Scanning probe visualization of electrostatically immobilized intercalating drug–nucleic acid complexes

J. E. Coury, L. McFail-Isom, S. Presnell, L. D. Williams[a] and L. A. Bottomley[a]
School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332-0400

(Received 29 September 1994; accepted 27 February 1995)

DNA intercalators are planar molecules that insert between the base pairs of duplex DNA. Intercalation into DNA necessarily results in changes in DNA conformation, separating base pairs along the helical axis; thus, intercalators are of great importance as probes of nucleic acid structure and as interferents of DNA replication, transcription, and/or topoisomerase activities. Relationships between structures of intercalators and the changes they cause in DNA conformation are subtle and remain unresolved. An intercalating drug molecule has been synthesized that incorporates markers easily distinguished by atomic force microscopy (AFM). This specially designed psoralen derivative has been intercalated into DNA. To facilitate recognition of the point of intercalation, the psoralen derivative contained a biotin moiety that was exposed and bound readily to streptavidin-coated colloidal gold beads. Atomic force micrographs were obtained following electrostatic immobilization of the DNA/psoralen complexes onto mica surfaces. This article presents AFM images of intercalating drug–DNA complexes which may provide further data for the study of structure/intercalator relationships. © *1995 American Vacuum Society.*

I. INTRODUCTION

Atomic force microscopy (AFM) can reliably image nucleic acids.[1–4] Structures visualized by AFM include nucleic acid complexes with macromolecules such as the large *T* antigen,[5] *Escherichia coli* RNA polymerase,[6] and staphylococcal protein.[7] In the *E. coli* RNA polymerase complex, a DNA bend increases upon transition from initiation to elongation. In addition, AFM imaging in solution has made possible the study of dynamics of aqueous nucleic acids.[8] Scanning tunneling microscopic (STM) studies have included those of synthetic oligonucleotides and intercalatively bound metal complexes.[10]

DNA intercalators are small planar molecules that insert between the base pairs of duplex DNA. Intercalation into DNA necessarily results in changes in DNA conformation while separating base pairs along the helical axis; thus, intercalators are of great importance as probes of nucleic acid structure and as interferents of DNA replication, transcription, and/or topoisomerase activities. Relationships between structures of intercalators and the changes they cause in DNA conformation are subtle and remain unresolved.[11] DNA intercalators are widely used photochemotherapeutic agents.[12] Psoralens have drawn attention as they are used in the treatment of psoriasis, vitiligo, and other skin disorders.[13] Psoralens photoreact with nucleic acids when irradiated with long wavelength ultraviolet light (365 nm) to form mono- and diadducts to adjacent pyrimidine bases.[14] Diaddition results in covalent interstrand bridges or crosslinks.[15,16] The psoralen DNA covalent adducts are stable and the products of the photoreaction of psoralens with nucleic acids have been isolated and well-characterized.[17] The sequence specificity of psoralens and their impact on the secondary structure of DNA has also been well-documented.[18–21] Psoralens are generally not sequence specific nor do they induce bends in DNA. Because of their ability to form crosslinks, psoralens have become useful probes in the study of nucleic acid structure and function. Biotinylated psoralen derivatives have been synthesized for use as a hybridization probe. [22,23]

Colloidal gold particles are used routinely in the marking of proteins and membranes for visualization in both scanning and transmission electron microscopy. Gold particles are now being used in AFM as standards for assessing tip shape and sample compressibility.[24] Recently, Henderson prepared biotinylated DNAs in which the biotin moiety was covalently linked to terminal[25] and internal[26] nucleotides. Treatment of these modified DNAs with streptavidin-coated colloidal gold beads enabled recognition of the point of biotinylation. This labeling procedure may be exploited to map regions of interest in chromosomal DNA.

Our interest is in the application of SPM to characterize the structure of intercalating drug–nucleic acid complexes. Psoralen is the focus of our initial studies because its interaction with nucleic acids has been well characterized. Since the resolution of AFM is currently not sufficient to locate a single intercalated psoralen molecule, we have developed a marking system based on colloidal gold beads to facilitate recognition of the site of intercalation. Figure 1 illustrates the concept.

