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AFM analysis of DNA–protamine complexes bound to mica

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ABSTRACT

A novel method for reconstituting sperm chromatin was used to investigate how protamine 1 condenses DNA. Complexes formed *in vitro* using linearized plasmid DNA were imaged and measured by atomic force microscopy (AFM). The structures formed were found to be highly dependent on the sample preparation method used for reconstitution. Interstrand, side-by-side fasiculation of DNA and toroidal-like structures only 1–2 DNA diameters thick were observed for complexes formed in solution following direct mixing of the DNA and protamine. Large chromatin aggregates were also observed on the mica. However, if the DNA was first allowed to attach to the mica prior to addition of the protamine, well-defined toroidal complexes were formed without any observed DNA fasiculation or aggregate formation. The diameter of the toroids measured 30.6–50.2 nm (mean 39.4 nm). The dimensions of these structures indicate that the condensed DNA is stacked vertically by four to five turns, with each coil containing as little as 360–370 bp of 'B'-form DNA. This approach for preparing and imaging DNA–protamine complexes permits the analysis of intermediate structures 'trapped' on the mica as partially formed toruses of nucleoprotamine.

INTRODUCTION

Analyses of sperm chromatin structure using biochemical and biophysical techniques (1–4) and various forms of microscopy (5–15) have revealed that the bulk of the DNA in the mammalian sperm nucleus is not packaged as nucleosomal subunits and solenoidal structures similar to those found in the nuclei of all somatic cells. While it appears that the overall condensation of DNA in the sperm nucleus is at least several times greater than for somatic nuclei (2,15), determining the exact structure and organization of chromatin in mammalian sperm has proved to be difficult. Structural models for mammalian sperm chromatin have been inferred from experiments involving the condensation of DNA by various multivalent cations (16,17), including spermidine and salmon protamine (14,18,19). Such experiments have shown that certain concentrations of multivalent cations collapse DNA into toroidal structures with circumferences independent of the starting DNA length (16,20). Toroids formed by complexation with spermidine and calf thymus or plasmid DNA in 150 mM NaCl had an average circumference of ~525–760 bp (16,19).

Recent atomic force microscopy (AFM) studies have shown that the protamine-complexed DNA (nucleoprotamine) in mature bull, mouse and human sperm is organized into nodular subunit structures much larger than nucleosomes (13,21,22). Other analyses of nucleoprotamine complexes formed *in vitro* (14) have revealed that protamine binding induces the coiling of DNA into toruses containing as much as 50–60 kb DNA. The physical process involved appears to be very similar to the induction of torus formation that occurs upon binding of other multivalent cations to DNA (16,23). In nature, the organization of DNA into these toroidal structures has been observed to occur only in sperm and certain viruses (bacteriophages).

AFM is well suited for studying the higher ordered packaging of DNA by protamine and other proteins because it offers a spatial resolution sufficient to visualize and accurately measure structures as small as an individual DNA molecule under a variety of conditions and environments without the use of stains or coatings. Studies of native (24,25) and reconstituted (26) somatic chromatin have demonstrated that the atomic force microscope can be used to examine the packaging of DNAs by histone core particles and investigate the role of histone H1 and counterions on higher ordered nucleosome packing. Our preliminary analyses of partially decondensed human sperm chromatin by AFM have also shown that the technique has sufficient resolution to identify two different populations of subunits within the chromatin (21,22), one the size and shape of nucleosomes and another larger, doughnut-shaped toroidal subunit.

