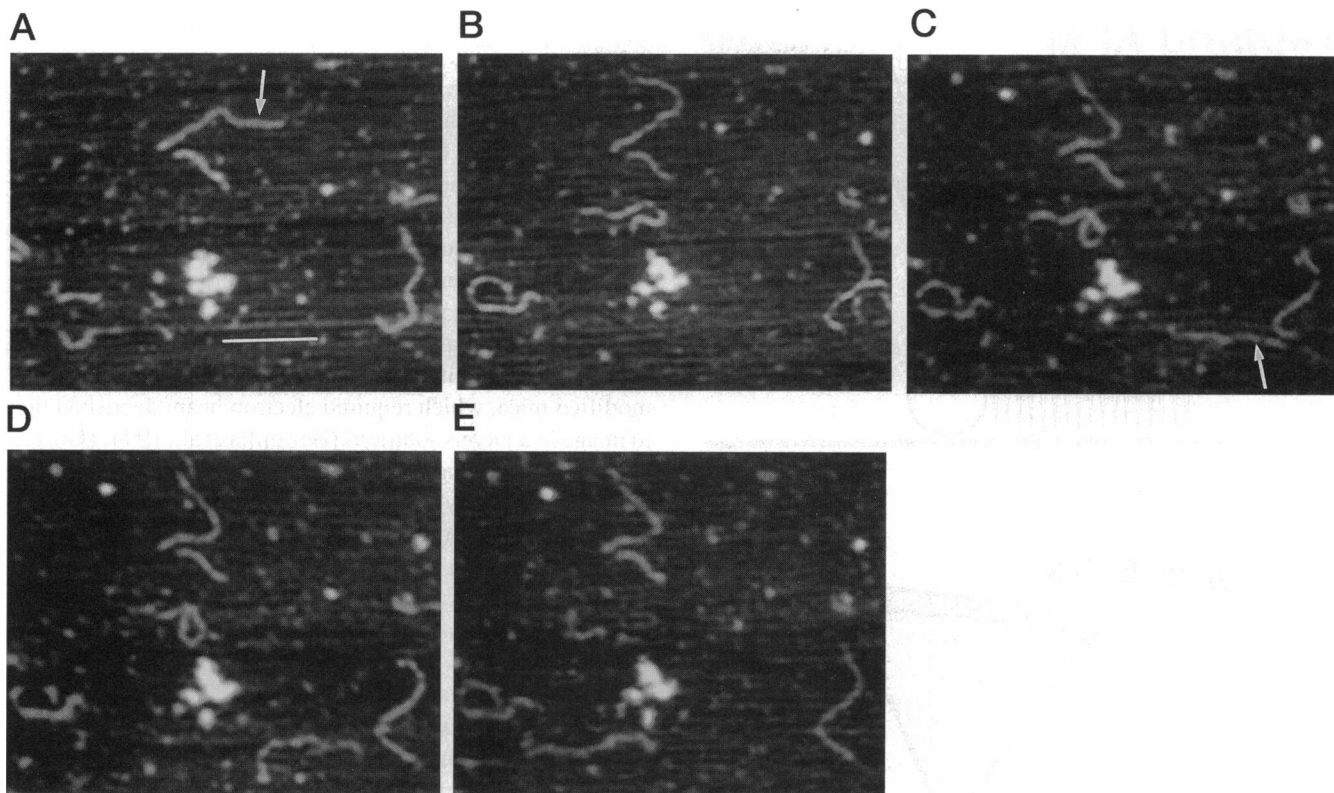


FIGURE 2 Small mobile pieces of DNA, 324 base pairs in length, can be imaged in buffer (5 mM HEPES, 5 mM KCl, 2 mM MgCl_2 pH 7.5) with the AFM tapping mode. (B–E) are a series of images taken 8, 11, 12, and 16 min, respectively, after A. The top half of the molecule indicated with an arrow in A changes orientation in the following image. In C, a new molecule, indicated with an arrow, appears in the field of view. In D it has changed its conformation, and in E it has moved to the other side of the field. The DNA was prepared as an elongation complex of His_6 RNA polymerase (Kashlev et al., 1993) and injected into the microscope. No intact elongation complexes were observed; the white spots near the center of the image appear to be an aggregate of RNA polymerase molecules. Bar in A is 100 nm. Tapping frequency, 17 kHz. Scan speed, 7 Hz.

