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T. Müller-Reichhert

*European Molecular Biology Laboratory, Germany*, reichert@embl-heidelberg.de

H. Gross

*Eidgenössische Technische Hochschule-Hönggerberg, Switzerland*

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## MICROSCOPIC ANALYSIS OF DNA AND DNA-PROTEIN ASSEMBLY BY TRANSMISSION ELECTRON MICROSCOPY, SCANNING TUNNELING MICROSCOPY AND SCANNING FORCE MICROSCOPY

T. Müller-Reichert<sup>1\*</sup> and H. Gross<sup>2</sup>

<sup>1</sup>European Molecular Biology Laboratory, Heidelberg, Germany,

<sup>2</sup>Institut für Zellbiologie, Eidgenössische Technische Hochschule-Hönggerberg, Zürich, Switzerland

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### Abstract

To investigate DNA and DNA-protein assembly, nucleic acids were adsorbed to freshly cleaved mica in the presence of magnesium ions. The efficiency of DNA adhesion and the distribution of the molecules on the mica surface were checked by transmission electron microscopy. In addition, various kinds of DNA-protein interactions including DNA wrapping and DNA supercoiling were analyzed using electron microscopy. In parallel, this Mg<sup>2+</sup>/mica method can be applied (1) to analyze embedded DNA by scanning tunneling microscopy, (2) to visualize freeze-dried, metal coated DNA-protein complexes by tunneling microscopy, and (3) to image DNA or DNA-protein interaction in air or in liquid by scanning force microscopy. An advantage of such a correlative approach is that parallel imaging can reveal complementary information. The benefit of such a combined approach in analysis of protein-induced DNA bending is discussed.

**Key Words:** Transmission electron microscopy, scanning electron microscopy, scanning tunneling microscopy, scanning force microscopy, DNA, DNA-protein interaction, Repressor Activator Protein (RAP) 1.

### Introduction

The various conformational states which can be adopted by DNA molecules have been intensively studied by X-ray diffraction [40]. Visualization of individual DNA molecules, however, was only possible after the development of the transmission electron microscope (TEM) (Fig. 1a). Techniques had to be developed to visualize immobilized, dehydrated molecules under high vacuum conditions. One of the most widely used techniques for the preparation of nucleic acids for TEM was reported by Kleinschmidt and Zahn [29]. Homogenous spreading of the molecules was achieved by binding them to a thin layer of an unspecific DNA-binding protein (i.e., cytochrome C). DNA was then adsorbed to carbon coated TEM-grids prior to contrast enhancement by rotary shadowing at low elevation angles. By application of this "Kleinschmidt" technique, the contour length of individual molecules could be measured. However, when the "Kleinschmidt" technique is applied, the DNA is covered by the cytochrome C and due to this concomitant broadening, details of DNA bound proteins underneath are obscured! A technique for protein free spreading of DNA was first proposed by Hall *et al.* [26]. DNA molecules were sprayed onto the surface of freshly cleaved mica, shadowed with heavy metal and replicated with C or SiO. The adsorption of DNA by this technique, however, was rather uncontrolled and occasional. The technique has been modified by the application of Al<sup>3+</sup> [24] and Mg<sup>2+</sup> [30] to make the molecules adhere to the solid support. With this Mg<sup>2+</sup>/mica technique, proteins bound specifically to nucleic acids could be visualized [30].

In the beginning of the 1980s a new class of microscope was invented, the scanning probe microscope (SPM). The most prominent microscopes of this new imaging technology are the scanning tunneling microscope (STM) [9] and the scanning force microscope (SFM) [7]. Common to both STM and SFM is the potential to measure surface related properties at atomic resolution. In general, topographic information of a

\*Address for correspondence:

T. Müller-Reichert

European Molecular Biology Laboratory

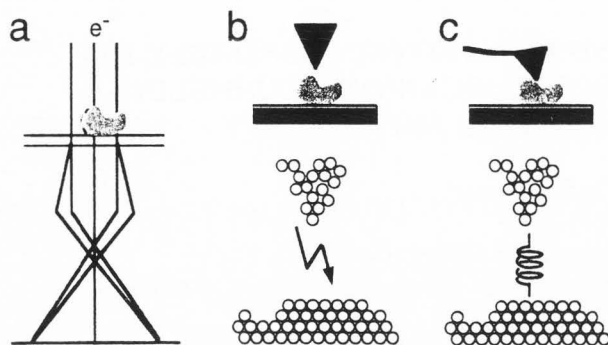
Postfach 10.2209

D-69112 Heidelberg, Germany

Telephone Number: +49-6221-387360

FAX Number: +49-6221-387 512

E-mail: reichert@embl-heidelberg.de



**Figure 1.** Imaging modes for analysis of DNA and DNA-protein complexes. (a) In TEM absorption and phase contrast are used to create a two-dimensional projection of a sample. (b) A tunneling current between metal tip and a conductive sample is recorded in STM to create a three-dimensional image of a scanned surface. (c) Three-dimensional image formation in SFM occurs by recording forces between a tip (cantilever) and a non-conducting surface.

given sample can be obtained in air and in liquid. This is in contrast to the TEM, where high vacuum conditions are necessary for imaging.

In STM a sharp, conductive probe (i.e., the tip) is attached to a piezoelectric XYZ-scanner and brought into close proximity to the surface of a conductive sample (Fig. 1b). During lateral scanning of biological objects, the tunneling tip is raised and lowered to keep the tunneling current constant (constant current mode). If the lateral position of the tip is plotted against the elevation of the probe, a three-dimensional non-contacting image of the surface features can be obtained. In contrast, SFM records interatomic forces between the atoms of the tip and the atoms of the sample during scanning of a cantilever over a given specimen (Fig. 1c). The tip, acting as a spring, is moved in raster fashion over the surface. Laser light is focussed on the back of the spring and deflections caused by the tip during scanning are measured by a segmented photodiode.

