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Atomic force microscopy of DNA-colloidal gold and DNA-protein complexes

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ABSTRACT

The atomic force microscope (AFM)\(^1\) is capable of imaging and manipulating nucleic acids in solution and in air\(^2\)\(^{-9}\),\(^{13}\). We are developing methods for random and site-specific labeling of individual DNA molecules to facilitate manipulation of fragments excised in the AFM and for localization of specific DNA domains, such as protein binding sites and origins of replication. One successful method was to incorporate biotinylated nucleotides at random internal locations or specifically at the ends of linearized DNA molecules \textit{in vitro}. Following complex formation with SAG diameter streptavidin-gold conjugates, chromatographic purification and passive adsorption of the complexes to mica, the biotinylated domains were easily localized in the AFM by virtue of the distinctive size and shape of the streptavidin-gold complex. In many cases unconjugated streptavidin (i.e., lacking gold) was also observed attached to the biotinylated DNA. A second approach to site-specific labeling of DNA for imaging in the AFM was to react DNA with restriction enzymes having sequence-specific binding properties. Like the unconjugated streptavidin-DNA complexes, these enzyme-DNA complexes were visible without attached colloidal gold. Efforts to image DNA labeled \textit{in vivo} using bromodeoxyuridine (BrdU) and anti-BrdU antibodies are ongoing.

1. INTRODUCTION

The atomic force microscope (AFM)\(^1\) is capable of generating high, sometimes atomic, resolution images of biological and non-biological surfaces. DNA has been one of the most widely utilized test substances for imaging of biological samples by AFM\(^2\)\(^{-9}\),\(^{13}\). These studies have shown that it is possible to immobilize and image DNA in air and under solutions and obtain images similar to those obtained by electron microscopy of metal shadowed samples. By controlling the imaging force individual DNA molecules can be dissected by the AFM at desired positions and small fragments manipulated\(^2\),\(^4\).

We have begun to develop techniques for localization of specific sequences and directed recovery and manipulation of DNA fragments by AFM. One approach has been to incorporate biotinylated nucleotides into DNA and subsequently react the biotinylated DNA molecules with a streptavidin-gold (SAG) conjugate, usually 5nm diameter gold spheres. We have shown that DNA can be labeled with gold spheres randomly at internal locations or specifically at the ends of linear molecules. These labeled molecules can be deposited in an oriented fashion and reliably imaged by AFM in a dry nitrogen atmosphere\(^10\),\(^11\). The gold spheres can be moved easily by changing their adhesive interaction with the mica substrate as a function of relative humidity, suggesting that they could be used as physical handles for manipulating attached DNA\(^10\). A second approach has been to localize sequence-specific DNA binding proteins (restriction endonucleases) bound to specific sequences on a DNA molecule. The degree of success of this method apparently depends upon the restriction enzyme used. Current work is focused on exploiting the methods we have developed to visualize specific genes and active domains
(e.g., origins of replication) in chromatin and to isolate tagged DNA fragments for subsequent molecular and biochemical analyses.

2. MATERIALS AND METHODS

2.1 DNA Purification, Labeling and Analysis

Detailed methods have been previously reported \(^{10,11}\). Briefly, plasmid DNA, pUC119 or pSK+ (Stratagene), was prepared by alkaline lysis and CsCl-EtBr gradient purification. DNA was 5' end-labeled with bio-dUTP (Enzo Biochem, NY) by using Klenow fragment of \(E. coli\) DNA polymerase I to fill in 5' overhanging ends generated by digestion with Hind III. For random internal labeling, purified DNA was nick-translated with biotin-dUTP in the presence of DNase I and \(E. coli\) DNA polymerase I for 20 minutes at 37°C. Unincorporated bio-dUTP was removed by ethanol precipitation, and the bio-dUTP labeled DNA was resuspended in 10mM Tris-HCl (pH 7.2), 5mM MgOAc, 50mM NH4OAc, 1mM EDTA (TMNE) and incubated with 1 μl streptavidin-gold conjugate (Amersham) for 60 minutes at 25°C. For restriction enzyme binding studies purified DNA (pSK+; Stratagene) was mixed with the enzyme in 10mM Tris-HCl (pH 7.5), 1mM EDTA (TE) and incubated for various lengths of time at room temperature. The optimal enzyme-DNA binding conditions were determined by gel shift analysis (Fig. 3) in which increasing amounts of enzyme were mixed with a fixed amount of DNA.

