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Atomic force microscopy of single- and double-stranded DNA

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ABSTRACT

A method has been developed for imaging single-stranded DNA with the atomic force microscope (AFM). ΦX174 single-stranded DNA in formaldehyde on mica can be imaged in the AFM under propanol or butanol or in air. Measured lengths of most molecules are on the order of 1 μ, although occasionally more extended molecules with lengths of 1.7 to 1.9 μ are seen. Single-stranded DNA in the AFM generally appears lumpier than double-stranded DNA, even when extended. Images of double-stranded lambda DNA in the AFM show more sharp kinks and bends than are typically observed in the electron microscope. Dense, aggregated fields of double-stranded plasmids can be converted by gentle rinsing with hot water to well spread fields.

INTRODUCTION

Atomic force microscopy of uncoated DNA molecules has great potential for contributing to a detailed knowledge of the substructure of individual DNA molecules and the understanding of processes involving DNA. Reports on the atomic force microscopy of double-stranded plasmid DNA are appearing at a rapid rate.[1–9] The images obtained correlate well with what has been seen in the electron microscope. Imaging under propanol shows, in addition, reproducible structure along the DNA strands as small as 3 to 5 nm[3,9], while imaging in air shows mainly structure at the level of super-coiling.[1,2,5–7]

Single-stranded DNA has proved to be a greater challenge for atomic force microscopy than double-stranded DNA. Single-stranded DNA from ΦX174 and M13 have now been imaged in the atomic force microscope (AFM). The DNA was extended by a modification of the method developed by Freifelder for the electron microscope[10].

The AFM, invented in 1986 by Binnig, Quate, and Gerber[11], images surfaces at sometimes even atomic resolution[12,13] by gently scanning a sharp tip across the surface at forces generally smaller than the forces between atoms. The tip is on a cantilever, which deflects as it scans over the bumps and pits on the surface. When the AFM is operated in the constant-force mode, a feedback loop holds the cantilever deflection nearly constant by adjusting the z-position of the sample. The AFM has been used to image the surfaces of live cells[14,15], the pores in gap junctions[16], the process of fibrin polymerization[17], and the individual molecules in lipid films[18–20], to name a few of its biological applications, whcih have been reviewed recently.[21]

MATERIALS AND METHODS

DNA

The following DNAs were used: ΦX174 virion DNA (single-stranded form, 5386 bases) from U.S. Biochemical Corp., Cleveland, OH, supplied at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA; lambda phage double-stranded DNA (48,502 base pairs) from Sigma, St. Louis, MO, supplied lyophilized from a concentration of 278 μg/ml in 1mM Tris-HCl pH 7.5, 1 mM NaCl, 1 mM EDTA; Bluescript II SK M13(+) double-stranded plasmid DNA (3204 base pairs) from Stratagene, LaJolla, CA, supplied at a concentration of 1 mg/ml. DNAs were diluted with water before use unless specified otherwise.

Preparation of DNA samples for the AFM

Single-stranded DNA. Freshly-cleaved ruby mica circles 1/2 inch in diameter (Unimica, New York, NY) were soaked 4 to 24 hours in 33 mM magnesium acetate, sonicated 5 min in Millipure water to remove excess magnesium acetate, dried with compressed air and exposed to an AC glow-discharge for 20 sec in 100 mtorr air.[2,3] Within a minute after the glow-discharge step, the mica circles were inverted onto 5- to 10-μl drops of single-stranded DNA, containing 50 to 300 ng DNA in 0.5% formaldehyde and 15 mM ammonium acetate, deposited onto Parafilm.After 2 to 5 minutes, the mica was rinsed with 2 to 3 drops of water, dried with compressed air, and stored in a desiccator over Drierite until use. More recent results show that the ammonium acetate can be omitted, and preliminary results suggest that the key to completely elongating the molecules may be to use 2% formaldehyde instead of 0.5% formaldehyde.