For both of the new imaging techniques, methods have to be available to immobilize biomolecules (i.e., make them adhere) onto solid supports. Moreover, when imaging under atmospheric conditions is intended, methods to properly dehydrate the specimens have to be applied. Techniques of preparation developed for TEM to immobilize and dehydrate biological specimens are of special importance in this respect. In this paper we discuss the application of mica as a support for TEM as well as for STM or SFM preparation. Particular attention will be paid to the fact that this technique offers the possibility to perform TEM and SPM experiments in

parallel. Different kinds of DNA-protein interactions will be discussed. As an example the binding of the yeast Repressor Activator Protein 1 (RAP1) [22] to cloned DNA molecules containing telomeric, sequence-specific protein recognition sites will be described. In addition, advantages and disadvantages of using TEM, STM or SFM in the analysis of DNA-protein assemblies will be discussed.

## Materials and Methods

### Film thickness measurements

The metal coat thickness was measured with a quartz monitor (Balzers QSC 301; Balzers, Liechtenstein) which was oriented always perpendicular to the coating source. The film thickness was determined in the direction of the metal deposition and calculated according to the density of bulk platinum.

### Preparation of DNA for TEM

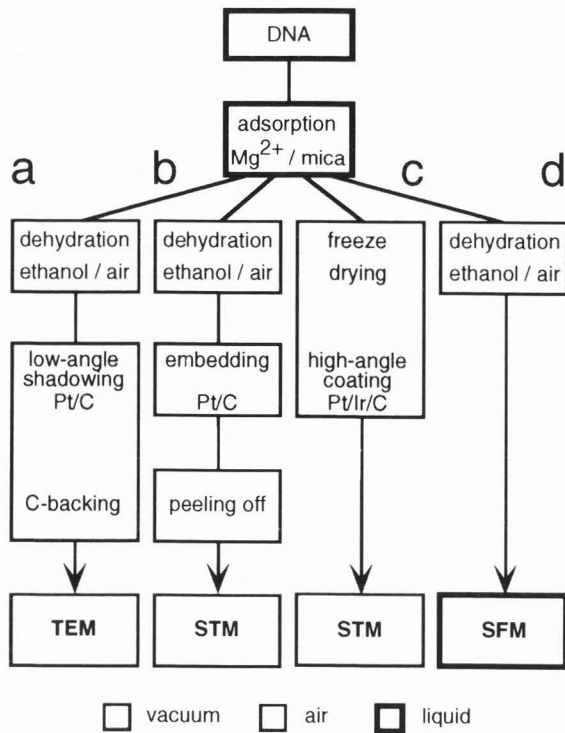
For standard electron microscopy linear DNA fragments (409 bp; 10 ng/ $\mu$ l, diluted 7 times in 8 mM Mg-acetate) were adsorbed to freshly cleaved mica (Ruby B, BAL-TEC, Liechtenstein) and washed in double-distilled water for 3 hours [41]. Dehydration of the samples was carried out using ethanol. The specimens were rotary shadowed (BAL-TEC BAF 400T) with 5nm Pt/C at an elevation angle of about 3° and stabilized at 90° with a thin layer of carbon (6-7 nm). Replicas from the mica were floated onto the surface of bidistilled water, collected on 400 mesh copper grids, and analyzed in a Philips CM 12 (Philips Electron Optics, Eindhoven, The Netherlands) transmission electron microscope operated at an acceleration voltage of 100 kV.

### Replica/anchoring technique for analysis of bare DNA

DNA fragments were deposited on freshly cleaved mica, washed, and dehydrated from ethanol in air as described above. Samples were rotary coated with 8 nm of Pt/C at room temperature at an elevation angle of 65° in the BAL-TEC BAF 400T. After evaporation of Pt/C a copper disk was glued with epoxy adhesive onto the Pt/C film. Finally, the copper disk with the DNA embedded in Pt/C was peeled off the mica. Imaging of the previously mica-exposed side of the Pt/C film allowed analysis of uncoated DNA molecules by STM. The mica exposed side has also been analyzed by SFM using a Nanoscope II [35].

