Mapping site-specific endonuclease binding to DNA by direct imaging with AFM

D. P. Allison*, T. Thudia†, P. Modrich‡, R. J. Isfort§, M. J. Doktycz*, P. S. Kerper*, and R. J. Warmack*

*Health Sciences Research Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6123
†Department of Biochemistry, Duke University Medical Center, Durham, NC 27710
‡CP&RSD/ISD, The Procter & Gamble Company, Cincinnati, OH 45239-8707

ABSTRACT

Physical mapping of DNA can be accomplished by direct AFM imaging of site specific proteins bound to DNA molecules. Using Gln-111, a mutant of EcoRI endonuclease with a specific affinity for EcoRI sites 1000 times greater than wild type enzyme but with cleavage rate constants reduced by a factor of 104, we demonstrate site-specific mapping by direct AFM imaging. Images are presented showing specific-site binding of Gln-111 to plasmids having either one (pBS*) or two (pMP*) EcoRI sites. Identification of the Gln-111/DNA complex is greatly enhanced by biotinylation of the complex followed by reaction with streptavidin gold prior to imaging. Image enhancement coupled with improvements in our preparation techniques for imaging large DNA molecules, such as lambda DNA (47 kb), has the potential to contribute to direct AFM restriction mapping of cosmid-sized genomic DNAs.

Keywords: DNA, DNA-protein interaction, Scanning Probe Microscopy, Mapping

1. INTRODUCTION

Sequencing genomes is one of the ultimate goals of genomic research. To sequence DNA using conventional methods, individual chromosomes must be cut into increasingly smaller fragments that can be replicated and characterized. The smallest fragments are the sequencing vectors, roughly 4000 bp in size, that after sequencing must be reassembled in the correct order, or mapped, to their correct position on the chromosome.

Physical mapping identifies physical or chemical characteristics on DNA molecules. The lowest resolution physical maps are the chromosomal or cytogenetic maps that identify, by light microscopy, specific banding patterns on stained intact chromosomes. This technique is useful both for identifying chromosomes that are similar in size and for locating genes or gene fragments to specific chromosomes. The latter is accomplished by tagging the DNA marker with a radioactive or fluorescent label and finding the position of the labeled probe bound to its complementary strand on the intact chromosome. The resolution of this technique, fluorescence in situ hybridization (FISH), is roughly 2 Mb on condensed chromosomes and 100 Kb or less on compact interphase chromosomes. Higher resolution physical maps are generated by cutting individual chromosomes into clonable cosmid or P1-sized fragments that are mapped and ordered into contiguous blocks of DNA (contigs).

High-resolution restriction mapping of cosmid and P1-sized genomic clones, 40 kb and 95 kb respectively, is labor intensive. Current mapping strategies rely upon multiple partial restriction endonuclease digestions, gel fractionation, Southern blotting, and hybridization with labeled end probes, or fingerprinting which requires multiple overlapping clones. Generation of a single high-resolution EcoRI and/or BamHI restriction map of a 95-kb P1 clone requires roughly three weeks work and may be compromised by the presence of “fast recognition sites.”

The primary goal of this study was to develop a new physical mapping technique combining the imaging capabilities of the atomic force microscope (AFM) with the documented site-specific attachment of restriction enzymes to DNA molecules. Using a mutant EcoRI endonuclease modified to bind, but not cut DNA, we have identified both single and double EcoRI sites on plasmid molecules. When fully developed, using this direct imaging technique, we anticipate restriction mapping one intact cosmid or P1 clone per day, a great savings of time over conventional methods.
2. MATERIALS AND METHODS

2.1 DNA

The plasmids used in this study were pBS+ (3204 bp, from Stratagene, LaJolla, CA), with one EcoRI site and pMP32 (4100 bp, ATCC Rockville, MD), with two EcoRI sites 1400 bp apart. The linearized pBS+ was prepared by treatment with Smal. All of the DNAs were maintained in 0.01 M ammonium acetate buffer at pH 7.2.