Following DNA-protein complex formation and, in some cases, ethanol precipitation, the DNA-protein complexes (± gold) were separated from unbound protein (± gold) by chromatography through Bio-gel A-50 (Biorad) in 20mM Tris-HCl (pH 7.5), 100mM NaOAc (TN). Fractions containing gold labeled DNA were pooled and ethanol precipitated. Fractions containing protein-DNA complexes without gold were made 5mM in MgCl\(_2\) and deposited immediately on freshly cleaved mica (see below).

2.2 Sample deposition and AFM imaging

Biotin-streptavidin-gold-DNA (BSG-DNA) was resuspended in 20mM Tris-HCl (pH 7.2), 100mM NaOAc and 5mM MgCl\(_2\) (TNM) and deposited directly onto freshly cleaved mica (Ted Pella, Inc.) for imaging in the AFM, or further concentrated by ethanol precipitation. Restriction enzyme-DNA complexes were brought to approximately 5mM MgCl\(_2\) and deposited as follows. The sample was allowed to adsorb for 5 minutes or less. The mica was rinsed with 1 ml ddH\(_2\)O and exhaustively dried with N\(_2\) gas with the gas flow orthogonal to the surface. DNA prepared in this way was imaged in air at relative humidity <10%. For this report, all images were collected on a Nanoscope II or Nanoscope III (Digital Instruments, Inc., Santa Barbara, CA) using Si\(_3\)N\(_4\) tips (Digital Instruments, Inc., Santa Barbara, CA). In height mode images, the gray scale represents the sample height with lighter features being taller. In error signal mode images, the error signal is displayed, rather than the true image height.

2.3 Data preparation
3. RESULTS AND DISCUSSION

AFM images of streptavidin-gold (SAG) DNA complexes were readily obtained in air at low humidity. Fig. 1 shows several examples of SAG-DNA complexes in which the DNA was labeled at random internal sites by nick translation in the presence of biotinylated-dUTP (bio-dUTP) and subsequently reacted with SAG as previously described. The DNA has an appearance similar to that seen with unlabeled DNA under these conditions, its apparent width being strongly dependent upon the geometry and sharpness of the scanning tip used. Both individual labeled molecules and clusters of molecules were frequently observed. The formation of clusters is a consequence of the multivalent nature of streptavidin (four biotin binding sites per tetrameric protein). In some cases, the DNA was obviously labeled with a particle smaller than the gold spheres. Since it is known from EM studies that streptavidin-colloidal gold conjugates readily dissociate under some conditions (E. Henderson, unpublished) it is likely that the smaller particles observed are unconjugated protein.

Images of linearized, end-labeled plasmids were also collected. Several examples are shown in Fig. 2. Linearized plasmid DNA was labeled at termini by filling in restriction enzyme cleavage sites using DNA Pol I from E. coli (Klenow fragment) and the appropriate nucleotides, substituting bio-dUTP for dTTP. Both individual molecules and clusters were again observed.

Images were readily and reproducibly collected in air at low humidity. A critical condition for good image quality was that the complexes be chromatographically purified prior to deposition and that the deposited sample be washed well with water to remove excess salt residue.

A striking and reproducible feature of these experiments was the non-random, polar orientation of the molecules in the field (Figs. 1 and 2). This is the result of forceful drying of the surface with nitrogen gas and the reduced mobility of the gold labeled regions. The nitrogen causes the DNA to flow across the surface as it dries while the gold apparently retards the flow of the DNA, resulting in alignment of the molecules in the direction of the flow with the gold labeled end trailing. In experiments with linearized plasmid, molecules with both ends labeled were rarely observed, although, in principle, both ends had the same probability of being labeled with bio-dUTP and SAG. The reason for primarily observing linear molecules with only one end labeled is not clear, however, it brings to light the possibility of labeling linear DNA molecules specifically at one end (commonly done in restriction mapping and sequencing experiments). Using the deposition method described here and previously with molecules labeled at a specified end would result in the linearized molecules being both oriented and polarized with respect to sequence, a situation that would facilitate sequencing attempts by scanned probe techniques.