We have synthesized a biotinylated trimethylpsoralen to enable the attachment of the colloidal gold bead marker to the intercalator (see Figure 2). In this article, we demonstrate that this approach enabled the visualization of the site of intercalation and present the first AFM images of intercalating drug–nucleic acid complexes.

II. EXPERIMENT

Unless otherwise noted, all reagents were used as received from Sigma Aldrich. Nanopure® water (18.3 MΩ cm) was exclusively used in this work. Buffer solutions for AFM sample preparation were 10 mM ammonium acetate and 5

[a]Authors to whom correspondence should be addressed.

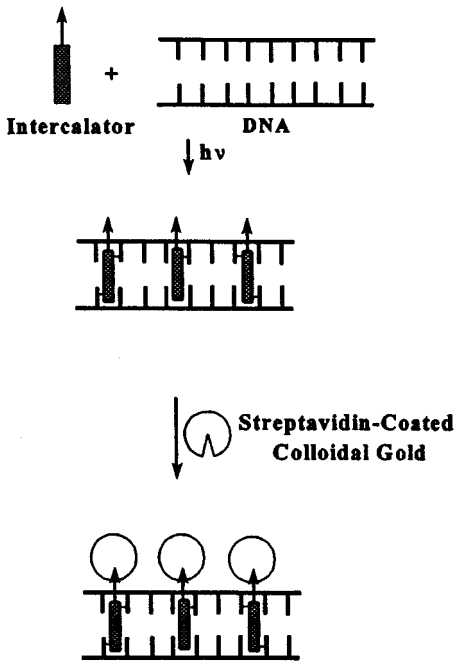

FIG. 1. Strategy for labeling sites of intercalation.

mM magnesium chloride, *p*H adjusted to 7.0 by addition of 5 M sodium hydroxide. All operations involving the intercalating drug were carried out under subdued lighting. *Caution: Psoralen and its derivatives are mutagenic. Appropriate precautions should be made when handling these materials.*

A. Synthesis of biotinylated trimethylpsoralen (5)

4′-chloromethyl-4,5′,8-trimethylpsoralen (2) was prepared from trioxsalen (Fluka)(1) as described by Isaacs *et al.*[27] ''Diamine psoralen'' (3) was prepared through reaction of (2) with N,N′-dimethylethylenediamine in accordance with the procedure described by Goldenberg *et al.*[28] A nearly quantitative yield was obtained by increasing the reaction time to 72 vs 24 h. The final step of the reaction sequence involved the reaction of NHS-Biotin (Pierce) (4) with (3) as delineated by Saffran *et al.*[22] The purified biotinylated trimethylpsoralen (5) ran as a single, sharp peak on a HPLC reverse phase column (Bondapak phenyl, Waters) in CH_3CN:0.05 M NH_4OAc 43:57. Fast atom bombardment (FAB) mass spectrometry in a ''magic bullet'' matrix exhibited an M+1 peak at m/z 555, consistent with the expected structure.

B. Preparation of plasmid DNA

The plasmid DNA used in this research was *p*BluBacHis b (In Vitrogen, V370-20) hereafter abbreviated as *p*BBHb. The circular 10.3 kb plasmid was transformed into competent HB101 cells. To amplify the plasmid, a 500 ml culture of the HB101 cells containing *p*BBHb was grown and the plasmid isolated according to the protocol of Maniatis *et al.*[29] The plasmid DNA was then purified using a CsCl gradient and concentrated by ethanol precipitation. After isolation, the DNA was then resuspended in 10 mM ammonium acetate/5 mM magnesium chloride/*p*H=7 buffer at a concentration of ~120 μg/ml as determined by ultraviolet (UV) absorbance at 260 nm.

