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POLYMERIZATION OF THE DNA BINDING FRAGMENT OF p53 ON DNA: ATOMIC FORCE MICROSCOPY STUDY

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Abstract

We performed atomic force microscopy (AFM) studies of complex formation between DNA and the DNA-binding fragment of p53 protein (p53F, amino acids 96-308) in a broad range of protein concentration. We have found that p53F coated DNA completely at high concentration. The coated filament had a width of 6.45 ± 1.0 nm and the length of the DNA in the complex was unaltered, in agreement with an earlier X ray study. As a control, we made similar measurements on RecA-DNA filaments. The unwinding of DNA by RecA was easily detected. We compared the stiffness of p53F/DNA filaments with DNA and RecA-DNA using the ratio of the end-to-end distance (L_e) to the contour length (Lc). We found L_e/L_c = 0.22 ± 0.15 for 1.07 Kb DNA, 0.49 ± 0.06 for p53-DNA and 0.87 ± 0.35 for RecA-DNA. Formation of filamentous complexes might play a role in the p53 regulation of DNA repair.

Key Words: Imaging, atomic force microscopy, nucleoprotein complexes, tumor supressor.

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Introduction

The tumor suppressor, p53, plays a key role in cell proliferation and human cancer (Hollstein *et al*., 1991; Harris, 1993). As an activator of transcription, it binds to the consensus sequence 5'-XXXC(A/T)(T/A)GYYY-3' (where X is a purine and Y is a pyrimidine; Kern *et al*., 1991; Funk *et al*., 1992; Cho *et al*., 1994; Clore *et al*., 1994). The protein products of these p53-activated genes regulate induced cell-cycle arrest. There is evidence that the protein plays a role both in repair of DNA lesions and in inducing apoptosis of a cell for the protection of the whole organism (Fritsche *et al*., 1993; Di Leonardo *et al*., 1994; Reed *et al*., 1995; Shivakumar *et al*., 1995). This activity of p53 protein requires high concentration of the protein in the cell.

Functionally, p53 is composed of three regions. The N-terminal domain interacts with transcription factors (Sancar, 1994; Chernova *et al*., 1995). Amino acids 96 through 308 form the DNA binding domain (Bargonetti *et al*., 1993; Harris, 1993; Pavletich *et al*., 1993; Wang *et al*., 1993, 1995; Cho *et al*., 1994). The C-terminal domain contains the region responsible for tetramerization of the protein (Jeffrey *et al*., 1995). It binds to single-stranded DNA (Bakalkin *et al*., 1995; Reed *et al*., 1995) and is probably involved in tetramerization of the protein on bulges (Lee *et al*., 1995).

The structure of the central DNA-binding domain, co-crystallized with a 21 bp oligomer containing a binding sequence, was solved recently (Cho *et al*., 1994). The DNA was only slightly perturbed from the canonical B-form. The oligomers were stacked in the crystal so as to form a continuous helix, coated with a continuous filament of protein. Electron microscopy (Lee *et al*., 1995) showed that p53 bound mostly at bulges and the ends of oligomers.

The atomic force microscope (AFM) has recently been applied to the study of a number of nucleoprotein complexes (Bustamante *et al*., 1993; Hansma and Hoh, 1994; Lyubchenko *et al*., 1995a). While the technique has lower resolution than X ray crystallography, it is capable of measuring bending and looping in single DNA molecules that are not part of a crystal. We performed AFM studies

of p53F-DNA complexes in a very broad range of protein concentration. Although we studied a fragment containing a binding sequence, there was little evidence of specific binding under the conditions in which we prepared our samples for microscopy. At high protein concentrations, we found that a continuous filament was formed. Its dimensions, and those of the DNA, were strikingly similar to those implied by the small assembly solved in the X ray study (Cho *et al*., 1994).

Materials and Methods

Protein

The DNA binding fragment p53F (amino acids 96-308) was prepared as described by Clore *et al*. (1994) and used in the AFM experiments without additional purification.

