

Mechanism of RecA-mediated homologous recombination revisited by single molecule nanomanipulation

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The mechanisms of RecA-mediated three-strand homologous recombination are investigated at the single-molecule level, using magnetic tweezers. Probing the mechanical response of DNA molecules and nucleoprotein filaments in tension and in torsion allows a monitoring of the progression of the exchange in real time, both from the point of view of the RecA-bound single-stranded DNA and from that of the naked double-stranded DNA (dsDNA). We show that strand exchange is able to generate torsion even along a molecule with freely rotating ends. RecA readily depolymerizes during the reaction, a process presenting numerous advantages for the cell's 'protein economy' and for the management of topological constraints. Invasion of an untwisted dsDNA by a nucleoprotein filament leads to an exchanged duplex that remains topologically linked to the exchanged single strand, suggesting multiple initiations of strand exchange on the same molecule. Overall, our results seem to support several important assumptions of the monomer redistribution model.

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Introduction

Homologous recombination is a subject of major interest in biology. *In vivo*, this mechanism is involved in DNA repair and in the generation of genetic diversity (Kowalczykowski and Eggleston, 1999). Understanding and controlling homologous recombination is also of the utmost importance for medical and biotechnological purposes because of its implication in cancer (D'Andrea and Grompe, 2003) and mutagenesis (Crameri *et al*, 1998; Kuzminov, 1999). The main actors of homologous recombination are a family of highly conserved proteins (Gupta *et al*, 1998; Egelman, 2001), among

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which are RecA for *Escherichia coli* and Rad51 for higher eukaryotes.

RecA alone is able to promote recombination *in vitro* over thousands of base pairs (bp). Yet, many aspects of the process are still poorly understood, and thus the subject of controversy. The three-strand reaction is the paradigm of homologous recombination. It is considered to feature in the early steps of the *in vivo* process. It consists of (i) the polymerization of RecA on a single-stranded DNA molecule (ssDNA) to form a nucleoprotein filament; (ii) the search for homology between the filament and neighboring double-stranded DNA molecules (dsDNA); (iii) the alignment and pairing of the filament (containing the *invading* strand) with a homologous dsDNA (formation of a *synapsis*); and (iv) the exchange of strands, resulting in the formation of a *heteroduplex* (wherein the invading strand is paired with its complementary strand) and in the ejection of the *displaced* strand. Except for the initial RecA polymerization, the current description of all of these steps retains some controversial aspects. For instance, Howard-Flanders *et al* (1984) proposed that the three DNA strands be intertwined in a triple helical way, but the size and detailed structure of the *synapsis* are still unclear (Rao *et al*, 1991; Adzuma, 1992). The role of ATP also raises questions: RecA hydrolyzes ATP throughout the recombination process, but strand exchange on hundreds of bp was demonstrated using adenosine-5'-[γ -thio]-triphosphate (ATP γ S) as a poorly hydrolyzable substitute for ATP (Menetski *et al*, 1990). ATP hydrolysis is nevertheless necessary to achieve strand exchange over more than a few kilobases (kb), to realign mispaired repeats (Sen *et al*, 2000; Zhang *et al*, 2001), to promote four-strand reactions (Kim *et al*, 1992) or to overcome heterologous sequences in the course of recombination (Rosselli and Stasiak, 1991).

Numerous efforts have been devoted in the last 20 years to the unraveling of the mechanisms of strand exchange, and several models (reviewed e.g. in Cox, 2000; Klapstein and Bruinsma, 2000) have been proposed. The treadmill model, in which formation of the *synapsis* and strand exchange are driven by RecA polymerization upstream and depolymerization downstream, was discarded because the rate of strand exchange seems uncorrelated with the polymerization and depolymerization rates of RecA (Lindsley and Cox, 1990). In the 'monomer redistribution' model (Kowalczykowski and Krupp, 1995), strand exchange occurs spontaneously, owing to different binding energies for ssDNA and dsDNA, but ATP hydrolysis is necessary to allow RecA redistribution and eliminate defects in the RecA coverage. Untwisting of the DNA associated with RecA depolymerization upstream of the heterologous zone would also provide the driving force for bypassing heterologous regions. This model, however, does not explain the bypass of heterologies in linear molecules (MacFarland *et al*, 1997).

The 'facilitated rotation' model (Shan and Cox, 1997) alternatively proposes that ATP hydrolysis is to rotate

non-incorporated duplex sections like in a 'rotary motor', ensuring both the directionality of strand exchange and the generation of the torsional stress instrumental to bypass heterologous sections. This model, however, implies a translocation between several DNA binding sites on the outside of the filament, whereas structural evidence suggest that DNA binding sites are rather located inside the filament (Klapstein and Bruinsma, 2000). Another version of 'facilitated rotation' (Burnett *et al*, 1994) rather views the nucleoprotein filament as a *coaxial* rotary motor that untwists the duplex DNA by rotating the whole triplex inside the RecA helix. Yet, such a concerted action on a rigid DNA triplex in the very crowded core of the nucleoprotein filament would imply that hundreds of ATP-hydrolysis associated translocation events occur simultaneously. Although not impossible conceptually, this type of collective behavior has to our knowledge never been observed.