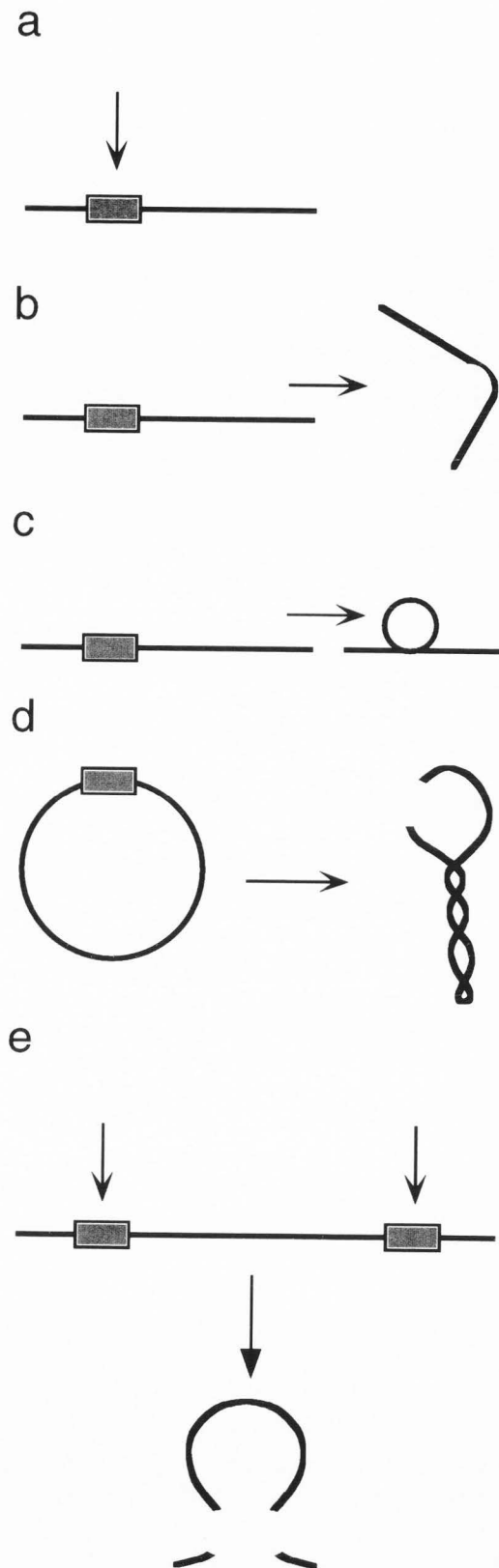
### Metal-coating of DNA for STM

With the Mg<sup>2+</sup>/mica technique DNA was immobilized on freshly cleaved mica. Samples were washed (3 hours in distilled water) and dehydrated by ethanol/air or by freeze-drying. For freeze-drying samples were mounted on a specimen table under liquid nitrogen prior



**Figure 2.** Flow diagram illustrating the main steps involved in the preparation of nucleic acids for TEM, STM and SFM. In the presence of magnesium ions DNA can routinely be adsorbed on freshly cleaved mica. (a) In standard TEM, DNA molecules then have to be dehydrated from ethanol in air, rotary shadowed at low elevation angles and coated with carbon (C backing). A replica of the preparation can be analyzed in the electron microscope. (b) For analysis by STM, DNA may be embedded in a thick layer of Pt/C after dehydration from ethanol in air. By peeling off the metal layer, the previously mica exposed side of the embedded DNA can be analyzed under atmospheric conditions. (c) Alternatively, freeze-drying can be applied to dehydrate the biomolecules. By high-angle metal coating reproducible imaging of DNA by STM in air can be achieved. (d) When analysis of DNA by SFM is intended imaging in buffer solution is the method of choice. To prevent lateral dislocation of the DNA on the support, dehydration of the DNA from ethanol in air prior to imaging might be necessary.

**Figure 3.** Types of DNA-protein interactions. Microscopic techniques may be applied to analyze the following features: (a) site specific occupation, (b) protein-induced DNA bending, (c) DNA wrapping, (d) DNA supercoiling, and (e) DNA higher order structure induced by protein-protein interactions. Black line: DNA molecule; grey rectangle: protein recognition site; grey circle: sequence specific DNA-binding protein.



to transferring the specimen onto the precooled cold stage (143K) of a freeze-etch unit (BAL-TEC BAF 400T). Freeze-drying was carried out for 3 hours at 193 K under high vacuum conditions ( $P < 10^{-6}$  mbar). Ethanol-dried samples were mounted on the specimen table at room temperature, transferred to the freeze-etch unit, and cooled down to 193 K prior to metal coating. Both ethanol-dried and freeze-dried samples were coated at 193 K by rotary shadowing them with 0.7-1 nm Pt/Ir/C at an elevation angle of  $65^\circ$  [3, 46]. The samples were warmed to room temperature after shadowing and removed from the vacuum.

### STM imaging conditions

STM measurements were performed in the constant current mode under atmospheric conditions. A modified Park Scientific (Sunnyvale, CA) Tunneling Microscope, operated at a tunneling voltage in the range of 350 mV was used. The tunneling current was kept below 25 pA. The scan rate was usually 0.5-2 Hz. Tungsten tips (0.5 mm diameter) were electrochemically etched in a 1 M KOH solution by applying a DC voltage of about 15 V between the positively biased tungsten wire and a grounded Pt electrode.

## Results and Discussion

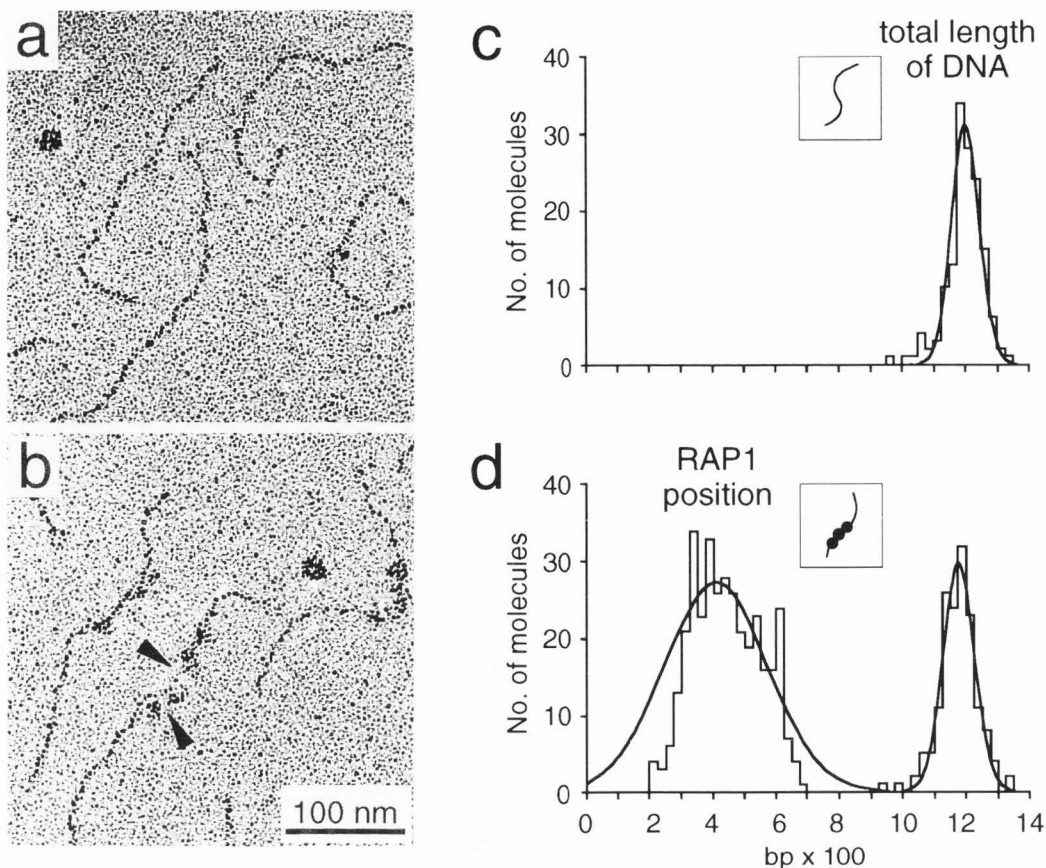
### Application of standard TEM to visualize DNA-protein assembly