DNA

A 32 base-pair oligonucleotide having a 20 bp Waf1 response element was cloned at the Bam HI site of pUC19. The recombinant plasmid (pWaf32) was digested with AlwNI and NdeI giving a 1.07 Kb fragment having Waf1 binding site 237 bp from the NdeI cut end. This fragment was extracted from agarose gel using a Gene Clean kit (Bio101, La Jolla, CA) and further purified through Centricon-3 columns (Amicon, Inc., Beverly, MA). Aminopropyl mica (AP-mica) substrates for the AFM studies were obtained by modification of freshly cleaved mica strips in vapors of aminopropyltriethoxy silane as described in Lyubchenko *et al*., 1992a,b; 1995a,b.

AFM Preparation of p53-DNA Complexes

One microliter of DNA (60 µg/ml) in TND buffer solution (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 10 mM DTT) was mixed with 0.2-3 µl of the protein solution (150 µg/ml) prepared by dilution of concentrated stock solution of the protein (~2 mg/ml) into TND buffer and a corresponding amount of TND buffer was added to make a final volume of 10 µl. The solution was incubated at 0°C for 30 minutes. 3 µl of the protein-DNA mixture was diluted in 30 µl of cooled buffer, the solution placed onto cooled AP-mica, and left for deposition for 3-5 minutes at 0°C. The mica was rinsed with cooled deionized water, dried in an argon flow and stored in an argon atmosphere. All manipulations with solutions of the protein and DNA-protein complexes were performed in an argon atmosphere to avoid protein oxidation.

AFM preparation of RecA-DNA complexes

Ten microliters of a mixture containing 1 µl DNA (concentration 6 µg/ml), 2 µl of 5 x RecA-buffer (5% glycerol, 35 mM Tris-acetate, pH 7.6, 1 mM DTT, 1 mM Mg acetate, Golub *et al.*, 1993), 1 µl ATP-γ-S (20 mM water solution), 6 µl deionized water and 0.2 µl RecA protein stock solution (concentration 19 µM) was incubated at 37°C for 15 minutes for formation of the complex (Revet *et al*., 1993). The sample was diluted two times with deionized water, the final solution (20 µl with DNA concentration ca. 0.2 µg/ml) was placed on AP-mica, and the sample was allowed to deposit for 2-4 minutes. The specimens were rinsed with deionized water, dried with argon and imaged immediately (or stored under argon).

AFM imaging

AFM Imaging was performed using NanoScope II and NanoScope III microscopes (Digital Instruments, Inc., Santa Barbara, CA) and a PicoSPM (Molecular Imaging, Tempe, AZ) in contact mode in ambient conditions in air and also in propanol (for details, see Lyubchenko *et al.*, 1993a,b; 1995a). We used silicone nitride tips (Microlevers, 0.6 µm) from Park Scientific (Sunnyvale, CA) with a loading force of ~10 nN.

Data acquisition

AFM data was translated to TIFF-format and measurements made using NIH Image 1.40 software. The dominant broadening mechanism arises from the finite radius of the probing tip. If the tip is modeled as a sphere of radius R_c and the molecule as a cylinder of radius R_m then the measured full-width of the image, w, is given by

$$w = 4\sqrt{R_c R_m} \qquad (1)$$

If the tip is modeled as a parabola of radius R_c, then the corresponding result is (Bustamante *et al*., 1992)

$$w = 4(R_c + R_m)\frac{\sqrt{R_m(R_c - R_m)}}{R_m} \quad R_c > R_m \qquad (2)$$

Results

Atomic force micrographs of the 1.07 Kb DNA are shown in Figures 1A and 1B. The molecules adopt a coiled conformation which is typical for samples deposited onto AP-mica (Lyubchenko *et al*., 1995a). The AFM results for a complex prepared at 4:1 molar protein/DNA ratio (number of monomers of the peptide per one 1.07 Kb DNA molecule) are shown in Figure 1C. One or more blobs can be found bound to the DNA filaments in these images (for example, 2 blobs on the molecule labeled "1"). At higher protein/DNA ratio (30:1) the number of such blobs bound to the DNA filaments increases (three blobs for the molecule labeled 1 in Fig. 1D), and in some cases large regions of the DNA are coated with the protein (Fig.