Mica has been determined to be a very good substrate for imaging DNA in that it is extremely flat (on the scale of the DNA molecule) and DNA binds to it in common buffer solutions. DNA binding to mica has been shown to be enhanced in the presence of millimolar concentrations of divalent metal counterions (e.g. Mg^{2+}). Details for reproducible imaging of individual DNA molecules have also been worked out independently in several laboratories and AFM images of DNA can be obtained quickly and routinely (27,28). In this study we used the atomic force microscope to examine the higher ordered organization of DNA that ensues when protamine binds to it. This has, in the past,

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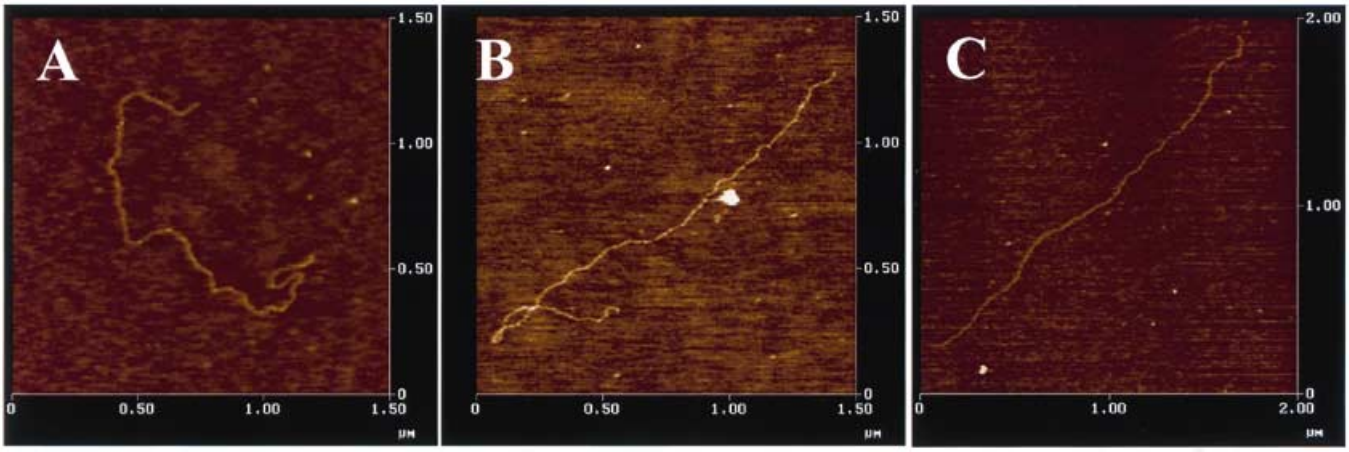

Figure 1. AFM images of linearized 7.5 kb plasmid DNA showing the structure of the DNA bound to mica without the addition of any protamine. (**A**–**C**) Three different DNA molecules.

proved very difficult because once protamine binding occurs, the charge on DNA is completely neutralized and the resulting complex rapidly aggregates with others to form entangled, insoluble mats of nucleoprotamine. The present studies show that DNA binds loosely to the surface of mica and proteins (such as protamine) added to mica-sequestered DNA can alter the structure of the molecule in a manner that is consistent with that found in native sperm chromatin. Variations in this method are described which facilitate the trapping of coiling or condensation 'intermediates' and provide a method for conducting a more detailed analysis of the process of toroid formation induced by protamine (or other polycations) binding to DNA.

MATERIALS AND METHODS

Preparation of free DNA on mica

Aliquots of 1–2 µl *Pst*I-linearized 7.5 kb DNA (1–10 ng/µl) isolated from the *Chlamydia trachomatis* plasmid in 5 mM $MgCl_2$ was applied to freshly cleaved mica (Pelcomica strips; Ted Pella Inc., Redding, CA) and allowed to bind for 20–120 s. The mica was then rinsed twice with 100 µl distilled water and the excess liquid wicked away at the mica edge with a tissue. The mica was then blown completely dry with a stream of nitrogen gas and put into a vacuum dessicator until it could be imaged by AFM.

Preparation of nucleoprotamine complexes

Bull protamine 1 was isolated from bull sperm and purified by HPLC as described previously (29). A 7.5 kb linearized plasmid DNA in Tris–EDTA buffer, pH 8, was provided by Dr R.Kaul (Department of Pediatrics, University of California, Davis, CA). Nucleoprotamine complexes were prepared directly on a mica substrate by three different methods.

Method 1. Aliquots of 2 µl containing 0.5 ng protamine in distilled water and 2 µl containing 20 ng 7.5 kb plasmid DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), 2.5 mM $MgCl_2$ were applied simultaneously to freshly cleaved mica and mixed briefly by pipeting. The droplet was allowed to incubate on the mica for 2 min.