C. Preparation of intercalated DNA

1. Method A

A 20 μL aliquot of the 120 μg/ml (2×10^{-8}) *p*BBHb plasmid in buffer was placed into a cap of a microcentrifuge tube that had been positioned upside down and glued to the bottom of a plastic Petri dish. The use of this plastic microcentrifuge tube cap reduces loss of DNA through adhesion to surfaces and facilitates the transfer of the intercalation reaction mixture once irradiation has been completed. A 20 μL aliquot of biotinylated trimethylpsoralen in buffer (whose concentration depended upon the desired ratio of intercalator to plasmid) was also placed into the cap and the solution was allowed to cool in a refrigerator (~4 °C) for 15 min. After cooling, the Petri dish containing the cap and solution was placed on ice and covered with a Corning filter No. 4303. This filter removes lower wavelength UV radiation that may photoreverse the psoralen crosslinking.[30] A hand held long wavelength UV lamp (Ultraviolet Products, Inc., model UVSL-58, λ_{max}=360 nm) was placed directly over the filter where it remained ~1 cm from the intercalation reaction mixture. The mixture was irradiated for 15 min before the lamp was removed. A 1 μL aliquot of 10 μg/ml (2×10^{-7} M) streptavidin-coated colloidal gold beads, 5 nm diam. (Amer-

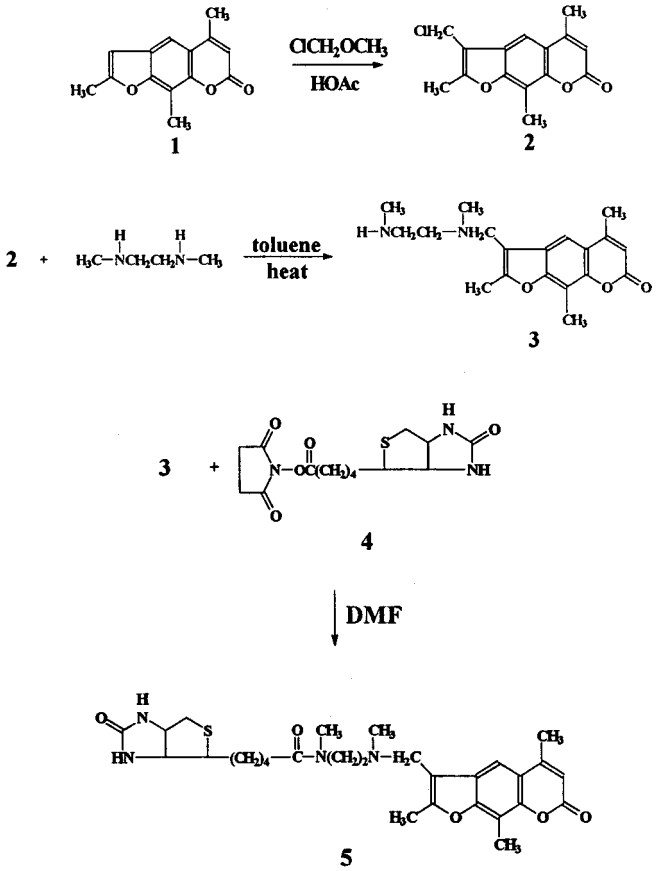

FIG. 2. Synthesis of biotinylated trimethylpsoralen.

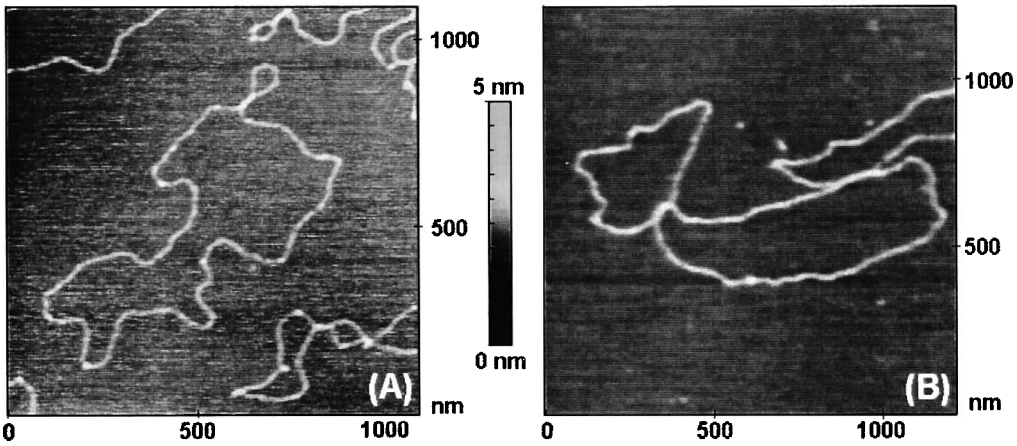

FIG. 3. (a) and (b) *p*BlueBacHis b circular plasmid DNA (10.3 kb) on mica.

sham), was added to the intercalated DNA solution and allowed to incubate for 1 h at room temperature.