The major steps involved in routine preparation of DNA for TEM are shown schematically in Fig. 2a. By addition of magnesium ions, DNA molecules are immobilized on a freshly cleaved mica surface. Dehydration of the sample is carried out from ethanol in air. To gain contrast, DNA has to be rotary shadowed with Pt/C at low elevation angles under high vacuum conditions. Carbon backing is applied for subsequent floating the carbon film on the surface of distilled water. Finally, the replica can be analyzed in the electron microscope [41]. When applying this routine preparation technique a homogenous spreading and a proper immobilization of DNA molecules on the solid support can be achieved.

Using low-angle shadowing by heavy metals to enhance the contrast of the small, fibrous DNA molecules on mica, a major disadvantage of this technique is the induction of self-shadowing. Owing to this effect, large metal clusters are growing not only in the background of the specimen, but also along the trace of the DNA molecules. As a consequence, the structure of the DNA itself (i.e., the sequence of bases) is obscured. Nevertheless, analysis of contour lengths can be carried out to determine the total length of linear DNA fragments and the correct positioning of bound protein complexes. Application of TEM imaging in analysis of different types of DNA-protein interactions is shown

schematically in Fig. 3. The following types of interaction can be analyzed: (a) site specific occupation, (b) DNA bending, (c) DNA wrapping, (d) DNA supercoiling, and (e) DNA higher order structure. Site specific occupation of proteins can be analyzed by binding of a sequence specific DNA-binding protein to cloned DNA fragments containing a single, sequence specific binding site [34]. By this kind of analysis not only the accuracy, but also the efficiency of protein binding can be determined. Cloned DNA fragments containing a single, asymmetrically located binding site are also applied to analyze protein-induced DNA bending [34]. Interpretation of the images, however, is limited, because of the low-angle shadowing procedure (see below: STM of metal-coated DNA and DNA-protein complexes). DNA wrapping can be analyzed by binding of proteins to multiple sequence specific binding sites on relatively short DNA fragments [23]. Depending on the size of the DNA binding protein, wrapping of the substrate double helix should be accompanied by a considerable shortening in the length of a DNA fragment when analyzed by TEM. By this kind of analysis a possible wrapping of telomeric DNA by RAP1 could be excluded (Fig. 4). No shortening of the DNA was observed after multiple occupation of RAP1 binding sites. In contrast, Gyrase, a 200 kDa protein of *E. coli*, for example, is known to wrap a segment of 120 to 155 bp of DNA around itself [39]. TEM may also be applied to analyze protein-induced supercoiling [23]. For this purpose proteins have to be bound to sequence specific binding sites on circular DNA molecules. Alternatively, TEM may also be used to investigate the role of protein-protein interactions in establishing higher order DNA structures [42, 43].

The  $Mg^{2+}$ /mica technique allows a routine, protein-free (i.e., cytochrome C-free) spreading of single DNA molecules. Moreover, by the application of low-angle rotary shadowing for TEM imaging, a number of different DNA-protein interactions can be analyzed. The limitations of this kind of contrast enhancement, however, are twofold: First, analysis of DNA binding proteins is limited to polypeptides of a size  $\geq 30$  kDa [41]. Using this standard technique, smaller proteins can not be visualized on DNA fragments. Second, the trace of the DNA in close proximity of the protein complex might be obscured due to the low angle of shadowing. However, shadowing has to be performed at low angles to obtain sufficient contrast. To avoid the disadvantages of the shadowing procedure STM was applied soon after the introduction of the instrument to analyze uncoated samples. The advantages and major drawbacks of STM in analyzing DNA structure and DNA-protein assembly are discussed in the next paragraph.



**Figure 4.** TEM analysis of DNA-protein assembly. (a,b) Electron micrographs illustrating the binding of the sequence specific, DNA-binding protein RAP1 to a cloned DNA fragment (1.2 kb) containing 14 binding sites for the yeast protein. (c,d) Statistical analysis of total DNA length and RAP1 positioning on the cloned DNA fragment. (a) Uncomplexed DNA fragment. (b) Multiple binding of RAP1 to the fragment. The trace of DNA between single complexes can not be followed (arrow heads). (c) Total length of uncomplexed DNA ( $1200 \text{ bp} \pm 35 \text{ bp}$ ;  $n = 45$ ). (d) Total length of fragments with multiple bound RAP1 molecules was  $1180 \pm 39 \text{ bp}$  ( $n = 155$ ). The RAP1 complexes peak at position  $408 \pm 130 \text{ bp}$  ( $n = 348$ ).