Method 2. An aliquot of 2 µl containing 20 ng 7.5 kb plasmid DNA in TE buffer, 2.5 mM $MgCl_2$ was applied to freshly cleaved mica for 1.5 min, followed by addition of 1 µl containing 10 ng protamine in distilled water for a total of 2 min.

Method 3. An aliquot of 5 ng protamine in 1 µl distilled water was incubated on mica for 1 min, followed by addition of 1 ng plasmid DNA in 2 µl TE buffer, 2.5 mM $MgCl_2$ for a total of 2 min. In all three methods the mica was then rinsed twice with a gentle flow of 100 µl distilled water, wicked dry with a tissue and then blown completely dry with a stream of nitrogen gas. The mica was then immediately stored in a vacuum dessicator until it could be imaged.

AFM imaging

A Nanoscope II atomic force microscope (Digital Instruments, Santa Barbara, CA) and 450 µm single beam etched silicon AFM probes were used to image the DNA and nucleoprotamine. Images were collected at 5–25% relative humidity (RH) at room temperature. To maintain a low RH, dry nitrogen gas was released slowly into a small plexiglass imaging chamber placed over the AFM sample. The 'height' or constant force mode was used and scan frequencies were typically 5 Hz. All images contain 400 × 400 data points. The piezoelectric tube scanner ('D' scanner), initially calibrated by the manufacturer, was calibrated by atomic level imaging of mica and various carbon, gold and silicon gratings with periods ranging from 0.463 to 10 µm. The raw data images were rendered using Nanoscope III software (version 3.01) with only background slopes corrected.

RESULTS

Uncomplexed DNA molecules readily attach to the surface of mica and remain sufficiently tightly bound to be imaged with an AFM tip (Fig. 1A–C). While regions of the duplex were often observed to fold back on themselves (Fig. 1C), individual molecules were not observed to bind to each other, aggregate or otherwise interact. Length measurements also confirmed that >90% of the DNA molecules were the correct length for 7.5 kb B-form DNA.