Another variant of the monomer redistribution model was subsequently proposed by Bianco *et al* (1998). In this model, RecA depolymerization releases a medial region of the dsDNA while the two flanking regions of the dsDNA remained bound to the filament. This depolymerization drives bypass of heterologous regions by inducing a negative torsion that transiently denaturates the heterologous zone. This model would thus provide an explanation for the bypass of heterologies, without postulating a hypothetical rotary motor activity of RecA.

A difficulty shared by all monomer redistribution and facilitated rotation models proposed so far, is that they imply the presence of several discontinuous RecA covered sections on the invaded dsDNA. Klapstein *et al* (2004) argued that simultaneous strand exchange at several independent locations along a nucleoprotein filament and a single dsDNA partner is very improbable, because of the differences in rise per base pair between the nucleoprotein filament and the dsDNA.

A 'traveling wave' model involving series of very short zones of RecA-free DNA travelling along a RecA covered nucleofilament following oriented RecA depolymerization and repolymerization was recently applied to the progression of the four-strand Holliday junction (Klapstein and Bruinsma, 2000). This model shares some features of the Bianco one, except that the length of the RecA-free zone is expected to be much shorter (only a few bases). In addition, the progression of a Holliday junction does not raise the topological puzzles involved in the three-strands exchange, and this issue was not considered in the Klapstein model.

In summary, in spite of more than 20 years of efforts and a large number of articles published on the subject, the molecular mechanisms of strand exchange are still not fully understood. The following questions, in particular, remain open:

- Is the bypass of heterologies promoted by torsional stress, and if so is this stress due to RecA depolymerization (as in monomer redistribution models), or to a DNA translocation process associated with a 'rotary motor' activity (as proposed in facilitated rotation models)?
- Is strand exchange occurring along a single, continuous synapsis, as suggested by Klapstein *et al* (2004) or are there several discontinuous interaction sites between the nucleoprotein filament and the invaded dsDNA, as suggested in Shan and Cox (1997) or Bianco *et al* (1998)?

- What ensures the irreversibility of strand exchange, and prevents the reverse process to occur between the newly created duplex and the released ssDNA?

The classical approaches for studying reaction intermediates, that is electron microscopy (Stasiak *et al*, 1984; Umlauf *et al*, 1990; Jain *et al*, 1995) or electrophoretic assays, have been applied to the strand exchange problem with perseverance and imagination, but they suffer from an intrinsic need to freeze the reaction. Consequently, they cannot provide information on fluctuations or on short time scale dynamics. Single molecule manipulations, in contrast, can provide real time information on DNA dynamics in physiological environments, and have already yielded invaluable information on the mechanics and molecular interactions of DNA. So far, however, only RecA-DNA interactions have been studied using single-molecule techniques (Léger *et al*, 1998; Bennink *et al*, 1999; Hegner *et al*, 1999; Shivashankar *et al*, 1999; Fulconis *et al*, 2004). Here, we study for the first time the topological and dynamical aspects of RecA-mediated homologous pairing and strand exchange, in real time and in physiological conditions. This is achieved with single molecule nanomanipulation by magnetic tweezers.

Magnetic tweezers (Strick *et al*, 1996) can stretch and twist a single DNA molecule, and allow a measure of its length with nanometer resolution, in response to these mechanical constraints. Since ssDNA, dsDNA and RecA nucleoprotein filaments have very different mechanical behaviors, we could use the mechanical 'signature' of these molecules and complexes to monitor various steps of the three-strand reaction. Two protocols, yielding complementary information on the recombination process, were used (Figure 1 and Materials and methods): (i) attaching an ssDNA in the tweezers, covering it with RecA, and then studying its interaction with homologous dsDNA (hereafter referred to as the (ssDNA-RecA + dsDNA in solution) assay); or (ii) manipulating a dsDNA in the tweezers, and studying its interaction with preformed homologous RecA-ssDNA nucleoprotein

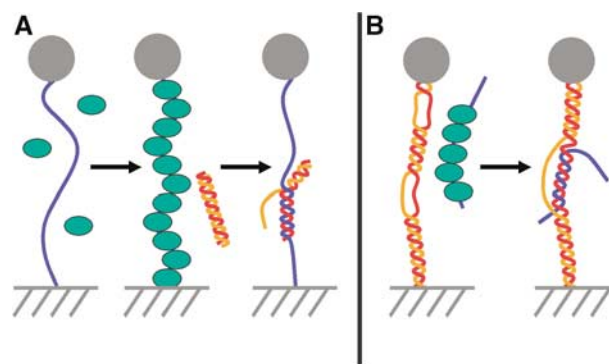


Figure 1 Experimental design: schematic view. (A) ssDNA-RecA + dsDNA assay. An ssDNA molecule (14 kb) is held in the magnetic tweezers, then covered by RecA and incubated with homologous dsDNA of various sizes. (B) dsDNA + ssDNA-RecA assay. A dsDNA molecule (14 kb) is held in the magnetic tweezers, partially untwisted, and incubated with preassembled RecA-ssDNA fibers, the ssDNA being homologous to the central part of the dsDNA. Please note that the number of molecules involved in the exchange reaction, and the topology of the formed complex are just provided as examples of what could happen, and do not imply any *a priori* assumption of the real mechanism of strand exchange, or of the actual final product of the exchange.

filaments (hereafter referred to as the (dsDNA + ssDNA-RecA in solution) assay).