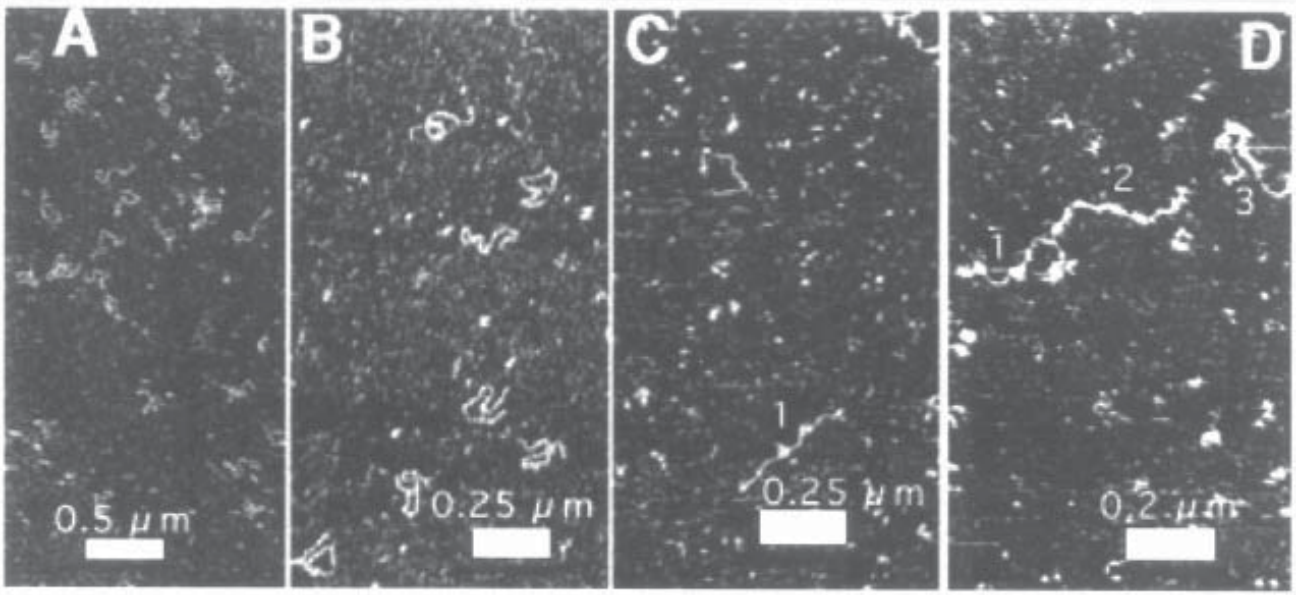

Figure 1. AFM images of: 1.07 Kb fragment of DNA (**A**, **B**); the same DNA fragment complexed with the p53F peptide at molar protein/DNA ratios 4:1 (**C**) and 30:1 (**D**). The images were obtained in propanol in contact mode. Z-range is 4 nm for images (A) and (B) and 5 nm for images (C) and (D).