#### Imaging of bare DNA by STM

Biologists were interested in applying STM to the study of macromolecular assemblies because of the potential of this novel imaging technique to provide atomic resolution on flat surfaces [10]. Due to this reason imaging of DNA by STM was started very early after the introduction of the microscope [8]. In most of the experiments where STM was applied to image DNA in air [1, 4, 5, 6, 17, 19, 28, 44], in vacuum [18] or in liquid [31, 32] highly oriented pyrolytic graphite (HOPG) was used as a support. Reproducibility in some of these experiments, however, was reported to be very poor. Most of the problems arose, because adsorption of nucleic acids to this conducting support was insufficient. In addition, chain-like structures mimicking nucleic acids were often observed on freshly cleaved graphite surfaces [16]. To avoid the HOPG-related problems, the follow-

ing approach has been developed for analysis of bare DNA by STM (Fig. 2b). DNA fragments in the presence of Mg-acetate were deposited on freshly cleaved mica. Dehydration of the specimen was carried out by drying from ethanol in air. Subsequently, Pt/C was evaporated to embed the molecules in a layer of metal. Using this replica/anchoring technique [11, 15] the metal film was peeled off the mica and the previously mica exposed side of the embedded biomolecules was accessible for imaging by STM.

Under the given atmospheric conditions bare DNA could not be imaged by STM (Fig. 5). Only "hollow trenches" averaging  $3.1 \pm 0.9 \text{ nm}$  wide and  $1 \pm 0.5 \text{ nm}$  deep were visualized in a very smooth metal surface. By SFM measurements, however, it could be confirmed that DNA remained in the metal film during the peeling off procedure. Applying this alternative imaging technique,

embedded DNA could be visualized using positive contrast [35]. Similar results were obtained by Dunlap *et al.* [20]. By partly masking the deposited DNA molecules before coating it was possible to produce adjacent segments of coated and uncoated regions. In uncoated regions DNA was observed as "empty furrows". In addition, DNA appeared as a "hollow trench" when adsorbed on gold surfaces [1]. In contrast, imaging of bare DNA molecules on mica with positive contrast has recently been reported by Guckenberger *et al.* [25]. By increasing the relative humidity of the ambient air to 70%, the authors demonstrated that a very thin film of water adsorbed to the surface was sufficiently conductive to allow STM imaging.

Application of mica for DNA immobilization turned out to be advantageous: (1) artefacts as described for HOPG have not been reported, (2) very clean, atomically flat areas can be obtained by cleaving the aluminum silicate structure along its crystalline planes [38], (3) the well established  $Mg^{2+}$ /mica technique can be applied for routine adsorption of the DNA [41], and (4) a control of the preparation (i.e., efficiency of DNA adhesion and distribution of molecules on the surface) can be obtained by standard TEM prior to imaging by STM or SFM [15, 34, 35]. Importantly, metal embedding by Pt/C appears to be sufficient to overcome the major disadvantage of mica (i.e., its low electrical conductivity). The surrounding Pt/C layer can provide sufficient conductivity for routine imaging. By applying this embedding technique, it becomes possible to use two major advantages of this method: First, the technique can be applied to establish the experimental conditions for imaging of bare DNA molecules. Because the intrinsic conductivity of DNA appears to be lower as expected, it is of importance to choose well defined, easy-to-use experimental conditions. Second, replica/anchoring can be used for studying artefacts accompanying adsorption of biomolecules. By application of freeze drying (see below) both the upper side and the previously mica exposed side of any biomolecule can be analyzed to investigate the structural implications of adhesion forces.

#### STM analysis of metal-coated DNA and DNA-protein complexes

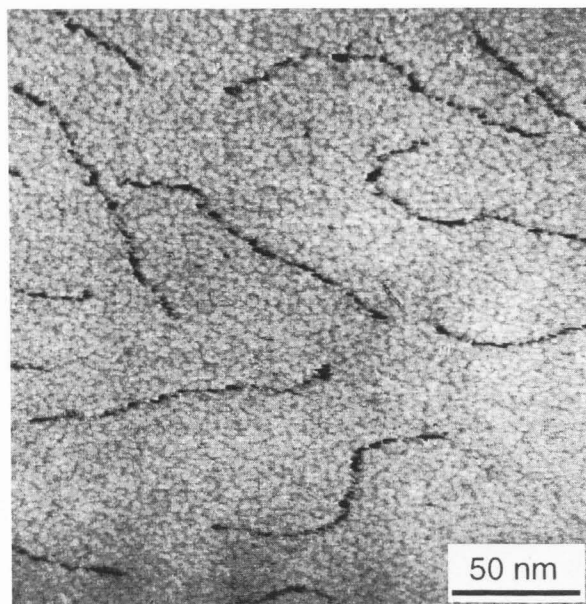
Because the STM cannot visualize bare, embedded DNA molecules in air, metal coating was applied to achieve reproducible imaging conditions. In addition, this coating offered the possibility to investigate structural artefacts accompanying dehydration. This is of importance, because surface tension forces were shown to cause severe damaging of biological structures [36, 47]. Using the  $Mg^{2+}$ /mica technique, DNA molecules were immobilized on a freshly cleaved mica surface. Specimens were freeze-dried and rotary shadowed with

Pt/Ir/C at a high angle of elevation and imaged by STM at atmospheric conditions (Fig. 2c). Alternatively, samples were dried from ethanol in air and coated with Pt/Ir/C under identical conditions. For ethanol/air-dried specimens measured values for DNA width and height were  $5.1 \pm 1.8$  nm and  $0.9 \pm 0.2$  nm, respectively. The width of freeze-dried DNA was  $4.2 \pm 1.3$  nm and the height was  $1.1 \pm 0.1$  nm [35]. Compared to freeze-drying, ethanol/air-drying appeared to broaden and flatten the DNA structure.