Double-stranded DNA. Double-stranded DNA was prepared in the same way, except that the formaldehyde and ammonium acetate were omitted. The glow discharge step is not essential; good samples have been prepared by evacuating the mica at 100 mtorr air without glow discharge. The important part of this step may be simply to dry the mica completely.Also, some samples with a good density of DNA were prepared with drops containing

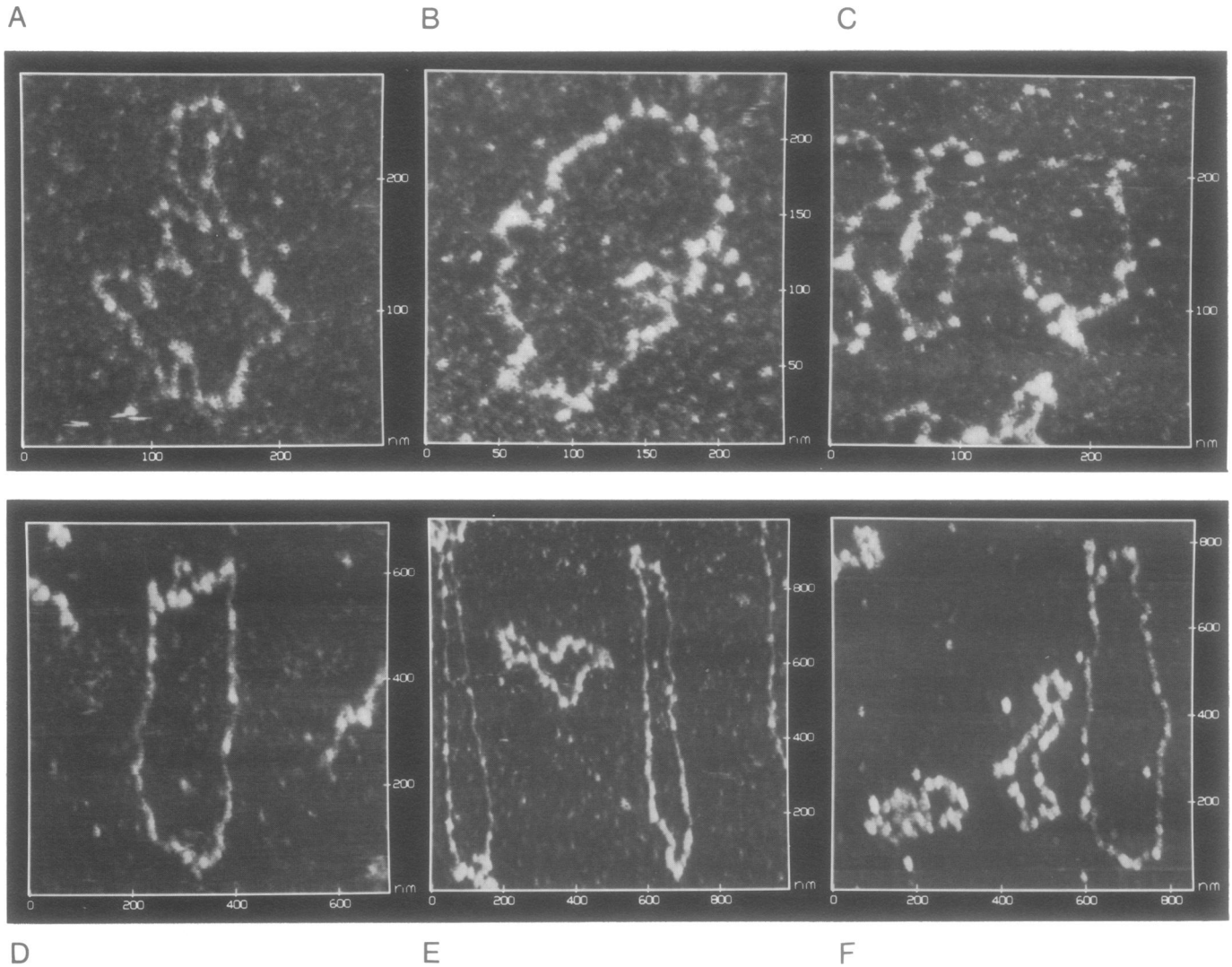

Figure 1. AFM images of several molecules of ΦX174 single-stranded DNA. Note the lumpy appearance of the strands and the differences in apparent molecular length, especially evident in E and F. Images were taken with a Nanoscope II under 1-propanol (A to C), 2-propanol (D, E) and 1-butanol (F) with ion-milled supertips. Image sizes are 300nm×300nm (A, C), 250nm×250nm (B), 700nm×700nm (D), 1000nm×1000nm (E) and 850nm×850nm (F).