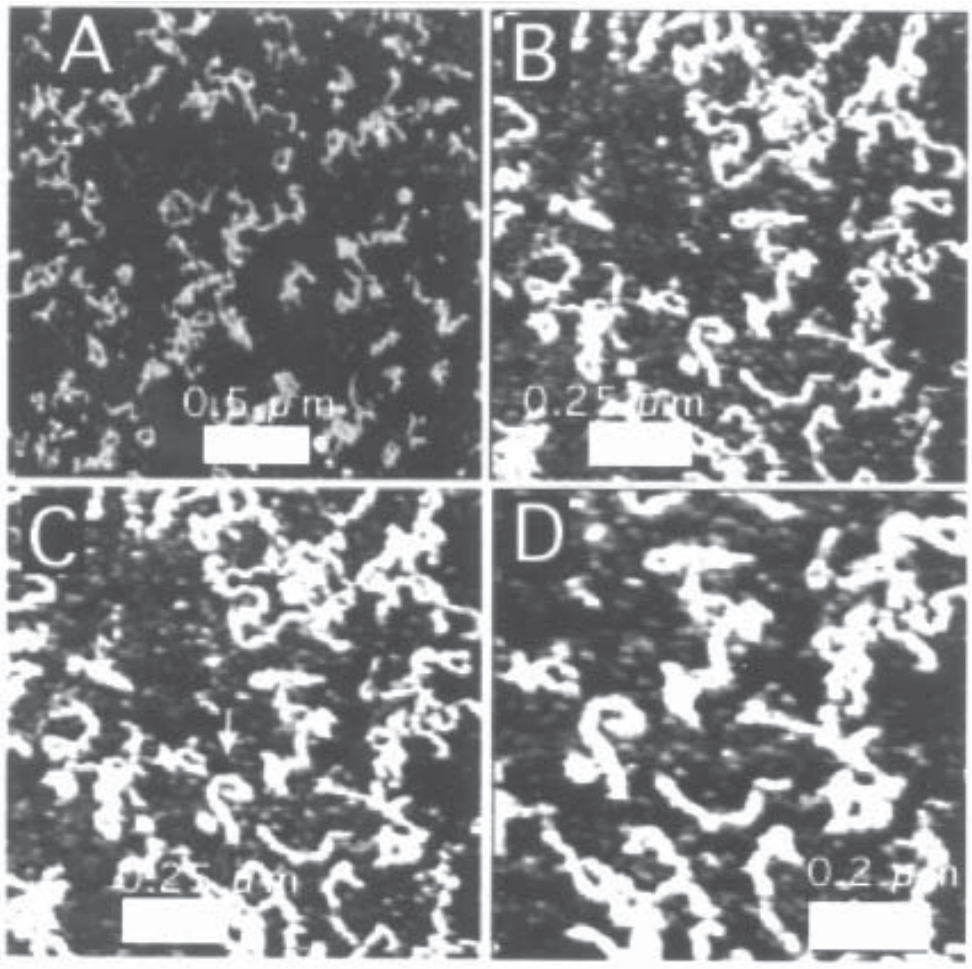

Figure 2. AFM images of complexes of 1.07 Kb DNA fragment with p53F polypeptide prepared at molar protein/DNA ratio 160:1. They demonstrate the stability of the sample. After scanning **A**, the microscope was zoomed to rescan twice (**B**, **C**) and zoomed again (**D**). All images were obtained in propanol in contact mode. Z-range for all images is 10 nm.