Results

(ssDNA-RecA + dsDNA in solution) assay

A single 14 kb ssDNA molecule was held in the magnetic tweezers, and a force versus extension measurement was first performed on the molecule in Binding Buffer (15 mM Tris-HCl at pH 7.5, 50 mM NaCl, 0.05% Tween-20; Figure 2A, black curve). After adding RecA and maintaining the molecule at a stretching force $F \sim 10$ pN for a few minutes (see Supplementary Methods), the molecule's mechanical behavior was dramatically altered (Figure 2A, blue curve). The filament's contour length increased to a value corresponding

to 1.5 times its crystallographic length, and its force versus extension behavior displayed a lower elastic modulus: a lower force was necessary for stretching the nucleoprotein filament than for stretching naked DNA by the same amount. In polymer elasticity theory, this is the signature of a larger bending persistence length. This behavior is similar to that reported previously for the formation of a ssDNA-RecA nucleofilament (Hegner *et al*, 1999; Shivashankar *et al*, 1999). The force versus extension response was quantitatively fitted by the extended series expansion expression for the worm-like chain model proposed in (Bouchiat *et al*, 1999). This fit yields typical persistence lengths of 700 and 450 nm, when ATP γ S and ATP were used as cofactors, respectively. These values, in qualitative agreement with earlier data, combined with a molecule's extension by a factor 1.5 ± 0.05 , provide a convincing check that a nucleoprotein filament covering 100% of the molecule (with an uncertainty of 10%), was indeed formed.

Homologous dsDNA was then injected into the channel (see Supplementary Methods). Four different homologous substrates were used: 14 kb dsDNA (homologous to the whole ssDNA), 3.5 kb dsDNA (homologous to the central part of the ssDNA), sonicated dsDNA (fragments of 200 ± 100 bp, typically two thirds of which are homologous to the ssDNA), and heterologous DNA. The ssDNA-RecA fiber was held under constant forces (ranging from 0.1 to 10 pN, depending on the experiment) during incubation, typically for one hour.

Monitoring the extension of the ssDNA-RecA-ATP fiber during incubation with homologous dsDNA at a force $F = 10$ pN, we observed a length reduction by a factor of about 1/3, associated with strong fluctuations (Figure 3A, blue curve). In the presence of heterologous dsDNA in solution (purple curve), we observed a constant length or