only 10 ng DNA.The DNA drops sat on the Parafilm for 30 to 60 min at room temperature before applying the mica; this may have introduced single-strand breaks that would account for the many relaxed circles seen, if nucleases were inadvertently present. More recent results show that good samples of double-stranded DNA can be prepared on untreated freshly split mica; it is not yet clear whether the same is true for single-stranded DNA.

Atomic force microscopy

AFM-imaging was done under 100% propanol or butanol using a Nanoscope II or Nanoscope III AFM (Digital Instruments, Santa Barbara, CA).To apply the propanol or butanol, an O-ring was laid on the sample in the AFM. The O-ring was filled with three or four drops of alcohol. One drop of alcohol was applied to the cantilever in the fluid cell, which was then positioned over the O-ring and clamped firmly in place. If there were no bubbles in the light path, this amount of fluid was sufficient.For changing fluids or removing bubbles, fluid was injected through the fluid cell with a syringe connected to tubing. The syringe and tubing then remained attached to the AFM for the duration of the experiment. No differences in AFM-image quality were seen between 1-propanol, 2-propanol, and 1-butanol.

Unless noted otherwise, imaging was done with 120-μ narrow silicon-nitride cantilevers with needle-shaped 'supertips' deposited onto the integrated pyramidal tips with the scanning electron microscope.[1,2,22,23] Most of the supertips were subsequently ion-milled[3,24] for 1 to 3 min in argon at 2.5 keV with an ion current density of 0.5 mA/mm^2. The tips were rotated around an axis parallel to the direction in which they pointed during ion milling. The ion beam was oriented 80° away from the axis of rotation and hit the tips from the side.

During AFM imaging, the force was reduced to the minimum force needed to prevent the cantilever from lifting off the sample, which was approximately 1 nanoNewton (nN). The scan rate was usually 7 to 9 Hz; height mode was used. Integral gain was adjusted to give sharp images; typical values for the Nanoscope II were 2 to 3. Proportional gain was 1.0; 2D gain was 0.05. Images were taken without on-line filtering and were subsequently processed only by flattening to remove the background slope.