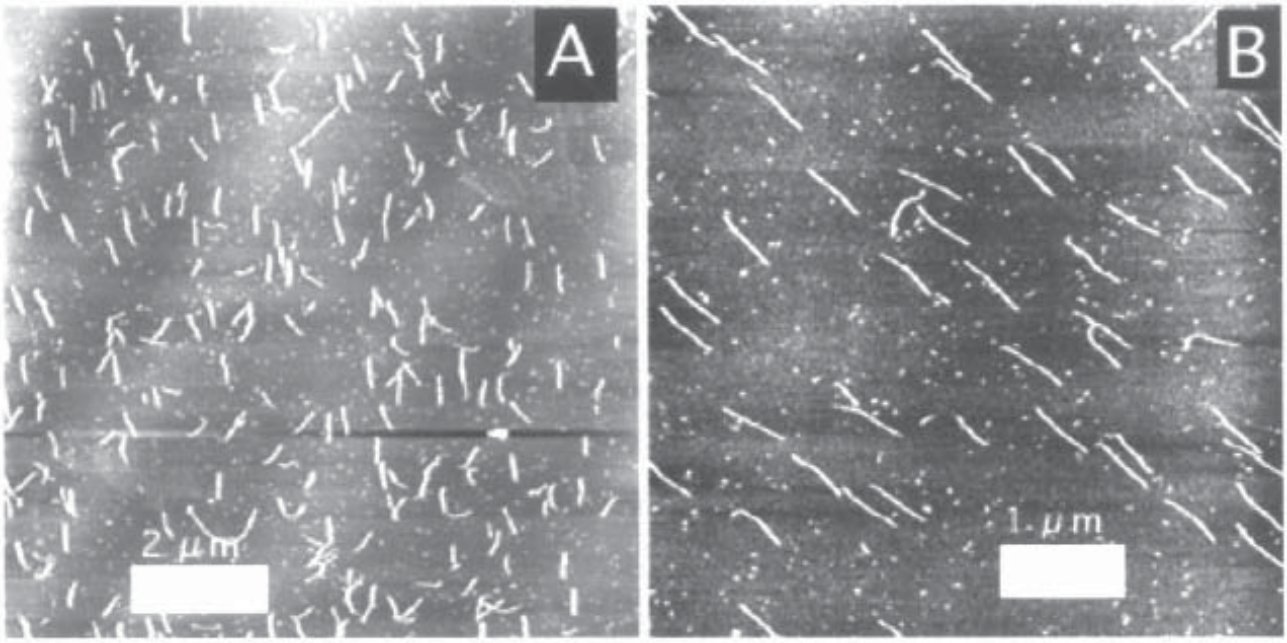

Figure 3. AFM images of the RecA-coated 1.07 Kb DNA shown in two different scans; the Z-range for (**A**) is 25 nm and for (**B**), 10 nm.

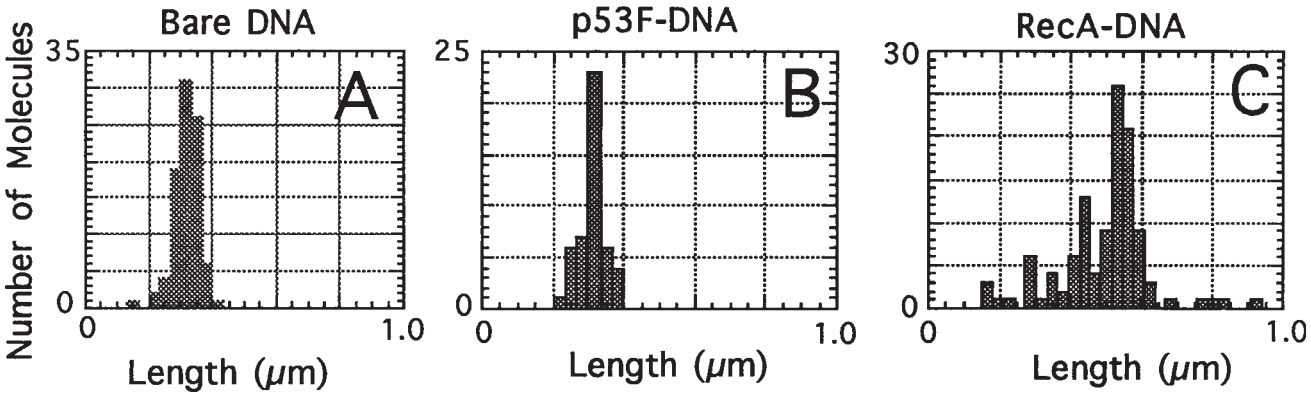

Figure 4. Histograms showing the length distributions of the bare DNA (**A**), p53F-coated DNA (**B**) and RecA-DNA complex (**C**).

1D, molecules 2 and 3). The Waf1 binding site was located at 237 bases from one end of the fragment but we did not find evidence of enhanced binding at this site. The dissociation constant for Waf1 element is (8.3 ± 1.4) x 10^{-8} M (Balagurumoorty *et al*., 1995). For comparison, the dissociation constant for *Cro* protein of lambda phage for its 17 bp operator region is more than two orders of magnitude lower (Kim *et al*., 1987), indicating a very high affinity of *Cro* protein to its operator region. Moreover, reliable AFM study of its specific interaction required the use of carefully chosen ionic conditions and, even then, specific binding was not easily differentiated from non-specific binding (Erie *et al*., 1994).