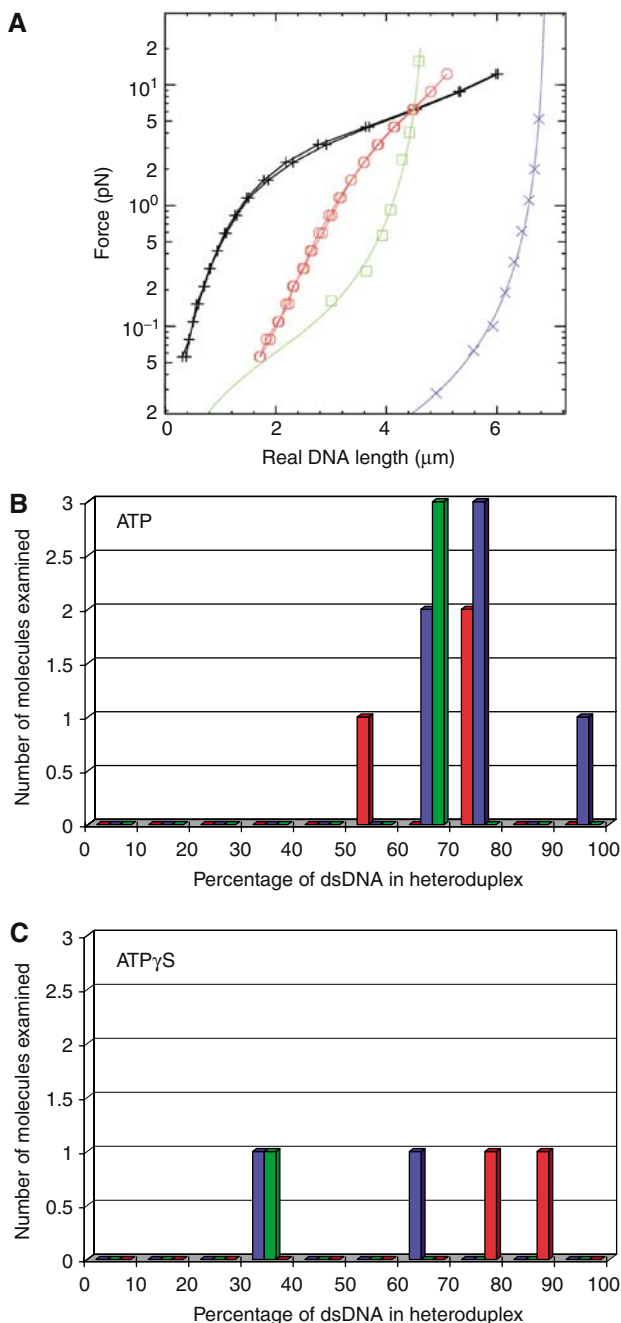


Figure 2 ssDNA-RecA + dsDNA assay: force versus extension curves. (A) Symbols represent the experimental data, and the full lines the best-fit using the extended analytical interpolation of the worm like chain model (Bouchiat *et al*, 1999). This approximation yields an analytical expression consistent with the exact numerical solution within better than 2%. Since we used forces at most 10 pN, corrections for elastic deformation of the DNA backbone are below 5% (Cizeau and Viovy, 1997) and were thus neglected; +, black: ssDNA before recombination (recorded in Binding Buffer); The best-fit parameters obtained using the worm like chain model are: $l = 5.4 \mu\text{m}$ and $\xi = 6.2 \text{ nm}$; \times , blue: same molecule after extensive RecA polymerization (recorded in Binding Buffer + 2 mM Mg^{2+} to ensure good nucleoprotein filament stability). Best-fit parameters: $l = 6.9 \mu\text{m}$ and $\xi = 449.2 \text{ nm}$; \circ , red: same molecule after recombination with 14 kb homologous dsDNA and rinsing to get rid of RecA (in Binding Buffer); \square , green: 14 kb dsDNA used as a reference (in Binding buffer); best-fit parameters: $l = 4.7 \mu\text{m}$ and $\xi = 56.7 \text{ nm}$. In this example, the reaction product curve (\circ) was fitted with an 'hybrid' curve consisting of 30% single-stranded DNA and 70% double-stranded DNA. Both increasing and decreasing force scans data are plotted, and were fitted independently with the model, leading to the two plain lines. They are almost superposed, showing the very weak hysteresis and good reproducibility of the experiments. $71 \pm 1\%$. (B) Histogram of the fraction of dsDNA in the hybrid molecule formed in presence of ATP, relative to the homology between the probe molecule and the substrate. Blue: experiments with 14 kb homologous DNA; red: experiments with 3.5 kb homologous DNA; green: experiments with sonicated 14 kb homologous DNA. (C) Histogram of the fraction of dsDNA in the hybrid molecule formed in the presence of ATP γ S, relative to the homology between the probe molecule and the substrate. Same color meaning as in (B).

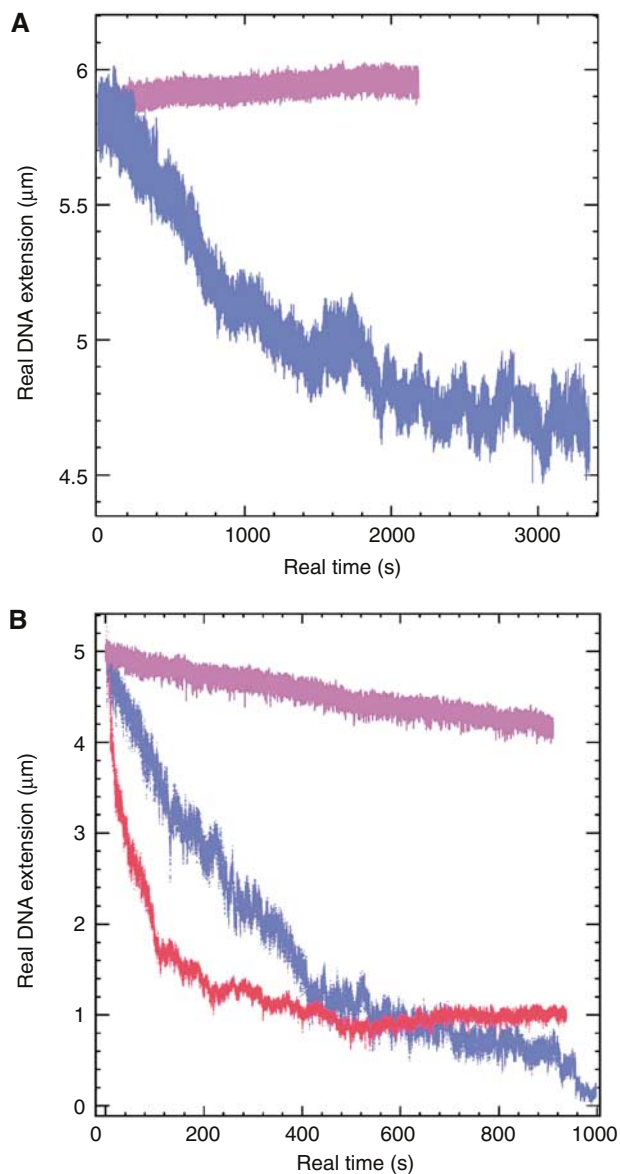


Figure 3 Kinetics of the ssDNA-RecA + dsDNA in solution assay. The filament length was recorded immediately after injection of Binding Buffer + 12 mM Mg^{2+} + 14 kb homologous dsDNA, except otherwise mentioned. (A) Blue line: Nucleoprotein filaments held in the tweezer at 10 pN; purple line: same with heterologous dsDNA. (B) Same under a stretching force of 0.1 pN; blue line: length recorded immediately after injecting buffer + homologous 14 kb dsDNA; red line: length recorded after having maintained the same molecule for 10 min at 10 pN and then lowered the force back to 0.1 pN (see text for details); purple line: control performed in the same buffer, at 0.1 pN in the absence of homologous dsDNA.