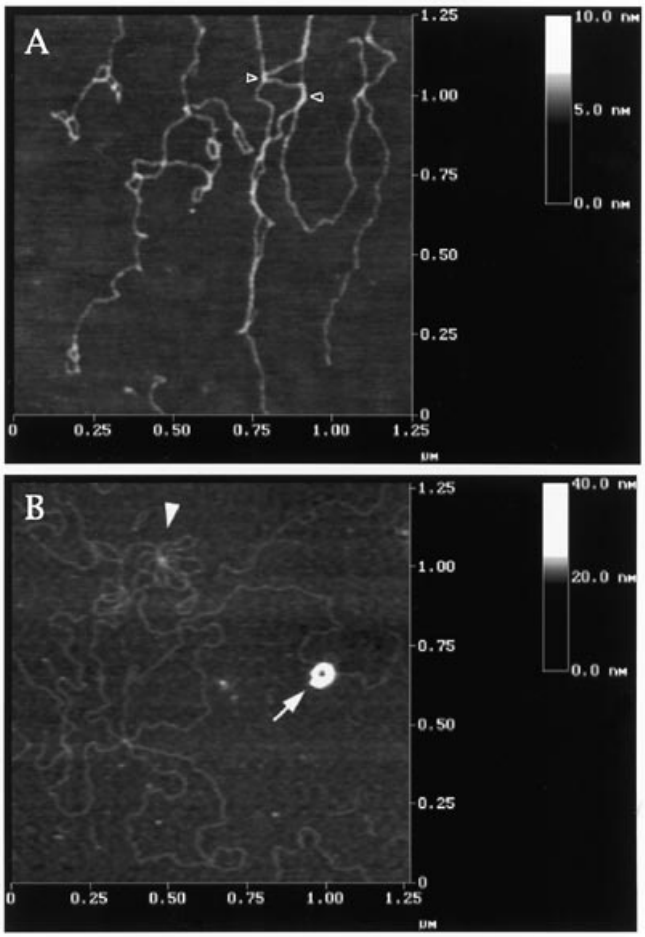

Figure 2. AFM images of sperm chromatin reconstituted by two different procedures (Methods 1 and 2) using dilute solutions of 7.5 kb plasmid DNA and protamine 1. (**A**) Nucleoprotamine complexes prepared by direct mixing of DNA and protamine in a small droplet applied to a mica surface (Method 1). Interstrand, side-by-side protamine-mediated DNA fasiculation was observed (open arrowheads) as well as the formation of large aggregates or clumps (not shown). As revealed by AFM thickness measurements, the toroidal structures formed using this procedure consist of only one to two DNA turns. (**B**) Nucleoprotamine prepared by first loosely attaching the DNA to the mica surface and then adding protamine to form the complexes (Method 2). The DNA has limited mobility and cannot fasiculate or aggregate appreciably upon addition of protamine. However, the DNA does form well-defined toruses ~40 nm in center-to-center diameter and nearly 10 nm in thickness (arrow in B).

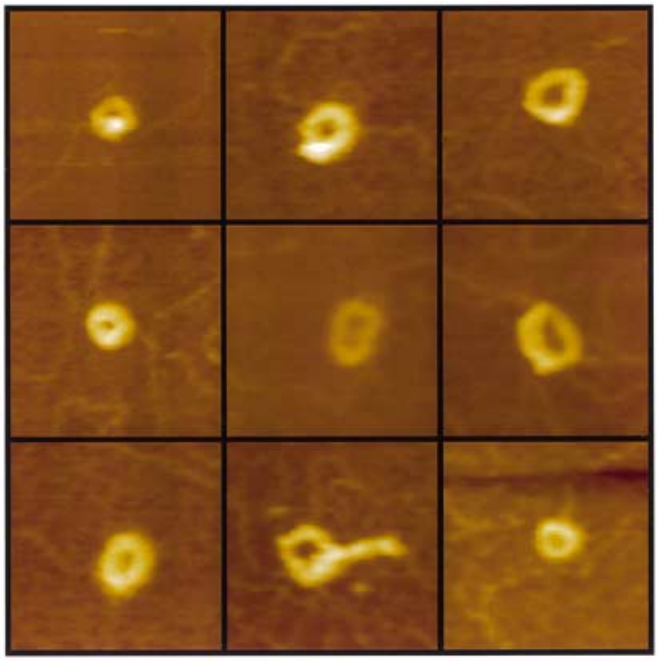

Figure 3. High resolution images of individual toruses produced by adding protamine to DNA sequestered on the surface of mica (Method 2). Image size is 325 nm. The measurements of the toruses indicate that the condensed DNA is stacked vertically by four to five turns with each turn averaging 39.4 nm in diameter. This corresponds to ~360–370 bp of B-form DNA/turn and a DNA compaction ratio of 3.14/turn. The average outer diameter of the toruses measured 83 nm.

In contrast, structures visualized for the nucleoprotamine complexes adsorbed onto mica were found to be highly dependent on the preparation method used (see Materials and Methods). Interstrand, side-by-side associations (i.e. fasiculation) of the DNA were observed for nucleoprotamine complexes formed in solution (arrows in Fig. 2A) by the direct mixing of DNA and protamine (Method 1). As expected, large aggregates of nucleoprotamine were also observed on the mica if direct mixing was performed (14). However, if the DNA was first allowed to attach to the mica substrate prior to addition of protamine (Method 2), well-defined toroidal complexes were formed (Figs 2B and 3). In addition, DNA fasiculation or aggregate formation was not observed in these preparations. The average diameter of 30 toroids measured from the center of one edge across the center of the hole to the center of the other edge was found to be 39.4 (± 6.9) nm, while the outer diameter was determined to be 82.8 (± 9.7) nm. Such center-to-center AFM measurements are reasonably accurate and provide good measures of the average toroid diameters. These measurements were used to calculate the circumference or length of DNA loop that forms the torus, because they are not hampered by uncertainties generated by probe–sample shape convolutions, as is the case for measurements of the outer diameter. The thickness of the toroids appeared somewhat variable, averaging 9.6 (± 1.6) nm. This may reflect differences in the regularity of coiling of the DNA to form the torus or variation in the physical properties of individual structures (e.g. compressibility). While height measurements obtained by AFM are very accurate, the toroids may shrink in thickness during drying or they may be compressed as they are imaged by the tip. The measurements obtained for the toroids indicate that the condensed DNA is coiled up to four or five DNA molecules thick with each coil consisting of ~360–370 bp of 'B'-form DNA. Thicker toroids generally had smaller center-of-edge to center-of-edge diameters.