Metal coating for STM was first applied to image DNA-recA complexes [3]. The aim of this coating was to render the surface of both the sample and the substrate uniformly conductive [2]. The metal coat has to cover the specimen homogeneously without blurring fine structural details of the biological sample underneath. After freeze-drying of the specimen the coating film has to stabilize the preserved conformation of the dehydrated sample when transferring the sample from vacuum to atmospheric conditions. Pt/Ir/C films have a fine granularity and have proven to remain three-dimensionally stable during the transfer from vacuum to air [46]. A minimum thickness of 0.7-1 nm Pt/Ir/C was sufficient to achieve stable tunneling conditions [34]. By comparing ethanol/air-dried and freeze-dried DNA under stable tunneling conditions it turned out that even the rather rigid DNA molecules were structurally better preserved after freeze-drying. Freeze-drying was therefore applied in further STM imaging.

A combined approach using TEM and STM was applied to analyze protein-induced DNA bending (Fig. 6) [34]. In the presence of magnesium ions, DNA-RAP1 complexes were immobilized on a freshly cleaved mica surface (Fig. 6a). Different parts of a single piece of mica were used for parallel imaging by standard TEM and STM. For TEM the samples were ethanol/air-dried and rotary shadowed with Pt/C at a low elevation angle. Samples for STM were freeze-dried and coated with Pt/Ir/C at a high angle of elevation. The efficiency and accuracy of RAP1 binding was checked by TEM. Protein-induced DNA bending was then investigated by analysis of TEM (Fig. 6b) and STM (Fig. 6c) images. Investigations on DNA bending revealed a different distribution of bent angles for DNA-RAP1 complexes imaged by TEM or STM [34]. Analysis of TEM images revealed a Gaussian-shaped distribution of bent angles. In contrast, STM images of DNA-RAP1 complexes showed an increase in the frequency of higher angles. About 50% of the molecules showed an induced bend by RAP1 of  $50^\circ$  or greater.

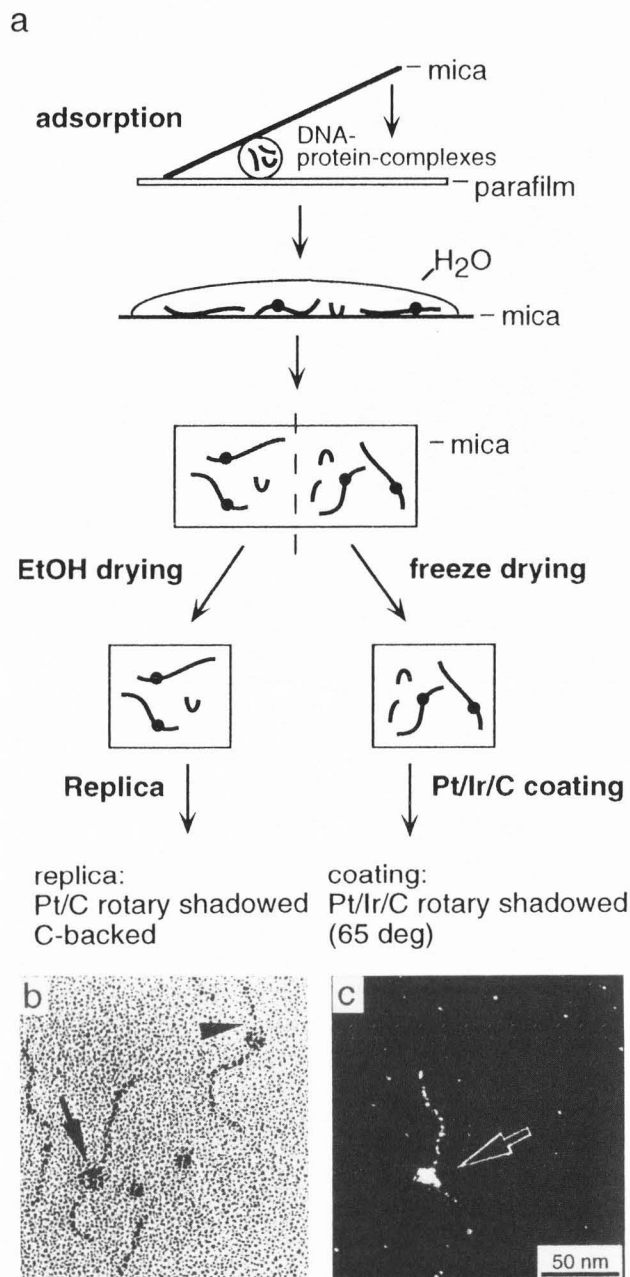
How can this difference in bent angles be explained? In standard TEM, the complexes are contrast-enhanced by rotary shadowing at low elevation angles in order to visualize small filamentous structures like DNA. This



**Figure 5.** Use of the replica/anchoring technique to visualize bare, metal embedded DNA fragments. Owing to the low intrinsic electrical conductivity, DNA molecules are only visible as "hollow trenches".