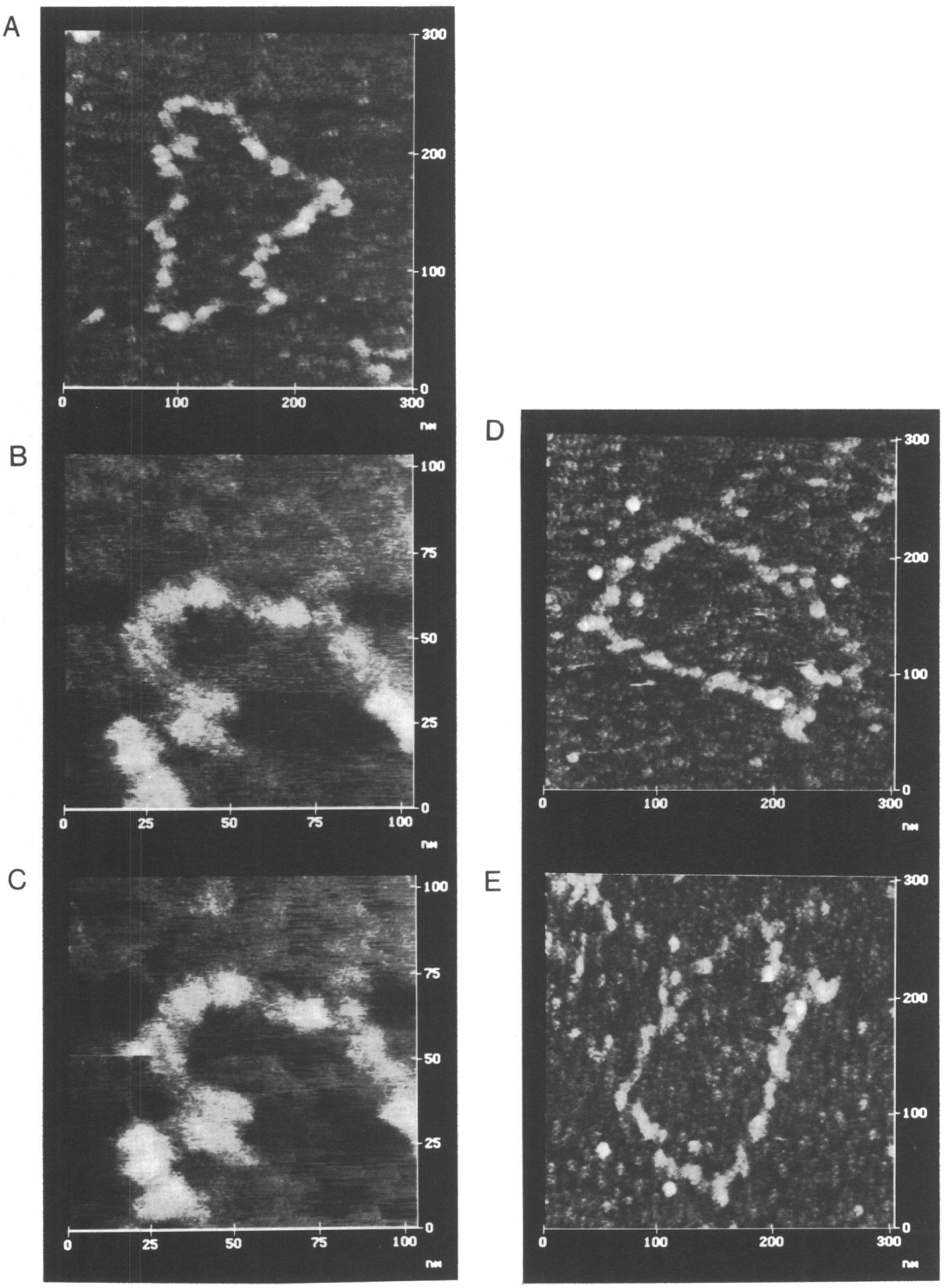

Figure 2. Reproducible detail is seen in AFM images of single-stranded ΦX174 DNA molecules in repeat scans (A to C) and rotation of the scan direction (D and E). B and C are repeat scans of a detail of the molecule in A. D and E are images of the same molecule at 0° (D) and 90° (E) rotation of the scan direction. The sample was imaged with a Nanoscope III under 1-propanol with an ion-milled supertip. Image sizes are 300nm×300nm (A, D, and E) and 100nm×100nm (B, C).

Information density of captured images was 400×400 points with the Nanoscope II and 512×512 points with the Nanoscope III.

Measuring apparent lengths and heights of DNA

Apparent lengths of DNA were measured by enlarging the images and laying a fine chain along the contour of the DNA image. Duplicate measurements usually differed by about 1 to 2%. A more convenient way to measure apparent lengths was discovered recently. 'Topview' images of DNA in the Nanoscope can be measured directly by summing the consecutive distances between the 2 '+'s' that are displayed on the screen along with the topview image.

Mean apparent heights of DNA were measured by using the Bearing command in the Nanoscope software to obtain a

histogram of the heights within an area of the image. The histogram typically shows 2 peaks representing the apparent heights (expressed as 'depths') of the DNA and of the background, relative to the height of the highest point in the area. The difference in height between the two peaks is approximately the mean apparent height of the DNA in this area. To obtain reliable data, one needs an area where the background is free of high debris that would be counted in the 'DNA' peak.