2.2 Restriction endonuclease

The EcoRI restriction enzyme used is a genetically engineered mutant having Gln substituted for Glu at position 111 in the amino acid sequence. The consequence of this mutation is that the specific affinity of the Gln mutant for EcoRI sites is 1000X greater than that of the wild type endonuclease while the rate constants for first and second strand cleavage are reduced by a 104-factor.12

2.3 Binding Gln-111 endonuclease to DNA

Binding of Gln-111 to pBS" or to pMP32 was accomplished by incubating 1 mg of DNA with 0.5 mg of enzyme in 0.1 M Tris + 0.001 M EDTA at pH 7.6. After 1 hr at room temperature the 40-ml reaction mixture was pipetted onto a Sephacryl 300 column (Pharmacia, Piscataway, NJ) constructed by cotton plugging a Pasteur pipette, adding an aqueous suspension of Sephacryl 300 to a height of 4.5 cm, and equilibrating the column with 0.005 M ammonium acetate pH 7.0. The column was eluted with 0.005 M ammonium acetate at pH 7.0, and collected in 7-drop fractions (~160 ml/fraction). Fractions 5 or 6, containing the bulk of the DNA, were used for AFM imaging.

2.4 DNA-EcoRI complex signal amplification

We amplified the signal from the mutant EcoRI-DNA complex by biotinylating the endonuclease and reacting with streptavidin/10-nm gold.3 The biotinylation of Gln-111 was accomplished by incubating 1.8 mg of either pBS" or pMP32 with 0.98 mg of Gln-111 in 40 ml of 0.1 M sodium bicarbonate at pH 8.5 for 60 min. at room temperature. Biotin-XX-NHS Ester (Clontech, Palo Alto, CA) was dissolved in n,n-dimethylformamide (Aldrich, Milwaukee, WI) at a concentration of 2 mg/ml and 3 ml was added to the DNA-endonuclease mixture, incubated at room temperature for 90 min. The solution was then placed on a Sephacryl 300 column, eluted with 10 mM sodium bicarbonate at pH 8.5, and collected in seven-drop fractions. A 100-ml aliquot of fraction 5 or 6 was mixed with 5-ml of streptavidin-10nm gold (Sigma, St. Louis, MO), incubated at room temperature for 90 min., and prepared for imaging with the AFM.

2.5 AFM imaging

DNA and DNA-endonuclease preparations at DNA concentrations of 0.2 to 0.4 mg/ml were prepared for imaging by adding magnesium chloride or magnesium acetate to a final concentration of 0.005 M to 100 ml of sample. The Mg2+ ion was added to stabilize the DNA on the substrate and to prevent subsequent removal by the AFM probe tip.4 A 30-ml aliquot was pipetted onto each of three 3/8-inch diameter freshly cleaned mica disks (punched out with a hole punch), incubated for 10 min. and, without drying, rinsed by plunging 10X into deionized distilled H2O, followed by rinsing with a stream of deionized distilled water, rinsed by plunging 10X into 1:1 H2O/EtOH, followed by 3 rinses in 100% EtOH, the last of which is critical point dried.5 After drying the samples were imaged in either a Nanoscope II (Digital Instruments, Santa Barbara, CA) operated in contact mode, or a Nanoscope III operated in tapping mode.

3. RESULTS AND DISCUSSION

3.1 DNA Strand Measurements

Accurate length measurements of DNA strands taken from AFM images of plasmids clearly demonstrate the feasibility of
making reliable assignments of marker positions along DNA molecules. Images of fields of 2-4 nm showed several molecules which were analyzed by software (NIH Image 1.47). Contour-length measurements proved to be very easy using the built-in tools of the program. Figure 1 shows the contour lengths of more than two hundred images of both circular and linearized pBS* plasmid. Although a distribution of sizes is observed, the peak is at 972 nm for the circular form and 957 nm for the linear form. This is very close to the 0.34 nm/bp expected for B-form DNA in solution. The standard deviation is 5.3% and 5.6%, respectively.

3.2 EcoRI binding to single site pBS*.

To determine the feasibility of directly mapping restriction enzyme sites on DNA molecules we chose Gln-111 a EcoRI mutant that specifically binds to the EcoRI site but effectively does not cleave DNA. EcoRI endonuclease is a small dimeric globular protein of known sequence with a molecular weight of 62,000 Daltons. The nucleic acid recognition site is GAATTC and the endonuclease binds as a dimer to this site and the complementary site on the duplex DNA molecule.

Crystallographic analysis shows the two subunits of the enzyme forming a globular structure with the DNA embedded in one side. The complex as a whole is 5.0-nm wide while the diameter of a DNA molecule is 2 nm. We would therefore predict, from previous studies that we have published on tip-sample interactions, that at the point where the enzyme is bound to the DNA molecule the imaged width and height should be ~50% larger, and therefore clearly resolved by AFM.