2. Method B

A 20 μL aliquot of the 0.1 μg/ml (1×10^{-8} M) biotinylated trimethylpsoralen in buffer was placed into a microcentrifuge tube cap positioned as described in method A. A 1 μL aliquot of the 40 ug/ml (8×10^{-7} M) streptavidin-coated colloidal gold beads solution was added and allowed to incubate for 1 h at room temperature. A 20 μL aliquot of the 24 μg/ml (4×10^{-9} M) *p*BBHb plasmid in buffer was added to the gold bead labeled intercalator solution. The mixture was allowed to cool in a refrigerator ($\sim$4 °C) for 15 min and then irradiated as described in method A.

D. AFM sample preparation

The mica used in the experiment was muscovite green mica (New York Mica Co.). Mica disks 3/16 in. in diameter were punched (Ralmike Tool-a-Rama®), blown free of mica dust, and freshly cleaved utilizing the ''tape-press''method immediately before application of the DNA sample. A portion of the DNA solution was successively diluted to provide a final DNA concentration of $\sim$0.3 μg/ml. A 5 μL aliquot was placed directly onto a piece of freshly cleaved mica and allowed to stand for 10 min. The mica disk was then gently rinsed by dipping once into water, once into 50/50 ethanol/water, and twice into anhydrous ethanol. After carefully ''wicking'' dry with a Kimwipe,® the disk was blown dry with a clean, compressed chlorofluorocarbon gas (electronics cleaner, Memorex) at an angle normal to the disk surface. The disk was then stored overnight in a desiccator containing P_2O_5 prior to imaging.

E. AFM imaging

A Nanoscope II® (Digital Instruments) was utilized for imaging purposes. The samples were imaged under a minimum constant force in the repulsive contact regime as indicated by force distance curves obtained frequently during the imaging procedure. Total forces encountered were typically $<$10 nN. Cantilevers with a force constant of 0.10 N/m and oxide etched (i.e., sharpened) pyramidal Si_3N_4 probe tips with a radius of $\sim$20–40 nm (sharpened microlevers,® Park Scientific) were utilized. Tip radii and force constants were provided by the manufacturer. All images acquired are high resolution (i.e., 400 data points per scan line X 400 scan lines). Scan direction was maintained at 180°. This scan direction collects imaging data as the tip is ''drawn over'' the DNA plasmid versus being ''pushed over.'' Dry N_2 at a flow rate of 1 L/min was passed through the imaging chamber while imaging was in progress to minimize the effects of humidity.[31] Dryness of the samples was confirmed by the monitoring of initial force curves to assure that a minimum total force existed.

III. RESULTS AND DISCUSSION

The AFM sample preparation procedure as described above resulted in very clean images with backgrounds essentially devoid of buffer salt deposits. Figures 3(a) and 3(b) are images typical of those routinely obtained of 10.3 kb *p*BBH circular plasmids on mica. The plasmids in Fig. 3 measured 3300 nm in length, consistent with the expected length of a 10.3 kb plasmid in the B-DNA form. In all instances, whether imaging unintercalated or intercalated DNA, the immobilization scheme resulted in no movement of the DNA by the probe tip.

The sample preparation protocol yielded very clean images of intercalated DNA–Au conjugates as well. Figures 4(a)–4(d) contain images typical of those routinely obtained of the DNA psoralen–Au conjugates. In each of these images, features we attribute to being a single gold bead are apparent either on top of or adjacent to each plasmid. Isolated gold beads were seldom observed in the background indicating that the rinsing procedure effectively removes unbound markers from the substrate. These features were never observed in the following control experiments: (1) imaging of unintercalated DNA, (2) imaging of unintercalated DNA with streptavidin-coated gold, and (3) imaging of intercalated DNA without streptavidin-coated gold beads. The beads are generally uniform in size and shape. Bead dimensions are identical to those found in images of uncomplexed streptavidin-coated colloidal gold on Mg^{2+}-modified mica.