RESULTS AND DISCUSSION

Molecules of single-stranded ΦX174 DNA in 0.5% formaldehyde and 15 mM ammonium acetate on mica show a variety of apparent contour lengths in the AFM (Fig. 1). Most common are circles that measure roughly 1 μ along their outline, or 1.3 μ, if the convolutions along the strand are followed more closely (Fig. 1A to C and the smaller molecules in Fig. 1E and F). These measurements correspond to 0.18 or 0.25 nm per base, respectively. Circles of this size were seen in many different samples. A few significantly longer circles were seen, 1.7 to 1.9 μ in length, (Fig.1D and the longer molecules in Fig. 1E and F), corresponding to 0.32 to 0.36 nm per base. Electron microscopy of single-stranded ΦX174 gives a measured length 1.8μ for cytochrome c-coated molecules.[25]

Substructure in single-stranded DNA is stable with repeated scanning and reproduces well when the scan direction is rotated (Fig. 2). The scan direction in Fig. 2D differs from the scan direction in Fig. 2E by 90°, but the two images show almost identical molecular substructure. The exact shape of the observed molecular substructure is a convolution of the AFM tip with the DNA. The convolution is the same in rotated images, since rotation changes the direction that the tip scans the sample but not the orientation of the tip relative to the sample.

The one-micron-long molecules have apparent widths of 5 to 18 nm and do not appear to be well extended (Fig. 1A-C, shorter molecules in Fig 1E and F, and Fig. 2). Since they are seen so frequently, they must have some structural stability, arising either from favorable intramolecular interactions or from DNA-mica interactions. Mica is an atomically flat crystal characterized by partially-negative hydroxyl groups in a hexagonal lattice with 0.5-nm spacing. The pre-treatment with magnesium acetate introduces magnesium ions into some of these sites for binding to the DNA. Under the conditions used, the mica is saturated with magnesium ions, which may provide a density of DNA binding sites that discourages the DNA from stretching out. An alternate explanation comes from electron microscopy, in which it was found that single-stranded DNA extended better when spread on a hypophase than when applied to the substrate as a drop[26]. Micron-long circles were also seen in samples prepared without ammonium acetate, while in samples prepared with 1M ammonium acetate, the DNA molecules show a highly condensed star-burst shape. The condensed shape in 1M ammonium acetate, which is the concentration recommended by Freifelder for electron microscopy of single-stranded DNA[10], is probably due to screening of the charges on the phosphate backbone by ammonium ions, which prevents the molecules from extending by intramolecular electrostatic repulsion.

In even the most extended single-stranded DNA, lumps are seen along the strands.(Fig. 3) These images are details from the single-stranded DNA molecule with the narrowest apparent width, 3 nm. Between the lumps are very fine DNA threads of uniform width.