The structure of the complexes changes dramatically if the protein concentration is increased further. A series of AFM images for complexes prepared at 160:1 protein/DNA ratio are shown in Figure 2. Thick filaments appear on the surface. Note that the Z-range for the images shown in Figure 2 is 2.5 times larger than that for non-complexed DNA (Figs. 1A and 1B). Binding of these filaments to the substrate is strong enough to withstand repeated scanning with the AFM tip as demonstrated by Figures 2B, 2C and

Table 1. Measured dimensions of DNA and DNA-protein complexes. The repeated set of DNA data labeled [(a)] was obtained with the same tip used for the RecA-DNA experiments.

Sample	Height (nm)	Width (nm)	Contour length, L_c (nm)	End-to-end length, L_e (nm)	L_e/L_c
DNA	0.47 ± 0.07	8.3 ± 1.2	320 ± 73	70 ± 45	0.22 ± 0.15
p53-DNA	2.2 ± 0.23	25.2 ± 3	311 ± 38	152 ± 53	0.49 ± 0.20
RecA-DNA	3.4 ± 0.24	31.1 ± 3.6	520 ± 131	453 ± 135	0.87 ± 0.35
DNA[(a)]	0.52 ± 0.11	8.25 ± 1.2	315 ± 63	72 ± 44	0.23 ± 0.15

2D. Clearly, the complexed DNA is wider, higher and stiffer than the bare DNA (Fig. 1).

In order to characterize the effects of tip broadening and test for artifacts owing to the substrate, we carried out a parallel study of RecA-DNA complex. The RecA protein coats the DNA molecules uniformly, leading to the formation of helical filaments ~10 nm wide with a ~10 nm helical pitch (Egelman and Stasiak, 1993). It is known that the DNA helix is unwound in the complex with RecA, so that the base-to-base distance is 5.1 Å versus 3.4 Å for canonical B-DNA (Egelman and Stasiak, 1993). The 1.07 Kb DNA sample was complexed with RecA protein in saturating conditions, and RecA-DNA filaments were deposited onto AP-mica in a manner similar to that used for p53-DNA filaments. AFM images are shown in Figure 3. The filaments are almost straight, consistent with the high rigidity of RecA-DNA filaments which have a persistence length of ~600 nm, 10-15 times larger than that for double stranded DNA (Egelman and Stasiak, 1993). The images are bright, consistent with the greater thickness of the complex compared to bare DNA.

Figure 4 shows histograms of the contour length measurements of bare DNA (Fig. 4A), p53-DNA complex (Fig. 4B) and RecA-DNA complex (Fig. 4C). The DNA and p53-DNA complex are both about 320 nm long, indicating no stretching or extension of DNA in the complex. These data are consistent with crystallographic data for a complex of the DNA binding fragment of p53 with oligonucleotides which showed that the DNA retained its normal geometry (Cho *et al*., 1994). In contrast, the RecA-DNA complex is considerably longer, the rise per base being 4.9 ± 1.2 Å, close to the 5.1 Å reported by Egelman and Stasiak (1993).

Data for the apparent height, width, contour length L_c, and end-to-end length L_e, for all samples are shown in Table 1. The uncertainties represent plus or minus one standard deviation of the measured data set and are dominated by the real molecule-to-molecule variations. The end-to-end length is determined by the shortest distance between the ends of each chain. The height values for DNA and RecA-DNA are substantially less than the real heights, an artifact of AFM that is well-known but not fully explained (Bustamante *et al*., 1992; Lyubchenko *et al*., 1992a, 1993b, 1995a; Yang *et al*., 1992; Marsh *et al*., 1995; Radmacher *et al*., 1995). The widths are also much greater than expected, predominantly as a result of the finite radius of the tip although other factors contribute (Yang *et al*., 1992; Lyubchenko *et al*., 1993b). We carried out a second study of the 1.07 Kb DNA fragment at the same time that we imaged the RecA-DNA. These data (last row of Table 1) were consistent with the original study (first row), indicating that the AFM tips (and other imaging parameters) did not change substantially over the course of this work.