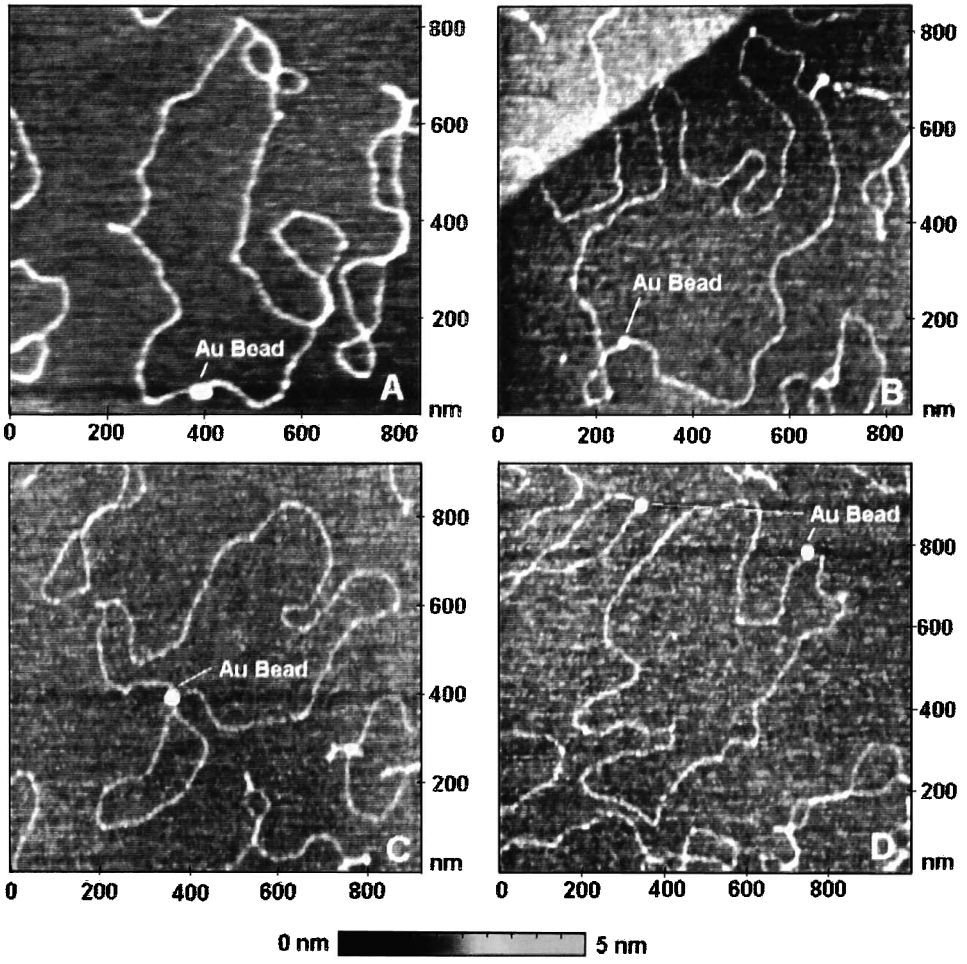

FIG. 4. (a)–(d) Intercalated plasmid DNA with colloidal gold marking the site of intercalation.

Widths of the DNA and gold markers are convoluted due to the size and geometry of the scanning probe tip. For a hemispherical tip, the apparent widths can be shown to be $(8dR)^{1/2}$ where R is the radius of the tip and d is the actual diameter of the feature being measured.[32] With this model, 5 nm diam. gold particles imaged with a probe tip radius of 20–40 nm should yield an image feature width of 28–40 nm. Similarly, DNA plasmids (with a Watson–Crick double helix diameter of 2 nm) imaged with a probe tip of the same radius should yield feature widths of 18–25 nm. In most instances, image feature widths were consistent with the expected ranges.