In certain preparations the DNA appeared to be only partially coiled by the protamine (Fig. 4). These nucleoprotamine structures appear to represent trapped kinetic intermediates in the early stages of torus formation. These structures were commonly observed in preparations where the protamine was first applied to the mica prior to addition of the DNA (Method 3). Individual complexes exhibiting multiple (Fig. 4A) DNA loops ~40 nm in diameter were frequently observed. Other more condensed complexes clearly show loose coiling of the DNA into toroid-like structures (Fig. 4B). Based on the length of DNA coiled in these

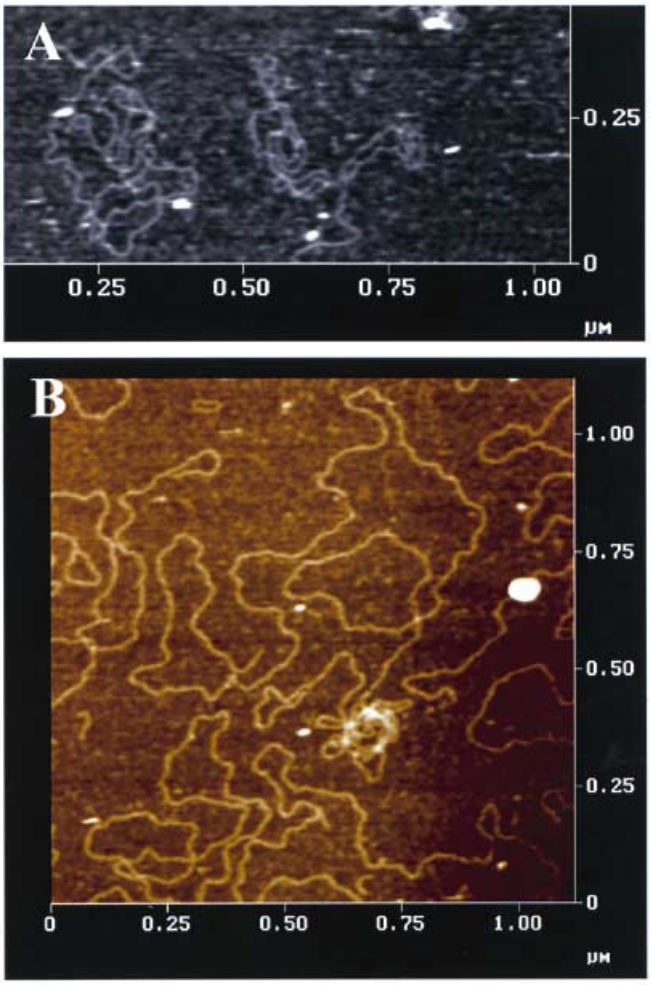

Figure 4. AFM images of 'trapped' kinetic intermediate structures of nucleoprotamine complexes formed on the surface of mica. Each of these samples was prepared by first pretreating mica with protamine prior to adding the DNA (Method 3). (**A**) In some preparations the DNA molecules exhibit extensive coiling and bending. (**B**) Trapped intermediate structure near completion of toroid formation.

structures, many of the complexes appeared to contain multiple DNA molecules.