NiCl_2 (Fig. 4). The DNA was imaged nondestructively for 25 min before introducing DNase I. (Fig. 4 A–C) Such stable DNA images in aqueous buffer have previously been seen only with samples that have been dried in vacuum (Bezanilla et al., 1994; Hansma et al., 1993a). Thus, it is a significant advance to be able to obtain stable images of DNA that has simply been injected into the AFM in a buffer containing Ni^{2+} . DNase I degraded this DNA progressively during the first 3 min after injection (Fig. 4 D–F). In other experiments, moving DNA molecules on mica rinsed with Ni^{2+} were also degraded with DNase I.

DISCUSSION

DNA in motion

Tapping AFM can monitor DNA dynamics in aqueous buffer much below the limit of optical resolution. With the light microscope, DNA dynamics have been observed previously with fluorescent-labeled DNAs of length 100 kbp or more (Bustamante, 1991; Gurrieri et al., 1990; Perkins et al., 1994a, b; Schwartz et al., 1993; Volkmuth and Austin, 1992). Tapping AFM can image uncoated DNA molecules of only 300 bp in motion in aqueous buffer on a substrate. Only a few anchor points are required to visualize these molecules, which appear to have the same length from image to image

even though they are extremely mobile on the surface. Some molecules appear to be shorter in one image but in the next image will have returned to their full length. This can be explained by the fact that part of the molecule may not have been close enough to the surface for the tip to image it. But in a subsequent scan, the molecule may have approached closer to the surface, restoring the molecular image to its expected length. The anchor points may also change over time as demonstrated by the molecule that is seen to move across the field of view in Fig. 2 C–E. The images can be viewed sequentially, thus animating the motion of the DNA on the surface with the time resolution limited by the AFM, which is on the order of one min per image in these experiments. The motion of the DNA is on a short time scale relative to the scan rate. This sometimes leads to a “double exposure” effect, in which a previously imaged piece of DNA translates to a new position ahead of the tip and is scanned again in the new position. Animation of the data allows one to follow molecules along the surface as well as to notice small conformational changes that occur near anchor points.

Enzymatic degradation

Observing DNA-enzyme interactions in action has long been a goal for AFM of DNA. In contact mode AFM, *Escherichia coli* RNA polymerase was seen to bind nonspecifically to

