

Dissecting Elastic Heterogeneity along DNA Molecules Coated Partly with Rad51 Using Concurrent Fluorescence Microscopy and Optical Tweezers

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ABSTRACT Nucleoprotein filament formation by recombinases is central to homologous recombination. To follow this process, we used fluorescent human Rad51 recombinase to visualize the interactions with double-stranded DNA (dsDNA). Fluorescence imaging revealed that Rad51 filament formation on dsDNA initiates from multiple nucleation points, resulting in Rad51-dsDNA nucleoprotein filaments interspersed with regions of bare DNA. The elastic properties of such heterogeneously coated DNA molecules were assessed by combining force-extension measurements using optical traps with fluorescence microscopy. This combination of single-molecule techniques allows discrimination of segments within an individual DNA molecule and determination of their elastic properties. The nonfluorescent zones of DNA-Rad51 constructs showed the well-known (over)stretching behavior of bare DNA. In contrast, the fluorescent, Rad51-coated zones did not overstretch and Rad51 remained stably bound in a structure that was ~50% longer than bare DNA. These results illustrate the power of adding sensitive fluorescence imaging to optical tweezers instrumentation.

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Optical tweezers have proved to be versatile tools to mechanically probe DNA and the interactions with DNA-binding proteins (1–5). Combining this technique with fluorescence microscopy is a powerful means to simultaneously observe proteins bound to the DNA and detect induced mechanical perturbations. However, only a few such applications have been reported (6–8). Here, we image fluorescence during force-extension measurements on double-stranded DNA (dsDNA) coated with fluorescently labeled recombinase proteins. This approach allows detailed analysis of elasticity of different segments on the same DNA molecule, either coated with fluorescently labeled protein or uncoated, rather than an average analysis over the whole molecule. Therefore, we can directly identify different elements of a complex structure and coherently dissect their separate elastic behavior, without assuming uniform molecular characteristics.

Using this combined approach, we have studied the mechanical aspects of human recombinase protein Rad51 binding to dsDNA. Rad51 forms the catalytic core of eukaryotic homologous recombination, an essential mechanism for maintaining genome integrity (9–11). Homologous recombination serves both as a crossover mechanism for chromatids during meiosis and as a reliable repair pathway for dsDNA breaks or stalled replication forks (12). Recombinase proteins drive DNA strand exchanges between homologous DNA molecules. Rad51 is structurally and functionally similar to the prokaryotic RecA and archaeal RadA recombinases (9–11). Like other recombinases, Rad51 forms nucleoprotein filaments on both single-stranded (ssDNA) and dsDNA (9).

To study Rad51 nucleoprotein formation on DNA using the combined trapping/fluorescence approach, we have generated functional single-cysteine variants of human Rad51 recombinase and labeled them with Alexa Fluor 555 (Molecular Probes, Eugene, OR); a detailed description of the variants and their biochemical functionality tests will appear elsewhere. Filaments assembled at multiple sites on dsDNA. Therefore, the dsDNA molecules become discontinuously coated with Rad51, reflected both in intermittent fluorescence emission along the DNA and heterogeneous elasticity (see below).

EXPERIMENTAL ASSAY

For a quantitative analysis of the mechanical properties of Rad51 nucleoprotein filaments, we used a double optical trap setup to manipulate individual Rad51-dsDNA complexes. To demonstrate the ability to extract features of an inhomogeneously coated single DNA molecule, filaments were assembled with Alexa Fluor 555-labeled Rad51 onto 48-kbp biotinylated λ -phage dsDNA (5). Assembly occurred in the presence of 1 mM ATP, 30 mM KCl, and with 2 mM CaCl₂ (instead of MgCl₂) to strongly reduce disassembly of the nucleoprotein filaments (13,14). By tuning the Rad51/bp stoichiometry during assembly, conditions were obtained resulting in incomplete coverage of the DNA molecules.