DISCUSSION

The three methods used to prepare nucleoprotamine complexes for analysis by AFM in this study provided results that appear to reflect differences in the accessibility of individual protamine-complexed DNA molecules, at the time of their formation, to other complexes and to mica. Nucleoprotamine complexes formed in solution before their introduction to the mica surface exhibit evidence of extensive aggregation between toroidal structures in various degrees of formation (Method 1). Similar structures were observed when complexes formed *in vitro* were examined by electron microscopy (14). In most cases, the coiling of a single DNA molecule into a torus appears to be terminated prior to completion by binding of the partially formed torus to other nucleoprotamine fibers or incomplete toroids, resulting in the formation of a highly fasiculated structure (Fig. 5A). At the concentrations of DNA and protamine used in most experiments the process of aggregation appears to occur more rapidly than completion of torus formation.

This is consistent with results we obtained in other studies which show, as one might expect, that the extent of aggregation of the nucleoprotamine complex varies with DNA concentration. At moderate DNA concentrations (~100 μg/ml) the DNA is observed to aggregate into large, densely packed masses (14). Individual toroids were detectable only when the concentration of DNA was decreased below 20 μg/ml (the DNA concentration used in the present study was 10 μg/ml). Even at a concentration of 1 μg/ml, however, we have found that protamine-complexed DNA molecules still aggregate (Hud *et al*, unpublished observations). The aggregates simply take a longer period of time to form.

Somewhat surprisingly, the coiling of DNA into toroids upon protamine addition was observed to occur most efficiently (i.e. completed single toroids are observed with the highest frequency) when protamine was added to DNA molecules that were loosely bound to mica (Method 2). DNA rapidly bound to mica when applied in millimolar concentrations of Mg^{2+}. However, the observed ability of the protamine to bind to the DNA and coil these extended molecules into completed toroids demonstrates that the DNA molecules were only loosely and not irreversibly attached to the surface. The mode of DNA binding to mica (mediated by the divalent cation Mg and its interaction with the negatively charged mica surface) appears to be sufficiently weak that the the arginine-rich domain of protamine can effectively compete for binding to the phosphate groups in the DNA backbone and wrap around each turn of DNA to form nucleoprotamine. The observation that the complexed DNA molecule then coils into a torus, presumably while loosely attached to the surface, indicates that the physical forces that drive the coiling process must be stronger than those that bind DNA or the protamine–DNA complex to the surface. Because the individual molecules of DNA (and the final complex) remain bound to the surface, they cannot interact with other molecules and the process of aggregation and formation of fasiculated structures is minimized (Fig. 5B).

The sizes of the toroids produced using this method (mean outer diameter 82.8 nm) were indistinguishable from the native structures observed in intact, demembraned and nanodissected bull sperm nuclei (50–100 nm) (13). Similar sized toroids have also been observed in partially decondensed human sperm chromatin (21). Interestingly, it is quite possible that the nodular structures observed in sperm chromatin *in situ* using AFM are in fact toroidal structures with small central openings (holes) positioned more 'edge on' than 'face up' at the chromatin surface.

The average diameter and circumference of the toroids formed on mica were also consistent with what we would expect if the persistance length of DNA is the primary factor that determines the size of the final torus (23). Because the toroids we analyzed in this study appeared to contain only a limited number (four to five) of turns of DNA (the DNA molecule is short), the circumference of the torus would be expected to be close to the persistence length of the DNA–protamine complex. While the number of toroids we measured to obtain this number was fairly small, the circumference (loop length) we obtained is close to what others have measured for the persistence length of free DNA (400–500 bp; 30). While studies by Porschke (31) have shown that divalent cations like Mg (up to 20 mM) do not alter the persistence length of DNA, such measurements have not been made previously for protamine. Thus it would appear that this