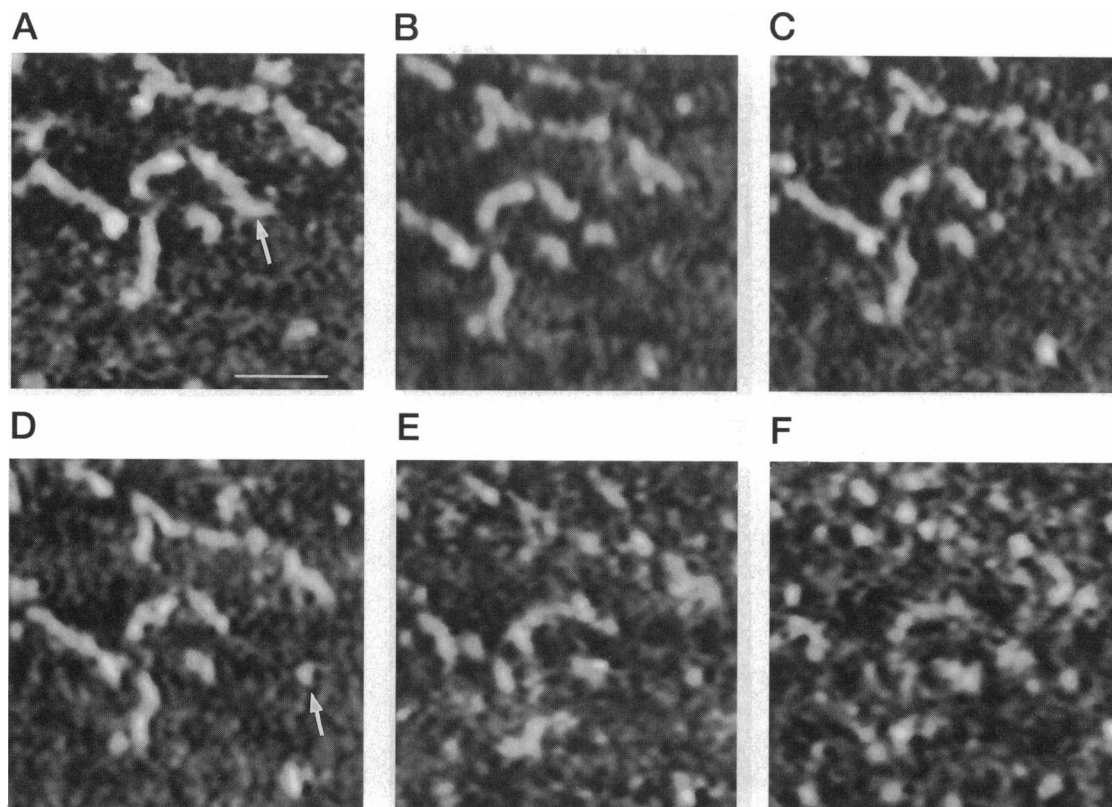


FIGURE 3 (A) A field of DNA molecules (ϕ X174 *Hae*III digest) after 15 min of imaging in a buffer containing 20 mM HEPES, 5 mM MnCl_2 , pH 7.6. This sample was made with a total of 2.5 ng of DNA; some of the DNA was removed by rinsing. (B–F) Degradation of the DNA in A after addition of DNase I (0.8 units/ml) at times 1.0, 1.5, 2, 7, and 14 min, respectively. Bar in A is 100 nm. Tapping frequency, 20 kHz; scan speed, 8 Hz.

DNA tightly adsorbed to mica but was not able to slide to the promoter site on the DNA (Guthold et al., 1994). Our previous attempts to image enzymatic degradation of DNA were unsuccessful, perhaps because the enzymes were unable to slide along tightly bound DNA. Specifically, the endonuclease *Pvu*II was introduced into the fluid cell onto tightly bound DNA (the DNA preparation used in Fig. 3). No cleavage was observed; this may be because the enzyme was not able to find its binding site when the DNA was tightly adsorbed to the mica. It is believed that nucleases like *Pvu*II bind non-specifically and then slide along the double helix to find the binding site (vonHippel, 1994). An exonuclease (Exo III) was also used to attempt to cleave double-stranded DNA starting from one end. Again, it is most likely that the enzyme needs to slide along the helix to find the end from which to start cleaving. With well adsorbed DNA this was impossible but now, with loosely adsorbed DNA, it might be possible.

On the other hand, DNase I is a better nuclease to use in these studies, because it is known to cleave even DNA that is tightly bound to a surface (Rhodes and Klug, 1980). The x-ray crystal structure shows that DNase I binds to the minor groove of the double helix and does not wrap around the DNA (Suck and Oefner, 1986). DNase I interacts with the phosphate backbone, cleaving the phospho-diester bonds. DNase I probably does not need to slide along the DNA because the active site is the phosphate backbone. Unlike type II endonucleases such as *Pvu*II, DNase I shows little

sequence specificity and, unlike exonucleases, it does not need to find the end of the DNA. In the present work, we have directly imaged the degradation by DNase I of DNA that is both loosely bound and tightly bound to surface. DNase I molecules (31 kDa) have not been clearly resolved on the DNA in buffer. It is easier to resolve small proteins on a hard substrate than small proteins on DNA with the AFM, especially in aqueous buffer. This could be because DNA forms secondary structures on the mica that are not easily distinguished from proteins bound to DNA (Hansma et al., 1993a). Another explanation may be that there is not enough temporal resolution to see the DNase. Because the DNase activity is on a shorter time scale than the imaging, it is not possible to image the action of cleaving. Faster image acquisition is possible, but the image quality is lower. Most enzyme processes are faster than the AFM imaging speed in tapping mode. It may be possible, however, to observe other enzyme processes where the enzyme involved is larger and the kinetics can be controlled, such as DNA polymerases and RNA polymerases (Kuriyan and O'Donnell, 1993).

Observing other DNA-enzyme interactions will involve progress in several areas. If the enzyme needs to slide along the DNA to recognize its binding site, then the adsorption of the DNA to the substrate must be carefully controlled. It is not known how free the DNA must be for enzymes other than DNase I to act on it. Also, buffers need to be found that allow both reliable AFM imaging and optimal enzymatic activity.