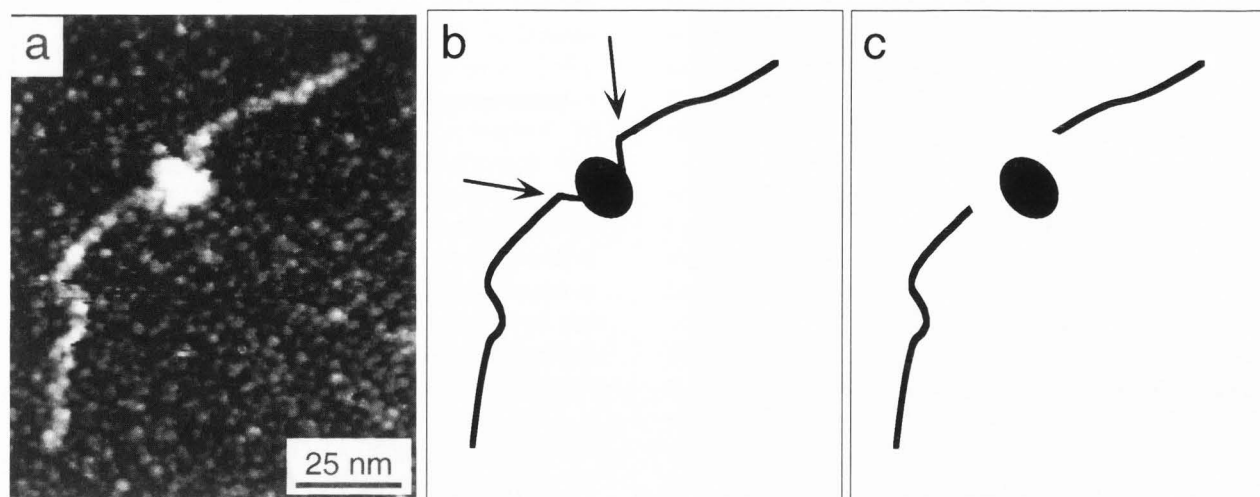
**Figure 6.** Combined approach for imaging of DNA-protein interaction by TEM and STM in parallel. (a) DNA-RAP1 complexes were immobilized on a freshly cleaved mica surface. Different parts of a single piece of mica were used for parallel imaging by TEM and STM. TEM samples were dried from ethanol in air and contrast enhanced with Pt/C at a low elevation angle. The carbon replica was analyzed in the TEM to check DNA adsorption and to determine correct positioning of the bound protein. STM analysis was carried out using freeze-dried specimens coated with Pt/Ir/C at a high elevation angle to investigate protein-induced DNA bending. (b) TEM micrograph of RAP1 molecules bound to a sequence specific binding site on the linear DNA fragment (arrow). Owing to the low-angle shadowing procedure the trace of the DNA close to the protein complex is obscured (arrowhead). (c) STM image of a DNA-RAP1 complex.

shadowing technique produced a relative large size of metal clusters and an indistinct protein structure due to "self-shadowing". The large metal grains can be followed along the DNA molecule, but are responsible for limiting the resolution of analysis near DNA-protein complexes. Owing to the protein size relative to the dimensions of the DNA, the trace of the fragment appears to be obscured in the vicinity of the polypeptide complex (Fig. 6b, arrowhead). Due to the high Z-resolu-



tion of the STM, a high elevation angle for coating of biological specimens can be applied. As a consequence, in STM images of DNA-protein complexes the trace of the DNA molecule can be followed in close proximity to the protein complex (Fig. 6c). This is of importance in view of the naturally occurring flexibility of DNA molecules as demonstrated in an STM image (Fig. 7a). The trace of the DNA close to the protein complex is shown schematically in Fig. 7b. In the vicinity of the RAP1 complex, a DNA bend of about 90° can be observed (arrows). Placing a "mask" at the position of the protein recognition site is considered to mimic conditions of low-angle shadowing (Fig. 7c). Owing to





**Figure 7.** Analysis of protein-induced DNA bending. (a) STM image of a freeze-dried, Pt/Ir/C coated DNA-RAP1 complex. (b) Cartoon illustrating the trace of the DNA. In the vicinity of the RAP1 complex, a DNA bend of about 90° can be observed (arrows). (c) By placing a "mask" at the position of the RAP1 recognition site no DNA bend can be determined. This situation is thought to "mimic" low-angle rotary shadowing.

this contrast enhancement procedure, information close to the protein complex is lost and no DNA bend can be observed (Fig. 7c). As a result, high-angle coating in combination with STM imaging appears to be advantageous in analysis of protein-induced DNA bending.

#### Imaging nucleic acids by SFM

For biologists the SFM is attractive because it combines a potentially atomic resolution [37] with the opportunity to image non-conductive surfaces in aqueous media under native conditions (Fig. 2d). Despite these promising features, high resolution imaging of DNA by SFM is hampered by several problems such as: (1) the specimen-substrate attachment, (2) the choice of the specimen environment, and (3) the influence of tip curvature on resolution [12, 13, 14, 27]. By SFM different kinds of DNA-protein interactions were imaged: (a) protein-induced DNA bending, (b) DNA loop formation due to protein-protein interactions, (c) targeting of sequence specific DNA sites, and (d) assembly of non-sequence specific nucleoprotein complexes (for a review see: [33]).