Figure 5 depicts the results of a typical line scan analysis performed on a high resolution image of a DNA psoralen–Au conjugate. The height of the gold marker in this particular line scan was 3.12 nm; gold marker heights ranged from 3 to 7 nm. Although frictional effects may introduce errors in the otherwise straightforward height determination of gold beads,[24] the observed range in height is consistent with the size distribution of the colloidal gold provided by the manufacturer. If one assumes that the measured height is an accurate estimate of the diameter of this particular gold bead, then the deconvoluted diameter of the DNA strand in Fig. 5 is 2.2 nm, in reasonable agreement with the crystallographically determined diameter of B-DNA.

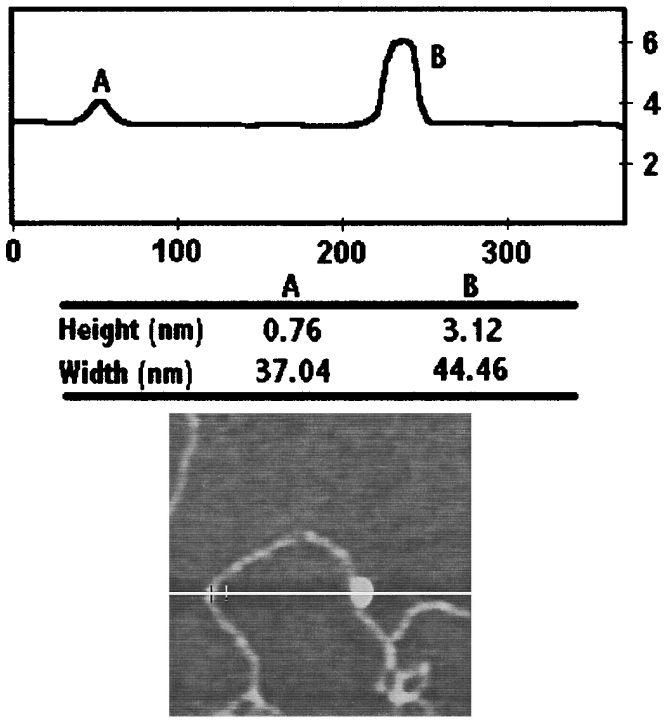

FIG. 5. Line scan analysis across a gold marker at high resolution.

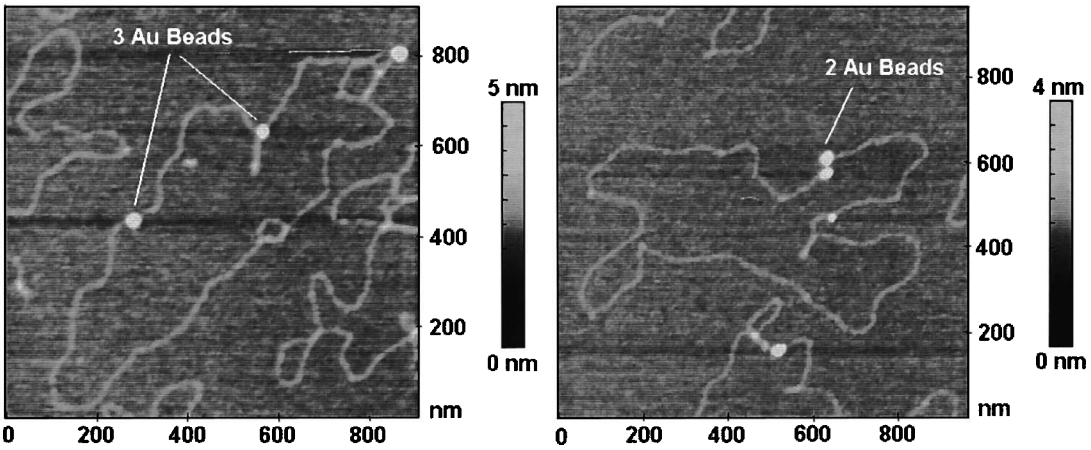

FIG. 6. (a) and (b) Plasmid DNA containing multiple gold markers.

Two different methods were used for preparing intercalated DNA samples. In both methods, a large ratio of biotinylated psoralen to DNA plasmid molecules was used to compensate for photodegradation of unbound psoralen that occurs during irradiation.[30] With method A, the biotinylated psoralen was intercalated prior to labeling with the gold marker. The ratio of marked plasmids to marker-free plasmids was always less than the ratio of gold beads to plasmids. This observation suggests either incomplete labeling of intercalated psoralen molecules or that the irradiation degrades a significantly higher than expected fraction of biotinylated psoralen.