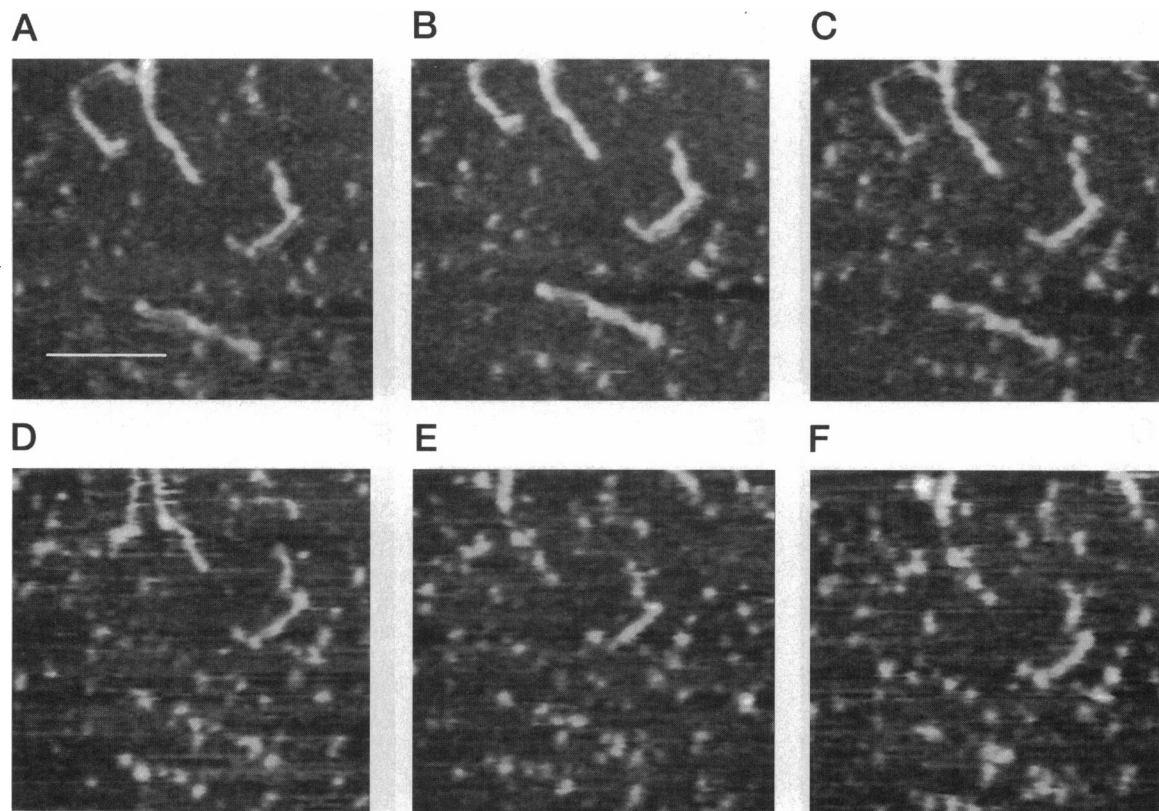


FIGURE 4 (A–C) Stable imaging of 324 bp DNA in a nickel-containing buffer injected into the AFM onto freshly cleaved mica. DNA, 0.5 $\mu\text{g}/\text{ml}$, in buffer containing 5 mM HEPES, 5 mM KCl, 2 mM MgCl_2 , 1 mM NiCl_2 , pH 7.4, was scanned continuously for 15 min, as shown here, before changing to buffer containing 20 mM HEPES, 5 mM MnCl_2 , pH 7.6. After 10 min of additional scanning, DNase I, 2 units/ml, in this buffer was added. (D–F) Degradation of the DNA molecules shown above at 1, 2, and 3 min, respectively, after addition of DNase I. Bar in A is 100 nm. Tapping frequency, 10 kHz. Scan speed, 7 Hz.

CONCLUSION

Tapping in liquid has enabled the AFM to image loosely adsorbed DNA molecules. This is a great advance because it is now possible to image enzymatic degradation of DNA. The dynamics of very small uncoated DNA molecules loosely associated with a surface were observed in aqueous buffer well below the limit of optical resolution. There may have been some tip-induced motion, but this demonstrates that the flexibility of small molecules can now be studied. It may also be possible to observe important DNA-enzyme interactions such as replication and transcription of individual DNA molecules with the AFM. To achieve this endeavor, future methods development can focus on quantifying and minimizing tip-induced motion as well as acquiring images at a faster rate.

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