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Single filaments were tethered from both ends to two streptavidin-coated beads, optically trapped in a custom-built flow cell (5) using a 3 W 1064 nm laser. By moving one of the traps, tension can be applied on the filament in a controlled manner. Combining back-focal-plane interferometry (15) with wide-field epifluorescence imaging (532 nm laser excitation) onto a charge-coupled device camera (Cascade 512B, Princeton Instruments, Monmouth Junction, NJ), both the force on the fixed bead and fluorescence images of the whole construct were recorded at 5 Hz (Movie 1, Supplementary Material). Moreover, the distance between the beads over time was tracked using transmitted bright-field video microscopy. Together, this allows determination of the elastic properties of distinct segments in a DNA molecule.

NONUNIFORM ELASTICITY

To dissect the force-extension data of the heterogeneous construct, kymographs (16) were constructed from the fluorescence images. The kymograph in Fig. 1 A shows that Rad51 remains stably bound even at forces that overstretch dsDNA. Moreover, it is locally anchored to the DNA: the relative position of the fluorescent zones did not change in several stretching cycles on the same construct. Although it is still possible that the dsDNA held within the filament is dynam-

ically melted, one or both strands must be tightly bound to Rad51. This behavior of Rad51, polymerized into ATP-coordinated nucleoprotein filaments, is thus very different from the one-dimensional sliding along dsDNA of Rad51 oligomers reported by Granéli et al. (17), a property that is ATP independent and presumably unrelated to nucleoprotein filament formation.

The force-extension curve of the entire construct in Fig. 1 (panel B) and comparison to the curve of bare DNA (shown in gray) immediately show the qualitative effect of Rad51 binding. First, the steep tension increase commences at larger extension than for bare dsDNA, indicative of a longer contour length. Second, the length increase during the overstretching transition (induced at tensions exceeding 65 pN) is less than the factor 1.7 increase for bare dsDNA (2), suggesting reduced overstretching for Rad51-coated dsDNA.

The kymograph was subjected to edge detection with sub-pixel resolution to discriminate fluorescent from dark zones and monitor them in time during extension. Force-extension curves were then generated for specific parts of the construct (Fig. 1, C–E). The force-extension behavior of a nonfluorescent zone (bare zone (i), Fig. 1 C) was, as expected from the apparent absence of Rad51 on this part of the dsDNA, indiscernible from that of published curves of dsDNA (2). It showed a steep increase of force when stretched up to a contour length of 2.8 μm . At forces exceeding 65 pN, a clear