To evaluate whether the intercalation-labeling reaction sequence was important for AFM visualization of the drug–DNA complex, the biotinylated psoralen was labeled with the gold marker prior to intercalation, i.e., method B. Image analysis showed that the ratio of marked plasmids to marker-free plasmids was always less than the ratio of gold beads to plasmids used in preparation of samples by this method. This observation supports the suggestion of significant photodegradation of the biotinylated psoralen. Alternatively, the reduced number of marked plasmids may also indicate that intercalation is inhibited by the short length of the spacer unit ($\sim$ 1.9 nm) separating the intercalating moiety and the biotin. We are currently synthesizing biotinylated psoralen molecules with longer spacer units to test this hypothesis. In either case, the observation of labeled plasmids prepared using method B provides convincing proof of concept that the site of intercalation can be identified by AFM.

A few plasmids were found with more than one gold bead. Figure 6(a) provides an image of a plasmid with three marked sites of intercalation. Figure 6(b) shows a plasmid with two marked sites of intercalation in close proximity, suggesting that the labeling approach used may provide useful information concerning the neighboring effects of intercalation. Plasmids with multiple intercalation sites may also provide information on the lengthening effects thought to occur with intercalation.

The preliminary results presented herein suggest that AFM may be a useful tool for determining the structural changes in nucleic acids induced by intercalation. Several important issues which remain include the length and chemical identity of the spacer separating the intercalating unit from the marker, the diameter of the marker as well as the mode of binding of the intercalator. In the present study, a very short, flexible ethylenediamine linker was used to couple the intercalating unit to the biotin-streptavidin marker. We are currently preparing biotinylated psoralens with longer linkers which may improve the efficiency of labeling as well as displace the marker from the plasmid in the image. This displacement may enable pinpointing of the site of intercalation with enhanced precision. We are also investigating the use of markers with smaller diameters; these will be essential for visualization of the neighboring effects of intercalation. Finally, the biotinylated psoralen used in this study was covalently bound to the plasmid. Yet, most intercalators are electrostatically bound. Thus, images of electrostatically bound intercalator–DNA complexes must be obtained before the potential of AFM in determining the structural changes in nucleic acids induced by intercalation can be properly assessed. Current efforts are directed towards this end.

IV. CONCLUSIONS

This article provides AFM images of intercalating drug–nucleic acid complexes providing proof of concept that colloidal gold markers enable recognition of the point of psoralen intercalation into nucleic acids. This approach should faciliate AFM studies of the subtle and currently unresolved relationship between intercalator structure and DNA conformation. Visualization of other intercalating drug–DNA complexes are currently under investigation using this approach.

ACKNOWLEDGMENTS

This research was supported by Bristol-Myers-Squibb company through a Research Opportunities Award from Research Corporation to one of the authors (L.A.B.). Partial support from the Georgia Tech Foundation, the Georgia Tech Research Corporation, and the Biomedical Research Program at Georgia Tech is gratefully acknowledged. The authors would also like to thank Tom Thundat, Bruce

Warmack, and David Allison of the Health Safety Research Division of Oak Ridge National Laboratory, Roger Wartell of Georgia Tech, and Ned Seeman of New York University for helpful advice and constructive comment.