slight increase (at the limit of experimental error), essentially devoid of the slow, large amplitude fluctuations occurring in the presence of homologous dsDNA. At $F=0.1$ pN, the molecule length decreased to essentially zero within ~ 30 min (Figure 3B, blue curve). This condensation behavior was partly reversible: when the stretching force was raised to 10 pN, the molecule length increased slowly again within ~ 30 min (see Supplementary data, for more details on force cycles).

After performing the above kinetic experiments, RecA, ATP and the DNA molecules not attached in the tweezer were

extensively rinsed by flowing in the cell Binding Buffer without RecA or ATP during 10 min (Supplementary Methods); and a force-extension characteristics in Binding Buffer was recorded again (Figure 2A, red curve) in the absence of magnesium since this protein tends to induce interactions of naked DNA with glass surfaces and reduce reproducibility. This response was fitted as a linear combination of the initial 14 kb ssDNA plot (Figure 2A, black curve) and a reference plot obtained with a 14 kb dsDNA (Figure 2A, green curve). The residual error of this fit was less than 5%. This result, similar to that previously described for torsionally induced hybridization of short oligonucleotides (Maier *et al*, 2000), provides strong evidence in favor of successful homologous recombination. The fit, averaged over at least four independent molecules, provided a measurement of the proportion of single-strand and double-strand DNA in the reaction product. For full-length homology (14 kb dsDNA in solution), the length of heteroduplex generated was $76 \pm 13\%$ of the immobilized ssDNA. The fraction of the molecule converted to dsDNA was independent of the stretching force exerted during incubation, within experimental error. This last feature is rather surprising, considering the molecule's length at the end of the incubation step: after incubation under a 10 pN force, the molecule's length was $4.7 \pm 0.2 \mu\text{m}$ (Figure 3A, blue curve), a range consistent with the expected length of a dsDNA-ssDNA hybrid at this force, but after incubation at 0.1 pN, the length decreases to $0.25 \pm 0.2 \mu\text{m}$ (Figure 3B, blue curve), whereas the expected length of a 70% dsDNA/30% ssDNA molecule was around $2 \mu\text{m}$. A value close to $1.5 \mu\text{m}$ was indeed obtained after cycling the same molecule several times between 10 and 0.1 pN (Figure 3B, red curve and Supplementary data). This point will be analyzed with more details in the Discussion.

Similar experiments were performed with a dsDNA substrate of 3.5 kb, homologous to the central part of the 14 kb ssDNA held in the tweezers, and with a 14 kb dsDNA, fully homologous to the ssDNA in the substrate but sonicated in order to reduce it into fragments of a few hundred bp. In both cases, a length decay with strong fluctuations was observed, and a partial conversion from ssDNA to dsDNA was observed. Overall, the conversion rate was around 70% of the homologous region, a figure comparable with that obtained with a fully homologous substrate (see Figure 2B and Supplementary data for more details).

Similar experiments were also performed with nucleoprotein filaments assembled in ATP- γ S. In this case, the length decay was totally suppressed (see Supplementary data and Supplementary Table I, line 3), but a hybrid molecule with an average of $50 \pm 13\%$ of the homologous zone transformed into dsDNA was nevertheless obtained (Figure 2C). Finally, controls studying the nucleoprotein filament dynamics in various solutions were performed, and the initial slope decay (linear fit over the first 5 min) was quantified for each case (see Supplementary Table I).

(dsDNA + ssDNA-RecA in solution) assay

In order to obtain complementary information on the recombination process, we then considered the progression of the exchange in real time from the point of view of the naked dsDNA. The principle of this experiment is to attach a dsDNA in the tweezers, and then study its interaction with homologous (or non-homologous for control) ssDNA