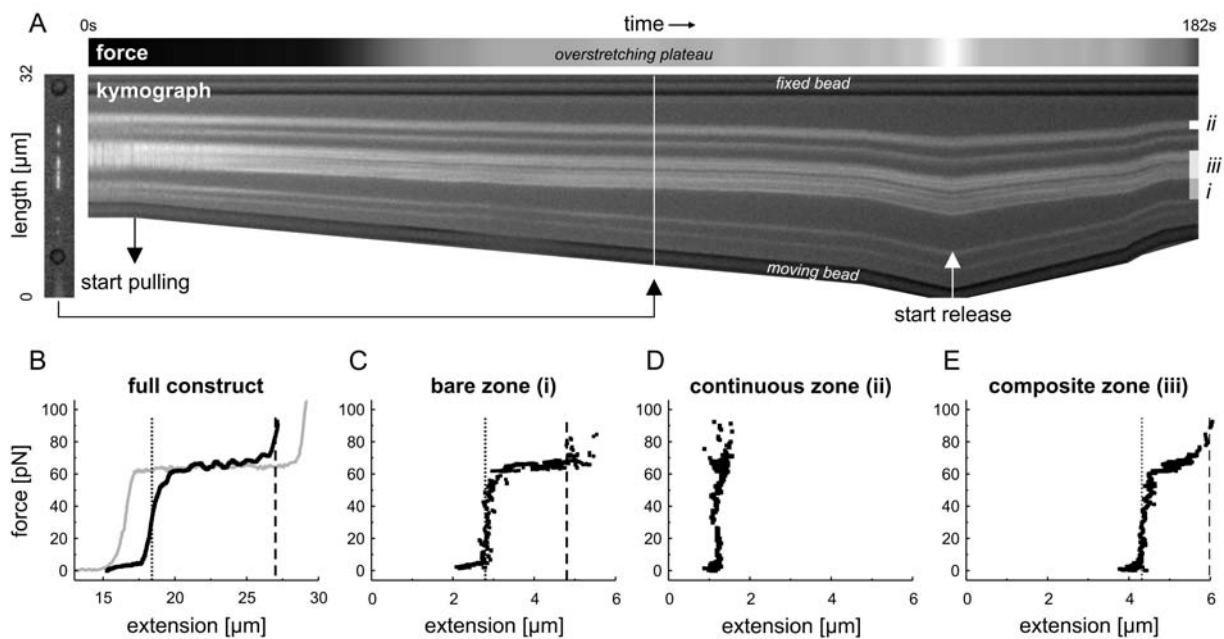


FIGURE 1 Elastic properties of a single 48 kbp λ -dsDNA molecule partly coated with fluorescent Rad51. (A) Fluorescence image (left) of such an assembly, tethered between two streptavidin-coated polystyrene beads. Kymograph (right) generated from the successive frames of the movie recorded during extension of the construct (Movie 1, Supplementary Material). Fluorescence decay is due to photobleaching and not Rad51 dissociation, as tested by varying the laser power. The corresponding force time trace is depicted in gray scales (top bar; white corresponds to 90 pN). (B) Force-extension curve corresponding to the construct in A. The gray trace shows a bare λ -DNA reference curve. (C–E) Force-extension curves of the bare zone (i), the continuous fluorescent zone (ii) and the composite fluorescent zone (iii) as indicated on the right of the kymograph.

overstretching plateau was reached that ended at a length of $4.8 \mu\text{m}$, i.e., $\sim 170\%$ of the contour length of this part of the dsDNA. In sharp contrast, the force-extension behavior of a continuously fluorescent zone, indicative of Rad51-coated DNA (continuous zone (ii), Fig. 1 D), showed that this zone did not overstretch under high tension (up to 90 pN). This has also been observed for RecA (3,18,19). The force-extension behavior of a composite zone ((iii), Fig. 1 E), apparently including zones with and without bound Rad51, was more complex. An overstretching plateau was observed, but the dsDNA could only be stretched to 140% of its contour length. This composite behavior observed in the force-extension curve can be accounted for by a linear combination of coated, rigid parts (cf. Fig. 1 D), and bare, elastic parts (cf. Fig. 1 C). With this assumption, the fraction of the dsDNA coated by Rad51 (f) and the Rad51-induced elongation factor of the coated parts (e) were estimated from the force-extension curve of the full construct (Fig. 1 B) as follows. The measured contour length of the partially coated and extended filament ($18.4 \mu\text{m}$, fit to worm-like chain (2), see Fig. 1 B) was expressed as the sum of an uncoated part of length $L_0(1-f)$ and a coated (and extended) fraction of length L_0fe , L_0 being the $16.4\text{-}\mu\text{m}$ contour length of relaxed uncoated λ -DNA. Similarly, assuming that only uncoated segments stretch under tension and knowing that bare dsDNA can be over-stretched to 170% of its relaxed contour length (2), the over-stretched length of the partially coated filament ($27.0 \mu\text{m}$, from inspection of Fig. 1 B) was expressed as the sum of $1.7 \cdot L_0(1-f)$ and L_0fe . From this set of equations, the values of f and e were solved, yielding a coated fraction f of 25% (for the DNA molecule in Fig. 1) and a Rad51-induced extension e of 148%. This latter value was reproduced within 4% (standard deviation) with other Rad51-dsDNA assemblies and is comparable to those determined from electron microscopy and scanning force microscopy images (9,14).

In this letter, we have shown that Rad51 nucleates at multiple sites along dsDNA, hence forming discrete segments. Moreover, the elasticity analysis showed that the DNA within the filament is stably bound to and held rigidly by Rad51. This information could only be obtained by combining fluorescence imaging with single-molecule manipulation with optical tweezers, hence avoiding the averaging over heterogeneous segments.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

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