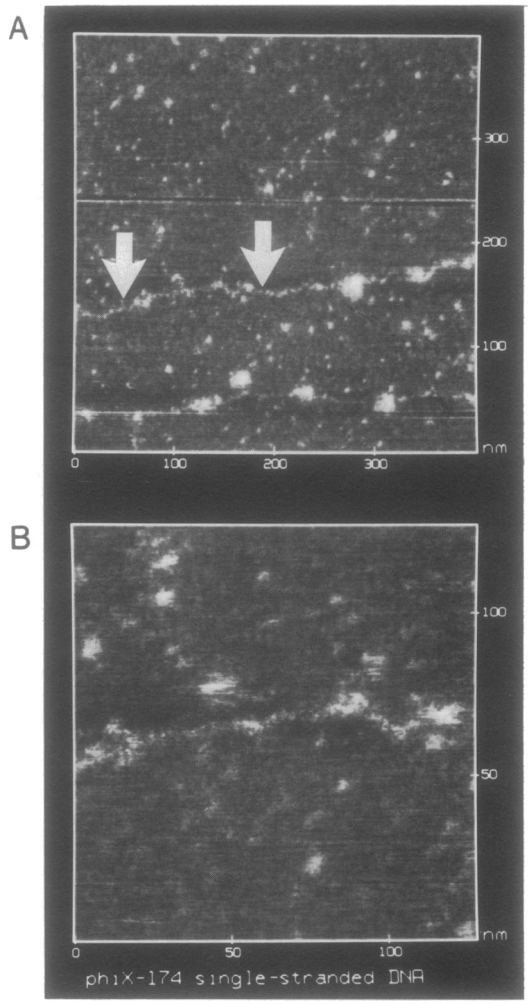

Figure 3. AFM images of detail from a molecule of ΦX174 with an unusually narrow apparent width (3 nm). Note the smooth regions of the strands and the intermittent lumps. B is an image of the region of A between the arrows. Images were taken with a Nanoscope II under n-butanol with an ion-milled supertip. Image sizes are 400nm×400nm (A) and 125nm×125nm (B).

The long DNA molecules in Fig. 1C to F were apparently stretched by flow, since all the long molecules are oriented in the same direction. Flow-induced elongation could have occurred either when liquid flowed through the fluid cell in the AFM or when the sample was prepared, caused by rinsing the sample or laying the mica onto the drop of DNA.

Double-stranded DNA usually appears more uniform along the strand than single-stranded DNA. The lambda DNA in Fig. 4B and C shows a fairly regular series of lumps along the strand, 6 to 8 nm apart, and the strand has an apparent width of 7 to 9 nm. Apparent widths depend strongly on the width of the AFM tip,[1,2,27] which is a source of variability that will be reduced when it becomes possible to make uniformly sharp tips. The minimum spacing of lumps that are imaged along the strand is also limited by the tip width, which may at present be preventing us from routinely imaging features as small as the turns of the double helix or the individual nucleotides. The imaged features are, however, reproducible and thus correspond to stable substructure or tightly bound ions. Double-stranded DNA, when constrained to a mica surface, may be pulled into a conformation significantly different from the typical A- or B-forms. This could also explain why substructure seen in the AFM is larger than