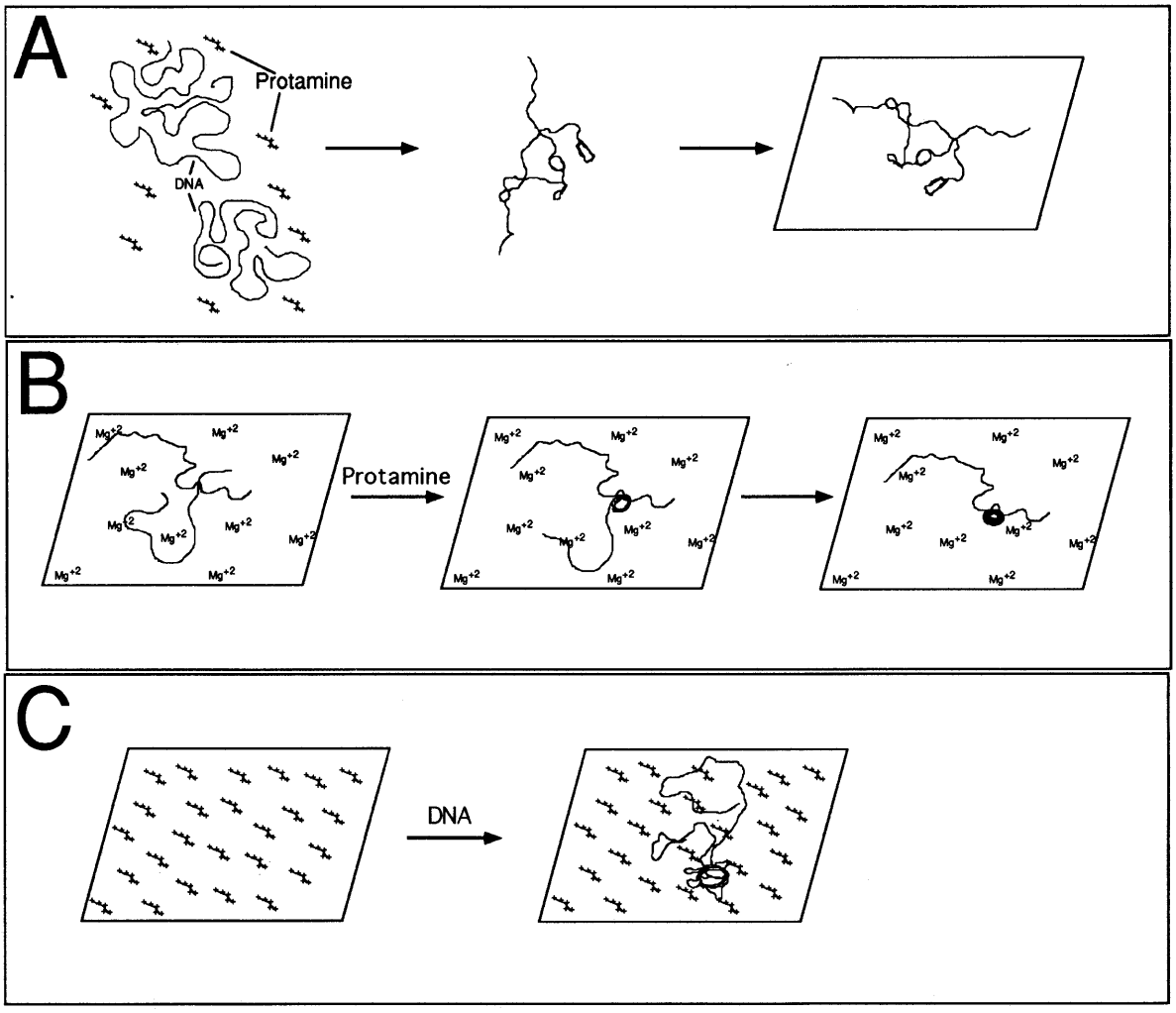

Figure 5. Schematic representation of the three different methods employed to produce DNA–protamine complexes on mica suitable for imaging by AFM. (**A**) *In vitro* (solution state) complexation of protamines and DNA, followed by attachment of these complexes to mica (Method 1). (**B**) DNA molecules applied to mica in the presence of Mg^{2+} ions bind loosely to the surface. The subsequent addition of protamine to the mica-sequestered DNA initiates coiling of the DNA into toroids without the associated aggregation that occurs when the complex is formed in solution (Method 2). (**C**) Mica pretreated with protamine presents a more highly charged surface to DNA molecules partially complexed by protamine. The complexes (intermediates) appear to be immobilized on the surface, trapped at various stages of torus formation (Method 3).

method can be used as a model system for studying protamine-induced toroid formation *in vitro*. The ability of protamine to bind to mica-sequestered DNA also indicates that this approach may be useful for characterizing the low resolution structure of other DNA–protein complexes by AFM.