The results of reacting the Gln-111 mutant EcoRI endonuclease with pBS* plasmid that has one EcoRI binding site is shown in Figure 2. This is a contact mode AFM image and three of the plasmid molecules clearly show the bound endonuclease (small arrows). As predicted, the DNA-enzyme complex appears to be ~50% wider than the DNA molecule and since the Z-scale (height) is from dark (low) to light (high) the complex appears lighter than the plasmid molecule. A fourth molecule (large arrow) cannot be scored for endonuclease binding due to background contamination. This is a problem inherent to probe microscopes where imaging is accomplished by sensing surface topography and as a consequence both sample and contaminants will be imaged. We are continuing to work toward reducing background contamination in DNA samples.

The AFM image in Figure 3 shows three circular pBS* molecules site specifically labeled with the mutant EcoRI endonuclease Gln-111. After forming site specific complexes...
with plasmid molecules, the enzyme was biotinylated and reacted with streptavidin-gold to amplify the signal. The single enzyme site on three plasmid molecules is clearly visible and could be accurately identified at lower magnifications which would greatly facilitate imaging and mapping larger DNA molecules. When compared to Figure 2 where only the enzyme-DNA complex was imaged, the effect of amplifying the signal with streptavidin-gold is remarkable.

3.3 EcoRI binding to double site pMP32

Our next experiment was to determine if more than one EcoRI site on a DNA molecule could be mapped by AFM imaging. For this purpose the Gln-111 endonuclease was incubated with the plasmid, biotinylated, reacted with streptavidin-gold, and prepared for imaging as previously described. In Figure 4a both of the EcoRI sites can be seen to be labeled with the expected distance between the two markers. A line scan through one of the gold spheres (Fig. 4b) shows the expected height of the gold sphere to be slightly less than 10 nm, well within experimental error, while the width of the sphere is greater than 10 nm due to the convolution effects introduced by the probe tip. In Figure 4c both of the EcoRI sites on the plasmid have been specifically labeled with the Gln-111 endonuclease complexed with biotin-streptavidin-gold. However, the gold spheres have become associated with one another and it is not possible to determine the distance between the EcoRI sites on this molecule. This is a problem that we have identified in these preliminary studies and one that we must address in future work.

3.4 AFM Imaging of large DNA molecules

Improvements in both instrumentation and in sample preparation techniques have made possible the routine imaging of large DNA molecules. Single-scan AFM images of a 50-kb DNA molecule, such as lambda phage DNA (Fig. 5), would have been impossible to accomplish with the technology that existed two or three years ago. Continued improvements in instrumentation include increasing the potential scan area from roughly 2-nm square to over 100-nm square. The implementation of a resonating cantilevered probe with cyclical contact (Tapping Mode, Digital Instruments, Inc. Santa Barbara, CA), has eliminated disturbances due to lateral forces introduced by direct
contact scanning. This mode of operation also greatly reduced annoying interference waves that obscured tiny structures such as DNA. Combining the results of our theoretical studies of cantilever resonance and damping\textsuperscript{13}, we found that a helium atmosphere greatly improved instrument performance and image quality. A further improvement involving phase detection was recently installed that allows very sensitive feedback to surface features and eliminates interference waves. The result is that high quality, high contrast images of DNA on mica can now be routinely obtained in minutes.

Sample preparation methodology has also been developed over the past few years. Basically the AFM is a topographical imaging system and both DNA and other structures that are either inherent or applied to the mounting surface will be imaged. In our laboratory we have deliberately focused our efforts to eliminate background adsorbates on mounting surfaces. We use volatile buffers such as ammonium acetate with our DNA preparations. We have systematically reduced other potential sources of background contamination, such as the minimum amount of Mg\textsuperscript{2+} that must be added to the mounting medium to stabilize DNA. Finally extensive rinsing of DNA preparations in both H\textsubscript{2}O and EtOH followed by critical point drying, in which the liquid goes to the gas phase at equal pressure, has eliminated spurious background salts and greatly reduced artifacts inherent in air dried preparations. These improvements have collectively made imaging routine for large DNA molecules, such as lambda\textsuperscript{4} or kinetoplast\textsuperscript{6} DNA and similar success in imaging cosmid or P1 clones should be expected.

4. SUMMARY

Our results support the idea that it would be both possible and practical to develop a direct physical mapping technology using AFM imaging to locate restriction enzymes bound to specific sites on large DNA molecules. The gold labels form a readily recognized signal that can be statistically verified for stable binding positions in a very straightforward manner. When developed it would be reasonable to expect, using this technology, that cosmid or P1-sized clones could be restriction mapped at high resolution on the intact molecules.

5. ACKNOWLEDGMENTS

This research was sponsored by the Office of Health and Environmental Research, U. S. Department of Energy, under Contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.
6. REFERENCES


DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.