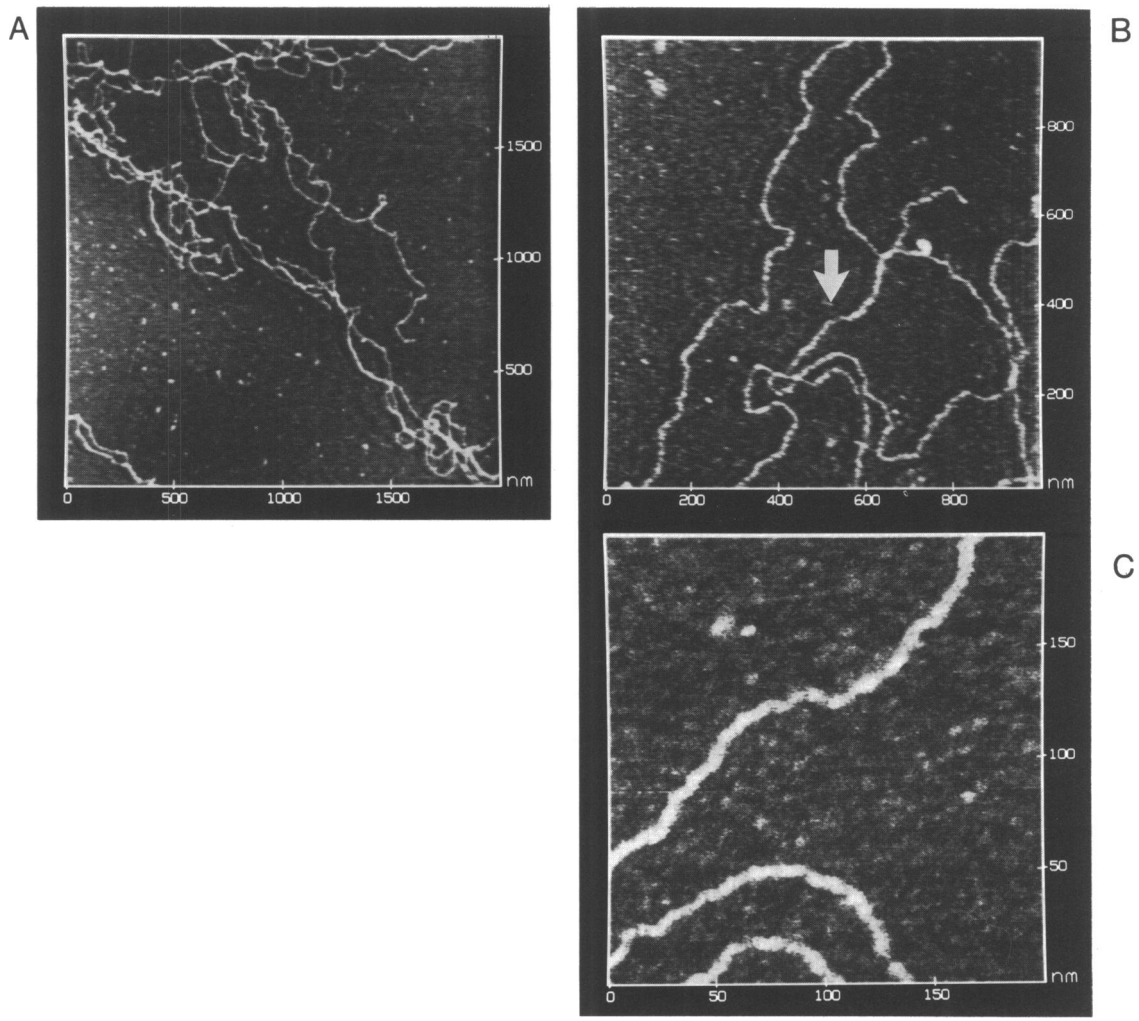

Figure 4. AFM images of lambda DNA under 2-propanol taken with a Nanoscope II. Note the sharp bends in the strands, especially in A, and the uniform appearance of the strands, with substructure evident, particularly in C. Image C is a small scan in the region of B indicated by the arrow. Image sizes are 2000nm×2000nm (A), 1000nm×1000nm (B), and 200nm×200nm (C). An ion-milled supertip was used in A; an unmodified pyramidal tip of unusual sharpness was used for B and C.