[1]Y. L. Lyubchenko, P. I. Oden, D. Lampner, S. M. Lindsay, and K. A. Dunker, Nucleic Acids Res. **21**, 1117 (1993).
[2]H. G. Hansma, R. L. Sinsheimer, M. Li, and P. K. Hansma, Nucleic Acids Res. **20**, 3585 (1992).
[3]C. Bustamante, J. Vesenka, C. L. Tang, W. Rees, M. Guthold, and R. Keller, Biochemistry **31**, 22 (1992).
[4]T. Thundat, D. P. Allison, R. J. Warmack, M. J. Doktycz, K. B. Jacobson, and G. M. Brown, J. Vac. Sci. Technol. A **11**, 824 (1993).
[5]I. A. Mastrangelo, M. Bezanilla, P. K. Hansma, P. V. C. Hough, and H. G. Hansma, Biophys. J. **66**, 293 (1994).
[6]W. A. Rees, R. W. Keller, J. P. Vesenka, G. Yang, and C. Bustamante, Science **260**, 1646 (1993).
[7]M. N. Murray, H. G. Hansma, M. Bezanilla, T. Sano, D. F. Ogletree, W. Kolbe, C. L. Smith, C. R. Cantor, S. Spengler, P. K. Hansma, and M. Salmeron, Proc. Natl. Acad. Sci. USA **90**, 3811 (1993).
[8]E. Henderson, Nucleic Acids Res. **20**, 445 (1992).
[9]Y. Kim and C. M. Lieber, Scan. Microsc. **5**, 311 (1991).
[10]Y. Kim, E. C. Long, J. K. Barton, and C. M. Lieber, Langmuir **8**, 496 (1992).
[11]J. C. Wang, Amu. Rev. Biochem. **54**, 665 (1985).
[12]L. D. Williams and A. Rich in *Molecular Structure and Life*, edited by Y. Kyogoku and Y. Nishimura (Japan Science Society, Tokyo/Chemical Rubber, Boca Raton, FL, 1992), pp. 3–27.
[13]J. A. Parrish, T. B. Fitzpatrick, L. Tanenbaum, and M. A. Pathak, N. Engl. J. Med. **291**, 206 (1974).
[14]L. Musajo, F. Bordin, G. Caporale, S. Marciani, and G. Rigatti, Photochem. Photobiol. **6**, 711 (1967).
[15]R. S. Cole, Biochim. Biophys. Acta **217**, 30 (1970).
[16]R. S. Cole, Biochim. Biophys. Acta **254**, 30 (1971).
[17]D. Kanne, K. Straub, J. E. Hearst, and H. Rapport, J. Am. Chem. Soc. **104**, 6754 (1982).
[18]R. R. Sinden and P. J. Hagerman, Biochemistry **23**, 6299 (1984).
[19]V. Boyer, E. Moustacchi, and E. Sage, Biochemistry **27**, 3011 (1988).
[20]T. E. Harran and D. M. Crothers, Biochemistry **26**, 3792 (1987).
[21]F. Gaboriau, P. Vigny, and J. Moron, Biochemistry **28**, 5801 (1989).
[22]W. A. Saffran, J. T. Welsh, R. M. Knobler, F. P. Gasparro, C. R. Cantor, and R. L. Edelson, Nucleic Acids Res. **16**, 7221 (1988).
[23]E. L. Sheldon, C. H. Levenson, K. B. Mullis, H. Rapoport, and R. M. Watson, U.S. Patent No. 4,617,261 (14 October 1986).
[24]J. Vesenka, S. Manne, R. Giberson, T. Marsh, and E. Henderson, Biophys. J. **65**, 992 (1993).
[25]W. Shaiu, D. D. Larson, J. Vesenka, and E. Henderson, Nucleic Acids Res., **21**, 99 (1993).
[26]W. Shaiu, J. Vesenka, D. Jondle, E. Henderson, and D. D. Larson, J. Vac. Sci. Technol. A **11**, 820 (1993).
[27]S. T. Isaacs, C. J. Shen, J. E. Hearst, and H. Rapoport, Biochemistry **16**, 1058 (1977).
[28]M. Goldenberg, J. T. Welsh, R. Haas, D. Rideout, and C. R. Cantor, Biochemistry **27**, 6967 (1988).
[29]*Molecular Cloning: A Laboratory Manual*, edited by J. Maniatis, J. Sambrook, and E. F. Fritsch (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).
[30]D. W. Ussery, R. W. Hoepfner, and R. R. Sinden, in *Methods in Enzymology* (Academic, New York), Vol. 212, pp. 242–301.
[31]T. Thundat, R. J. Warmack, D. P. Allison, L. A. Bottomley, A. J. Lourenco, and T. L. Ferrell, J. Vac. Sci. Technol. A **10**, 630 (1992).
[32]T. Thundat, X. Y. Zheng, S. L. Sharp, D. P. Allison, R. J. Warmack, D. C. Coy, and T. L. Ferrell, Scan. Microsc. **6**, 903 (1992).