A measure of the relative flexibility of these samples may be obtained by comparing the end-to-end length to the contour length, and this ratio, L_e/L_c, is shown in the last column of Table 1. RecA-DNA is almost fully extended, implying that the persistence length is longer than the molecules imaged here, consistent with an earlier report (Egelman and Stasiak, 1993). The p53-DNA complex is intermediate in stiffness between the DNA and the RecA-DNA complex.

The width of the p53-DNA complex may be estimated using eqs. (1) or (2). The spherical tip model yields R_c = 4.3 nm (assuming R_m for DNA is 1 nm) and R_c = 12.1 nm (assuming R_m for RecA-DNA complex is 5 nm). Similar values (3.6 and 10.6 nm, respectively) are obtained from the parabolic tip model {eq. (2)}. An appropriate tip radius is best determined from a known sample of similar width and height, so we choose to use the values determined from the RecA complex. Although the mechanism of the apparent height reduction is not understood, it seems reasonable to assume that the magnitude of the effect will be similar when similar molecules are imaged in similar conditions. Thus, we can arrive at an estimate for the height of the complex by assuming that the p53 images are distorted by the same factor (3.4/10 or 0.34) that the RecA complex is (Table 1). The three values of molecular diameter for the p53-

DNA complex obtained from the two broadening models and the height data are 6.34 nm {eq. (1)}, 6.56 nm {eq. (2)} and 6.47 nm (height data). The values are remarkably consistent. Taking the average of the values for the two broadening models and folding the spread in these values into the measured standard deviation gives a value of 6.45 ± 1 nm for the width/height of the complex.

Discussion

We have found that the DNA-binding fragment of p53 protein polymerizes onto a DNA template to form thick filaments at high concentration. There are two broadly different classes of structure for nucleoprotein filaments. For chromatin-like structures, DNA is outside the complex, wrapping around the protein core. In the other type, protein molecules are outside, and in many cases, they form a shell around the DNA core. A majority of filamentous bacteriophages and, for example, regular RecA-DNA filament, form complexes of this type. The chromatin-like complexes are considerably shorter than the free-DNA contour length. Complexes of the second (RecA) type may be shorter than DNA (e.g., bacteriophage fd, Frank and Day, 1970; Lyubchenko *et al*., 1993b, 1995a) or longer than DNA (e.g., RecA-DNA complex, Stasiak and Egelman, 1993). The p53-DNA filament is of the same contour length as DNA, eliminating the possibility of a chromatin-like structure. In the crystal complex of the p53 DNA binding domain (102-292 amino acids in that case) with 21 bp DNA duplex, one protein monomers binds a consensus site near the center of the DNA duplex and another binds 11 bp away in a region of a weak homology with the consensus sequence. The consequent model for the p53 tetramer-DNA complex yields a width of about 6.5 nm (Cho *et al*., 1994). Our AFM measurements yielded an estimated width of the filament of 6.45 ± 1.0 nm. We found that the DNA in p53F-DNA filament has a rise per base indistinguishable from that of the canonical B-form, which is also consistent with the crystallographic data of Cho *et al.* (1994).

Both the continuous coating observed here and the X ray data indicate that protein-protein interactions are important in formation of the assembly, and this, in turn, suggests that p53 binding at high concentrations will be cooperative. Early evidence from chemical footprinting data indicates that this is indeed the case (A. Nagaich, unpublished results).

The formation of filaments with characteristics similar to that for the p53F-DNA complex was studied with EM and AFM for Fur repressor from *E. coli* (Le Cam *et al*., 1994). They found that these complexes are stiffer than DNA, and that these changes may be an important factor in the repression process regulated by Fur. The formation of such filaments is not universal. For example, even at high concentration, TATA binding protein (TBP) from yeast accumulates at specific sites, forming highly compacted nucleoprotein structures (Griffith *et al*., 1995).