The results obtained by adding DNA to mica that had been preincubated with protamine were quite different (Method 3). Completed toroids were only rarely observed. Instead, the majority of the structures imaged appeared to represent intermediates at various stages of torus formation. This difference could be explained if, during the pretreatment step, some of the highly charged protamine molecules (the DNA binding domain contains 22 positive charges) bound to the negatively charged surface of mica in a fashion similar to that observed for Mg^{2+}. The excess charge on these molecules (each mica bound-protamine would have 20 times more excess charge than a Mg ion) would still be available for binding to the partially formed DNA–protamine complexes, seqestering them on the surface of the mica (Fig. 5C). This would trap the partially formed complexes at intermediate stages of completion and inhibit torus formation.

It has been calculated that the surface of mica contains a slight negative charge (approximately one negative charge per 60 nm^2; 32) and it has been hypothesized that magnesium mediates DNA binding to mica through its interaction with these negatively charged groups. This paucity of binding sites for magnesium is consistent with the apparent 'loose' binding of DNA to mica we have observed. Upon adding protamine to the surface, the positively charged arginine residues would be expected to bind to the surface and present a much more highly charged surface to the DNA. Each bull protamine molecule contains 22 arginine residues (each with a positive charge) that would be spread across 70 of the surface [this is based on experimental data that identified the length of DNA covered by protamine (33) and models of the DNA–protamine complex (34)].

The success of this method of preparing well-defined 40 nm nucleoprotamine toruses using a defined length fragment of DNA

and protamine 1 appears to be due to the fact that the DNA used for reconstitution is partially immobilized and sequestered as individual molecules on the surface of mica when it is presented to protamine. This attachment of DNA to the mica appears to prevent the protamine-mediated DNA fasiculation or aggregation that occurs when the DNA is freely mobile and accessible to neighboring DNA molecules in solution. DNA is also packed inside the sperm nucleus DNA in a constrained state as a result of its tethering to a scaffold, attachment to a nuclear matrix or confinement in loop domains. This limits the degrees of freedom of the DNA molecule and its interactions *in vivo* in comparison with DNA molecules in solution, which are free to move throughout the solution and form aggregates with a variety of other molecules (or other regions of the same molecule) in a disorderly or chaotic fashion. In this respect, DNA molecules loosely bound on the surface of mica appear to be similar to the state of nuclear DNA when it comes to torus formation. Further, it is reasonable that the sperm genomic DNA does not undergo simple, random aggregation as protamines are deposited during the DNA condensation process. Such an uncontrolled condensation reaction would leave the sperm genome in a highly unstructured state. It is clear, however, that protamine must be introduced to DNA *in vivo* in a reasonably organized fashion, since condensation of chromatin in maturing spermatids has been observed to occur in a particular pattern, beginning at the apical end of the nucleus and progressing toward the implantation fossa.

The DNA strand of one chromsome becoming tightly complexed with the DNA strand(s) of one or more different chromosomes would represent an example of such disordering in the sperm genome. One would more reasonably envision protamine deposition occurring in a stepwise fashion, with the protamine–DNA reaction being restricted to local sites, possibly at chromatin loop domains. In support of this assertion of specific sperm chromatin structuring, Blow and Laskey (35) showed that replication of sperm DNA was dependent on the structural form of the DNA exposed to oocyte extracts. Only demembraned sperm heads and not isolated naked sperm DNA could be replicated. Furthermore, Zalensky *et al.* (36), using fluorescence *in situ* DNA labeling techniques, have recently established a specific structural organization of subchromosomal domains in human sperm. Ward *et al.* (37) have proposed a similar model for maintaining somatic loop domain organization in sperm.

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