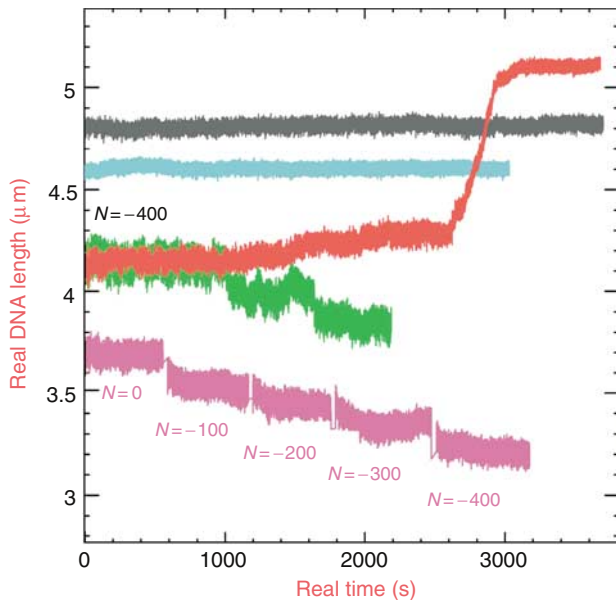


Figure 4 Kinetics of the dsDNA + ssDNA-RecA in solution assay. A 14 kb dsDNA is held at a stretching force of 2.3 pN, and 3.5 kb preformed homologous nucleoprotein filaments are injected at the beginning of recording. N is the number of turns applied on the dsDNA using the tweezers. Purple: applying negative supercoiling progressively by 100 turns steps every 600 s, in order to compensate for the untwisting action of the double strand invasion by the nucleofilament (see text). The degree of supercoiling corresponding to different sections of the curve are indicated in the figure; green: same conditions, except for imposing 400 turns negative supercoiling in one step at the beginning of incubation; red: same as green, except that the nucleoprotein filaments were assembled in the presence of ATP- γ -S instead of ATP; blue: control: same conditions as for the green curve, in the absence of nucleoprotein filament. Gray: control: same conditions as for the green curve, in the presence of heterologous nucleoprotein filament.

nucleoprotein filaments introduced into the dsDNA environment (Figure 1B; also see Materials and methods for more details).

A single 14 kb dsDNA molecule was first attached in the tweezers. An extension versus supercoiling measurement was performed at $F = 0.3$ pN for reference. Molecules without any single-strand nick were selected by their ability to yield a bell-shaped extension versus supercoiling plot, characteristic of the formation of plectonemes (Strick *et al*, 1996). ssDNA-RecA filaments (3.5 kb) were separately prepared (in the presence of the facilitating single-strand binding protein SSB and ATP at 37°C): they were then introduced into the channel (see Materials and methods). When the dsDNA was kept under a 2 pN stretching force in the relaxed state (no supercoiling) in the presence of these nucleoprotein filaments, its length remained stable (first section of purple line, Figure 4). This stability was maintained for hours (data not shown). Since the duplex must untwist for strand exchange to occur, and since our dsDNA molecules were torsionally blocked, we untwisted the dsDNA by -100 turns (i.e. to $\sigma \sim -0.075$), 600 s after the beginning of incubation in order to compensate for the negative twist expected to result from strand invasion (the degree of supercoiling σ is defined as $\sigma = (\text{Lk} - \text{Lk}_0) / \text{Lk}_0$, where Lk is the linking number of the manipulated DNA molecule and Lk_0 is the linking number of the same DNA molecule when it is torsionally relaxed). The length of the molecule readily decreased after supercoiling,

and continued its decrease more slowly. Three further negative supercoiling steps of -100 turns were applied, and the molecule continued to shorten, with an increased rate after each supercoiling step. In another set of experiments, -400 turns were imposed readily at the beginning of incubation (Figure 4, green curve), and the length of the molecule also decreased. Overall, the length decreased by typically ~ 350 nm over 30 min, whether supercoiling had been applied in one step or in four. This decrease in the dsDNA length is surprising, since the incorporation of dsDNA into a nucleoprotein filament is expected to extend, rather than shorten, the molecule. In contrast, in the absence of nucleoprotein filament, or in the absence of supercoiling, the length remained essentially stable within typically 50 nm over several hours. Strong fluctuations were superposed on the molecule's length decay in the presence of nucleoprotein filament, somewhat stronger when the total supercoiling was applied in one step. They were much stronger than those of dsDNA untwisted by -400 turns in the absence of nucleoprotein filament (Figure 4, blue curve).

After a 1 h incubation with homologous nucleoprotein filaments under negative supercoiling, RecA was eliminated by extensive rinsing, and the reaction product was characterized by measuring the molecule's extension versus supercoiling at 0.3 pN, and comparing it with the initial dsDNA reference plot. This study as a function of twist is a major advantage of magnetic tweezers, and it could be applied here to the characterization of the invaded duplex before, during and after strand exchange. The profile obtained after extensive rinsing features a well-defined plateau (Figure 5A, red curve). A similar plateau has been already observed (Strick *et al*, 1998) after hybridization of dsDNA with naked short ssDNA fragments: it was interpreted as the consequence of the formation of D-loops (see Figure 1B for an illustration of a D-loop). This similarity suggests that incubation with nucleoprotein filament in our case also yields one (or several) D-loop(s). The plateau in Figure 5A extends between $\sigma \sim -0.17$ and ~ 0 (corresponding to 230 ± 20 turns). It provides a measurement of the length of DNA involved in D-loops (Strick *et al*, 1998), in this case 2400 ± 200 bp. As ssDNA-RecA filaments were 3.5 kb long, this corresponds to $70 \pm 10\%$ of the homologous zone, in agreement with the results obtained in the (ssDNA-RecA + dsDNA in solution) assay. Notably, in 25% of the experiments, we observed no dynamics of the dsDNA held in the tweezer during incubation with ssDNA-RecA nucleoprotein filaments; in such cases, after extensive rinsing, we measured the same extension versus supercoiling curve as the initial dsDNA (data not shown). The occurrence of enhanced dynamics during incubation was thus correlated with the presence of D-loops on the dsDNA at the end of incubation.