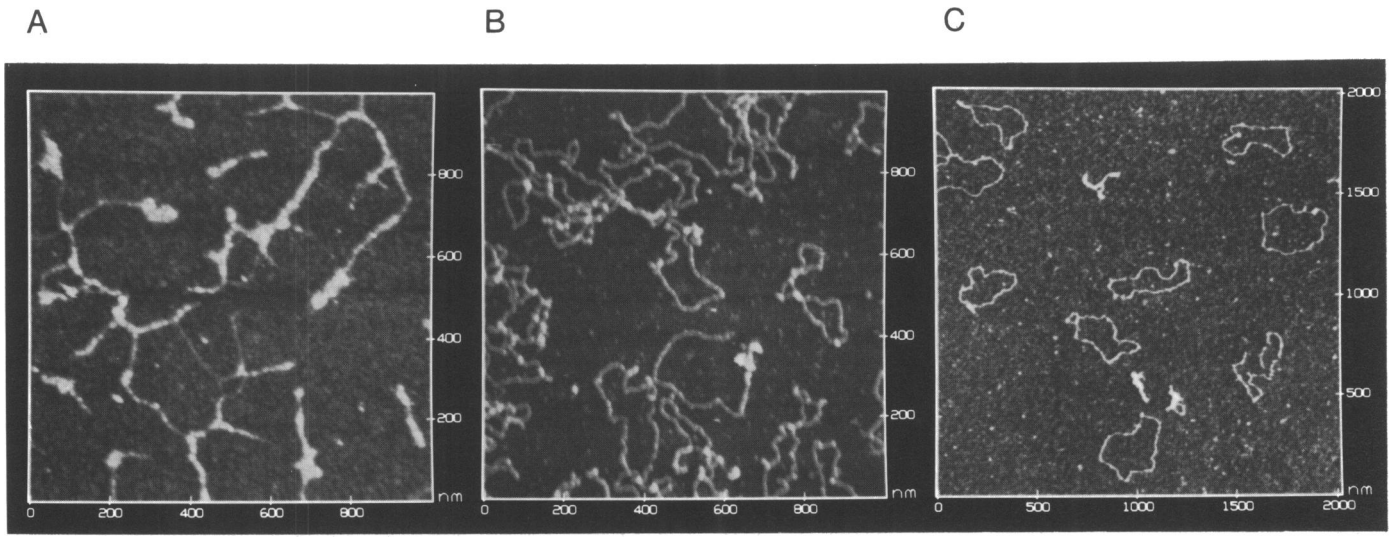

Figure 5. AFM images of Bluescript DNA under 1-propanol, showing the effects of hot water washing. Unwashed sample (A) and samples after washing with several drops of hot water (B, C). Images were taken with a Nanoscope II. The tip used for A and B was a pyramidal tip shadowed at 10° with aluminum to produce an aluminum micro-tip; the tip for C was an ion-milled supertip. Image sizes are 1000nm×1000nm (A, B) and 2000nm×2000nm (C).

the periodicity of helix turns in solution: 2.6 nm for A-DNA, the dehydrated form seen in ethanol or low humidity[28] and probably also in propanol.

In contrast to double-stranded DNA, the single-stranded DNA molecules in Figs.1 to 3 have broader lumps, sometimes with gaps or fine strands between them. Spacing between the lumps varies from 8 to 50 nm; apparent width varies over a two-fold range, even within a single molecule and even in elongated molecules. Apparent heights of both single- and double-stranded DNAs were approximately 1 nm. The appearance of single-stranded M13 DNA in the AFM is essentially the same as that of ΦX174.

In a particularly dense aggregate of double-stranded Bluescript plasmid DNA (Fig 5A), the individual molecules cannot be seen. The molecular aggregate shows evidence of super-coiling. This aggregate can be partially (Fig. 5B) or completely (Fig. 5C) dissociated by rinsing the sample with several drops of hot water and drying it with compressed air. The hot water appears to wash some of the DNA from the surface, which is consistent with the observation that DNA is rapidly scraped off the mica surface when imaged under water. DNA *can* be imaged under water, however, if it has first been imaged under propanol; DNA molecules can remain on the surface for a half hour or more of continuous scanning in the AFM, though they are more easily damaged or moved in water than in propanol. The reason for this propanol-induced stabilization of DNA in water is not known; but it may be that the propanol, in dehydrating the DNA, allows it to bind to the mica more firmly. The images of Fig. 5 demonstrate the AFM's special ability for before-and-after imaging of samples at high resolution, since the sample surface can be imaged directly in the AFM without being metal coated.

We have recently developed a way for finding the same area to re-image in the AFM. An electron-microscope locator grid is glued onto the steel disc underneath the transparent mica (or glass) sample substrate. With the aid of the locator disc, one can relocate a particular region when using a top-view AFM under a light microscope.

Questions have been raised about the relevance of propanol to studies of DNA, which naturally exists in aqueous solutions. This is an important concern, especially since one hopes eventually to observe DNA-enzyme interactions in progress with the AFM. Similarly, it is important to remember that DNA imaged in a scanning probe microscope will always of necessity be bound to a surface, unlike the DNA in a cell. The miracle of molecular biology, however, is that one *can* actually discover the incredible complexities of DNA structure and function with relatively artificial laboratory experiment The DNA molecules used for X-ray crystallography and electron microscopy are in a no more natural state than the DNA molecules in the AFM, yet they have contributed immeasurably to our scientific knowledge; it is not unreasonable to expect that DNA in the AFM will make a comparable contribution.

CONCLUSION

It is a challenge for the emerging field of AFM of DNA to equal, and hopefully surpass, the beautiful and useful images of DNA that have come from electron microscopy over the past many decades. As the techniques improve for preparing tips and samples for the AFM, this new method can also contribute to the knowledge of DNA structure and function.

Single- and double-stranded DNA in the AFM show different fine structures in the 5- to 10-nm size range, and the DNA is imaged without a coating of cytochrome c or metal shadowing. One exciting application of the AFM that is just beginning to be explored is the ability to image the same region of the same sample before and after making a modification of the sample.

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