Is the filamentous structure observed with the binding domain fragment, p53F, relevant to the behavior of intact p53? Recent studies (Balagurumoorty *et al*., 1995) have shown that p53F is able to bind to DNA as a tetramer in a manner similar to complete p53 protein, even in the absence of the tetramerization domain. Based on their structure for the tetramerization domain, Jeffrey *et al*. (1995) proposed a model for the interaction of this domain with DNA in which this region lies on the *opposite* side of the helix to the DNA binding domain. In an electron microscopic study, Stenger *et al*. (1994) found oligomerization of wild-type p53 on DNA containing a repeated binding sequence. Therefore, it appears that wild-type P53 may be able to polymerize on DNA in much the same way as p53F.

If wild-type p53 is able to polymerize on DNA, then what are the possible biological consequences? Severe DNA damage (by ultraviolet or ionizing radiation) is accompanied by increased synthesis of p53 which controls the propagation of damaged DNA (Nelson and Kastan, 1994). The p53 protein has a high affinity for DNA lesions (e.g., Lee *et al*., 1995). Thus, a polymerization process which occurred at high p53 concentration might play a role in recognition and repair of damaged DNA while also delaying replication (Cox *et al*., 1996). If the DNA damage is beyond the capabilities of the repair machinery, apoptosis occurs (Martin *et al*., 1994); Enoch and Norbury (1995) suggested that a high level of p53 is an important signal for this. The possibility that the filamentous form of the DNA-p53 complex plays a role in these processes deserves further study.

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Discussion with Reviewers

D.P. Allison: Why are the data obtained with RecA referred to as a control experiment?
Authors: RecA-DNA complex has been very well-characterized (Egelman and Stasiak, 1993) and the complex is of similar dimension to the p53-DNA complexes used here. For this reason, the RecA-DNA complex is a useful control (**a**) for determining tip broadening effects and (**b**) for determining if our sample preparation method alters the length of the complex. The fact that it does not do so for RecA-DNA suggests that our conclusion that the DNA in the p53-DNA complex is in the B-form is correct.

D.P. Allison: What is the connection between the high affinity of p53 to bind UV-damaged DNA and the coating of undamaged DNA revealed in this work?
Authors: p53 has a range of functions which include a role in repair as well as regulation of transcription. It also slows or stops the propagation of cells with damaged DNA. Because p53 binds with high affinity to damaged sites, and because it binds cooperatively, we speculate that the initial binding at a lesion may be followed by rapid coating of the neighboring DNA at high p53 concentration. This coating of the otherwise normal neighboring DNA may be one way in which p53 shuts down transcription.

D.P. Allison: Why does there appear to be a preferential orientation of molecules in Figure 3?
Authors: We believe this to be a consequence of the rinsing step.

H.J.K. Hörber and P. Becker: The drying of samples for AFM investigations always negates the major advantage of the AFM over the electron microscope, namely, that samples in general do not have to be dehydrated. Why did you not work in liquid?
Authors: Studies in liquid are planned using a gentler form of AFM (Han *et al*., 1996).

H.J.K. Hörber and P. Becker: The data presented here does not support cooperative binding of the p53. Please comment.
Authors: The cooperatively of binding was established

independently (Balagurumoorty *et al*., 1995).

R. Balhorn: What percentage of the DNA molecules in the 4:1 p53F treated samples contain blobs similar to those shown in Figure 1? Of those, are any located at the Waf1 site?

Authors: Eighty percent of the molecules treated at this concentration of p53F have "blobs" associated with them, most often at the ends (which have single stranded regions) and this demonstrates the high affinity of p53F for single stranded regions (Bakalkin *et al*., 1995). Only a small fraction (less than 10%) of these have molecules near enough to the Waf1 site for the binding to be considered specific. Clearly, sample preparation methods were not conducive to specific binding. Erie *et al*. (1994) had to go to considerable lengths to bind the DNA to a substrate in a salt solution of concentration high enough to see specific binding of *Cro* protein to lambda DNA, a more specific binding than p53F-DNA.

Additional Reference

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