To label specific sites within a DNA molecule we attempted to attach a sequence specific DNA binding protein (the restriction endonuclease EcoRI) to circular DNA molecules. The optimal binding conditions were determined by electrophoretic gel mobility shift analysis using linear DNA fragments. Fig. 3 shows a typical example of a gel shift analysis for the restriction endonuclease EcoRI. The samples migrated from top (cathode) to bottom (anode) in the figure. A shift in the smaller (faster migrating) DNA fragment containing the EcoRI binding site to a position higher in the gel (i.e., with retarded mobility) is evident at the higher enzyme concentrations, indicative of protein-DNA complex formation.
Fig. 1. A gallery of AFM images of circular biotinylated DNA molecules labeled at internal sites with streptavidin-colloidal gold\textsuperscript{10}. The field and three of the four individual images were collected in error signal mode \textsuperscript{12}. The middle, right panel is a height mode presentation. The non-random orientation of the molecules is a consequence of the deposition/drying procedure (see text). Similar data have been presented in a previous publication \textsuperscript{10}. Bar = 100 nm.

Fig. 2. A gallery of AFM images of linearized, biotinylated DNA molecules labeled at the ends with streptavidin-colloidal gold\textsuperscript{11}. All data are presented as height mode images. As in Fig. 1, the molecules are aligned in the direction of flow of the stream of nitrogen used to dry the sample prior to imaging. Similar data have been presented in a previous publication \textsuperscript{10}. Bar = 100 nm.
Fig. 3. (above) Electrophoretic gel mobility shift assay to determine conditions for site-specific binding of EcoRI to DNA without hydrolysis. The lower band has a single EcoRI binding site. The shifted band, corresponding to the EcoRI-DNA complex, is indicated with a black arrowhead. Lane M contains DNA size markers. Numbers to the left indicate marker lengths in kilobase pairs. Units of EcoRI added to each reaction are indicated at the top of the gel. The direction of electrophoresis is from top to bottom. The conditions in lane 4, 80 units of EcoRI in TE buffer (see Materials and Methods) were used for this study.

Fig. 4. (right) A gallery of AFM images of EcoRI-DNA complexes. The field and top four individual molecules are error signal mode 12 images. The bottom four individual molecules are presented as height mode images. The protein appears as a bright spot, roughly circular in shape. The high background level of protein precludes definitive judgment, but the bending of the DNA upon putative association with the protein is consistent with the binding characteristics of EcoRI (and other DNA binding proteins). There is a single EcoRI site on each molecule. Multiple binding events to some molecules is probably due to "star" activity; altered binding specificity of the enzyme under non-standard incubation conditions.
formation. It is important to note that Mg$^{2+}$ was omitted from the binding reaction since the hydrolytic activity of the enzyme is strongly magnesium dependent, though its binding activity apparently is not. In these pilot experiments no attempt was made to reduce non-specific binding of the enzyme to the DNA. Moreover, this and other restriction enzymes are known to have altered sequence recognition characteristics under different reaction conditions, so some binding to the larger DNA fragment, which lacks an EcoRI site, would not be completely unexpected. However, in the figure it is apparent that the lower band is well shifted relative to its unbound position in the gel, therefore, the binding appears to be site-specific. We note that some apparent shifting of the upper band, lacking an EcoRI site, occurred at the highest enzyme concentration used. This could be the result of relaxed binding specificity due to the non-standard reaction conditions used or the presence of excess glycerol (which can cause migration artifacts during electrophoresis) in the samples with higher enzyme concentrations.

AFM images of the EcoRI-DNA complexes are shown in Fig. 4. A large number of molecules are observed with protein apparently attached to them. Although the incomplete separation of protein-DNA complexes from unbound protein precludes detailed and definitive analysis of the putative complexes, we note that the protein appears to bend the DNA upon binding, a characteristic expected for EcoRI and some other DNA binding proteins (e.g., RNA polymerase). Experiments to improve the sample preparation and purification method, and demonstrate binding site specificity, are ongoing.

4. SUMMARY

Complexes of SAG-DNA and EcoRI-DNA have been prepared and imaged by atomic force microscopy. These and other studies illustrate the ease with which DNA-protein complexes can be imaged in the AFM. We have imaged these complexes both in dry air and in solution (propanol; data not shown). Recent work from another laboratory strongly suggests that similar images can be obtained in aqueous environments (H. Hansma, personal communication).

Given the simplicity of sample preparation and image acquisition demonstrated by this and many other studies, it is clear that the AFM has much to offer in the area of structural biology. While major strides have been made in this area, the ability to rapidly prepare and characterize samples by AFM, coupled with the ability to manipulate them and measure forces at the single molecule scale, indicates that the AFM and its descendants will make important contributions in many areas of the life sciences including genetic engineering, molecular cell biology, molecular development, and neurobiology.

5. ACKNOWLEDGMENTS

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6. REFERENCES