For imaging of nucleic acids, the major advantage of the SFM is that imaging can be performed in electrolyte solutions. Beside the fact that electrolyte solutions are the natural environment of nucleic acids and proteins, the force between tip and sample can be reduced by a factor of 10 to 100 when imaging in liquids [45]. In most SFM experiments, however, it was reported that complexed or uncomplexed nucleic acids had to be completely dried and/or imaged in an alcohol solution to

prevent translocation during scanning. Despite reducing forces between tip and sample, the major advantage of the SFM is lost, when dehydrated specimens are imaged in unphysiological solutions such as alcohol. In this respect the SFM offers no advantage compared to standard TEM or STM of metal-coated specimens, where freeze-drying can be applied to structurally preserve the DNA-protein complexes [3, 34]. On the other hand, SFM has been shown to be advantageous in imaging the interaction of nucleic acids with small proteins ( $\leq 30$  kDa). Assembly of Cro protein from bacteriophage lambda with specific and non-specific DNA sequences has been imaged in air [21]. Cro, which has a molecular weight of 14.7 kDa, was visualized bound to the operator sites on a 1 kb DNA fragment. Owing to its size, Cro cannot be imaged applying standard, low-angle shadowing for TEM.

#### Conclusions

DNA can routinely be immobilized on a freshly cleaved mica surface in the presence of magnesium ions. This method opens up the possibility to perform STM or SFM experiments with TEM analysis in parallel. Applying the standard method of low-angle rotary shadowing various kinds of DNA-protein interactions can be analyzed. Using STM DNA embedded in a layer of metal can be visualized as a "hollow trench" only. However, this method is especially useful in establishing the experimental conditions for imaging of bare DNA in air. High-angle metal-coating of freeze-dried specimens

appears to be useful in determination of protein-induced DNA bending. Both STM imaging of metal coated specimens as well as SFM analysis of DNA in solution appears to be advantageous in analyzing small proteins ( $\leq 30$  kDa) bound to cloned DNA molecules.

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#### Discussion with Reviewers

**J. Zasadzinski:** What is the ultimate resolution you expect for STM of shadowed or replicated materials and what is the main factor limiting that resolution?

**Authors:** The expected resolution is highly dependent on the type of specimen imaged and the kind of image analysis applied. TEM experiments with 2-D protein crystals have shown that after coating with 0.3-0.5 nm Ta/W followed by image averaging a lateral resolution better than 1 nm can be obtained [51]. To my mind, the strength of STM imaging, however, is the analysis of those objects where averaging is not possible and thus a high signal-to-noise ratio is necessary (i.e., filamentous molecules). Unfortunately, due to the low electrical conductivity of biomolecules, reproducibility is hard to achieve in STM and interpretation of the data is not a trivial task. I speculate that the ultimate resolution on coated DNA samples is to resolve the pitch of the DNA helix. Nevertheless, structural details will be obscured by the metal coat. Despite of this fact metal coating can be applied to analyze protein-induced DNA bending by STM [34]. It has been shown recently that the STM information is in good agreement with data obtained from crystal structure [49]. In STM, metal shadowing is not the method of choice when high resolution imaging of biomolecules (e.g., DNA) is intended. Ultimate goal has to be the imaging of bare molecules. For this reason, an easy-to-use method was developed to establish the experimental conditions for analysis of uncoated samples [35]. This approach can be applied for analysis under both atmospheric and vacuum conditions. Broadening of specimens caused by the shape of the tip has also to be taken into account under both imaging conditions. Convolution is especially evident when imaging

elevated or deepened structures [14].

**J. Zasadzinski:** How does STM resolution of shadowed materials compare to that of STM on hydrated materials as recently reported by Guckenberger *et al.* [48] or to the best new tapping mode SFM images? How does the resolution compare to standard TEM techniques?

**Authors:** The report on STM of biological specimens based on lateral conductivity of ultrathin water films [25] describes an interesting physical phenomenon. However, from the specimen preparation standpoint it is not clear to which extent the biological specimen is damaged during the preparation procedure. It seems likely that dehydration plays some role during imaging (i.e., analysis in a humid environment). So far no structural details of the DNA double helix could be detected. To my knowledge proper adhesion of the DNA to a solid support and translocation of the molecules during scanning is not fully solved even in tapping mode SFM. To my mind high resolution on DNA structure can not be achieved in SFM when disadvantageous air drying cannot be completely avoided. Structurally, SFM of air-dried DNA is of no advantage compared to standard TEM techniques.

**J. Zasadzinski:** Given that the STM or SFM resolution will likely never approach individual base pairs, what are the best questions to ask and expect to answer with probe microscopies?

**Authors:** In the beginning of STM imaging most of the researchers started out with the intention to sequence short DNA fragments. To date DNA sequencing can be performed fast and reliably using biochemical techniques. In my opinion sequencing of nucleic acids is not a question to be answered by scanning probe microscopy. However, questions can be answered concerning the conformational states of DNA molecules. Our knowledge on DNA structure is mainly based on analysis of DNA crystal structure. It will be interesting to analyse individual DNA molecules and the regularity of the helical arrangement under physiological conditions. Moreover, it would be of advantage to detect local regions in DNA fragments that flip from a right-handed B form to a left-handed Z form. To date Z-DNA regions can be detected using an anti-Z-DNA antibody [50]. Another interesting topic might be the structural analysis of local DNA unwinding due to binding of sequence specific transcription factors as reported for RAP1 [23]. Structural details of this kind of protein-DNA interaction cannot be expected by low-angle shadowing following TEM analysis.

**J. Zasadzinski:** Do you recommend that DNA researchers go out and invest in a STM, SFM or hybrid instru-

ment, if they have never yet done so?

**Authors:** This strongly depends on the research project. Because of the low electrical conductivity of biological specimens most of the STM researchers continued to work with SFM [48]. However, it appears that the preparation of the biomolecules seems to limit also the analysis by SFM. Before high resolution data on DNA structure can be expected by SFM the proper attachment of the biomolecules without prior dehydration has to be solved. In parallel, imaging of bare DNA by STM has to be established for routine analysis.

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