In these experiments, as in supercoiling assays with circular DNAs without nicks, the duplex strand complementary to the nucleoprotein filament and the exchanged one remain attached at both ends. In contrast with conventional supercoiling assays, though, here we are able to control reversibly the topology of the molecule. Notably, in our experiments, the modification of the torsion profile obtained was in general irreversible, and in all experiments except one on a total of 7, the plateau could be followed repetitively after several excursions upon positive supercoiling. It even resisted stretching the molecule at 12 pN under positive supercoiling

($\sigma \sim +0.16$) for several minutes. This is in striking contrast with the hybridization experiments achieved with oligonucleotides: in the latter case, the plateau disappeared after the first excursion at positive supercoiling, a behavior inter-

preted as a consequence of the ejection of the invading ssDNA (Strick *et al*, 1998).

The above enlarged profile was obtained with comparable efficiency, whether negative supercoiling was applied in one or several steps (Figure 4, green and purple curves, respectively). In the absence of supercoiling, however, no such profile could be obtained, and the naked dsDNA profile was always recovered after incubation and rinsing. Several other controls were performed: when the dsDNA in the tweezers, untwisted by -400 turns was incubated with naked ssDNA without RecA or with heterologous nucleoprotein filament, no significant length decay was observed after 90 min (see e.g. Figure 4, blue and gray curve, respectively), and no modification of the dsDNA appeared in the length versus supercoiling assay. Finally, we also incubated the dsDNA, untwisted by -400 turns, with homologous ssDNA preincubated with SSB in the absence of RecA. In that case too, no significant length decay was observed during 90 min (data not shown); after rinsing the proteins, however, an asymmetric length versus rotation was obtained (Figure 5B, red). The molecule had clearly been modified during incubation with ssDNA/SSB, but the extent of profile broadening was smaller than in the presence of RecA. Also, in contrast with the behavior obtained after incubation with nucleoprotein filaments, this widening disappeared when twisting the dsDNA from -400 turns to $+100$ turns: a typical naked DNA profile was obtained when returning from $+100$ turns to -400 turns and during subsequent cycles from -400 turns to $+100$ turns and back (Figure 5B, green).

Finally, we also performed experiments in which the dsDNA was incubated in the presence of nucleoprotein filaments prepared with ATP γ S instead of ATP. In this case, the reaction was much more difficult to initiate, with a typical lag time of 60 min before initiation. The overall success rate of assays with ATP γ S was also lower than with ATP: in more than one half of the attempted experiments, it was impossible to identify any significant change in the length versus supercoiling characteristics of the molecule after more than 90 min incubation. Remarkably, though, in contrast with the ATP case, in some experiments a clear increase in the molecule's length was observed during incubation with nucleoprotein filaments (see Figure 4, red curve, and Discussion). In these cases, the supercoiling profile after RecA rinsing was modified with regards to that of naked dsDNA, but the profile was less reproducible over several experiments than with ATP. In one experiment only (among a total of 8 in which the

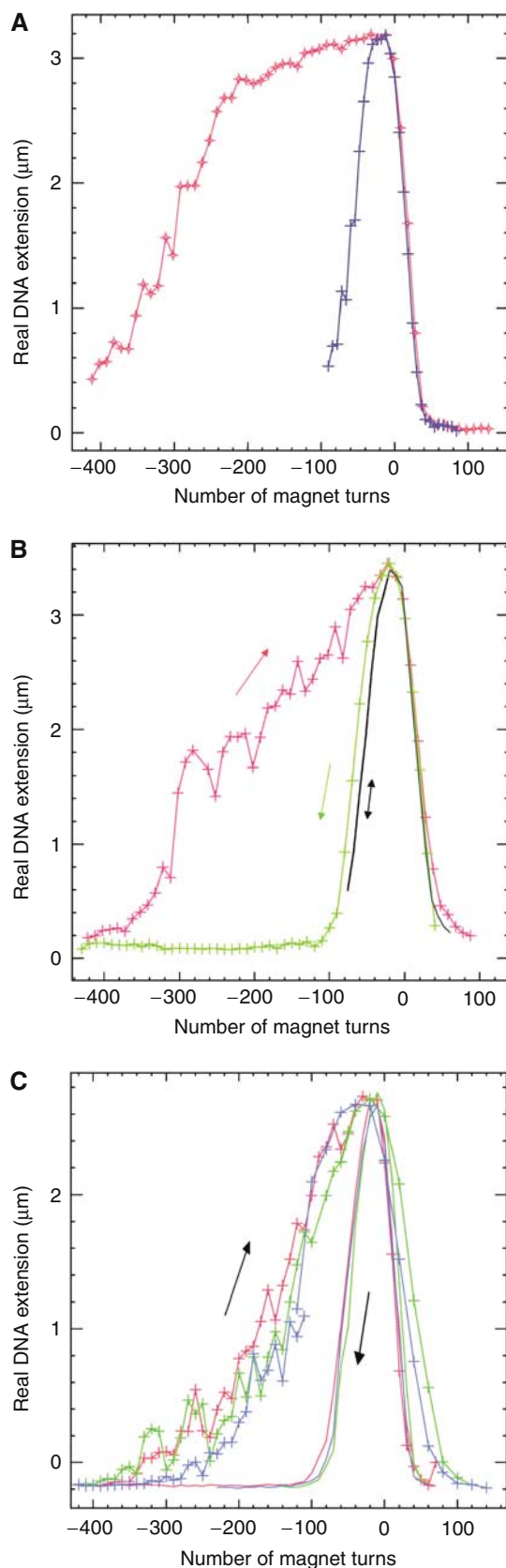


Figure 5 dsDNA + ssDNA-RecA assay. Extension versus supercoiling curves. The measures are carried out in Binding Buffer, under 0.3 pN force. (A) Blue, dsDNA profile before incubation with nucleoprotein filaments; red, same molecule after 30 min incubation with 3.5 kb homologous preformed nucleoprotein filaments. The red plot is followed reversibly upon series of increasing and decreasing scans (increasing and decreasing σ , respectively). (B) Plot obtained after incubation with the same ssDNA, in the presence of SSB and in the absence of RecA. Black: naked DNA; red: first increasing scan from -420 to $+100$ turns. Green: 'return' scan from $+100$ to -420 turns; After the first return scan, the green curve, identical to that of naked dsDNA, is reversibly followed during both increasing and decreasing scans. (C) Plots obtained after incubation with nucleoprotein filament assembled in the presence of ATP γ S. Each color represents a different sequence of one increasing scan (plots with symbols) and one decreasing (full line without symbols) scan. This series of curves displays a stable hysteresis loop.

DNA profile was altered after incubation with ATP γ S nucleoprotein filaments), we recovered a profile comparable to that obtained with ATP (Figure 5A). The other experiments leading to a change in the torsion profile yielded a behavior as plotted in Figure 5C. The first scan from negative to positive supercoiling showed the same progressive and irregular length increase as seen with SSB and no RecA (Figure 5B), and the return to negative supercoiling also followed the same curve as for naked DNA. However, in contrast to the experiment without RecA, repeating this sequence of scans always led to hysteresis, with a progressive length increase for increasing σ and a profile identical to that of naked DNA for decreasing σ . Overall, a correlation between the kinetics during incubation and the final state of the molecule was observed, length versus supercoiling curves being wider and more reversible when the extension during incubation was larger.

Qualitative summary of direct results

In a first series of experiments, ssDNA held in the tweezer was incubated with RecA in the presence of ATP to yield a nucleoprotein filament, and homologous dsDNA was introduced. The length of the molecule decreased during incubation under a stretching force of 10 pN, down to values between 4 and 4.5 μ m, corresponding to the expected length of the molecule in its naked, dsDNA form under such a force. At 0.1 pN, the length decreased to a non-measurable, close to zero value, but a length around 1.5 μ m, consistent with that of dsDNA at 0.1 pN, was recovered after several stretching cycles. In all cases, strong length fluctuations were observed during incubation, indicating that the dynamics of ssDNA-RecA nucleoprotein filaments are strongly increased in the presence of homologous dsDNA. This behavior was not obtained with heterologous DNA, or with a nucleoprotein filaments assembled with ATP γ S. After extensive rinsing of RecA, the reaction product was characterized by measuring the force versus length response of the molecule, which could be accurately fitted as that of a 'hybrid' DNA molecule with single strand and duplex sections. The fraction of dsDNA in the product, as deduced from this fit, was 76% on average, and once reached 100%.

An experiment dual from the above was also performed, holding a naked, un-nicked dsDNA molecule in the tweezers, and injecting into the microchannel preassembled 3.5 kb nucleoprotein filaments homologous to the middle section of the dsDNA. Again, a length decrease and strong fluctuations were observed during incubation. The final product was characterized by performing a length versus rotation supercoiling assay after extensive RecA rinsing, and comparing it with that of the initial dsDNA. The curves exhibited a plateau on the negative supercoiling side, suggesting the presence of one or several D-loops. In contrast to the behavior obtained with oligonucleotides (Strick *et al*, 1998), the invading ssDNA could not be released by positive supercoiling. This behavior was not observed in the absence of RecA, or with heterologous nucleoprotein filaments. When the nucleoprotein filaments were prepared with ATP γ S instead of ATP, a length increase of the dsDNA was observed during incubation. The length versus supercoiling profile after protein rinsing was larger than that of naked DNA, but in general less regular, less reproducible and narrower than that obtained after incubation with ATP nucleoprotein filaments.

Discussion

Evidence for single-molecule recombination

The proof that recombination actually occurred was provided independently in the two (ssDNA-RecA + dsDNA in solution) and (dsDNA + ssDNA-RecA in solution) assays. When the initial substrate was a ssDNA, the hybrid product was probed by a force versus extension measurement, which yielded the percentage of the molecule converted to dsDNA. This percentage fluctuated from one experiment to the other. In the most favorable case (14 kb substrate), the efficiency reached 100% once over seven experiments. The average value over all experiments was 76% with a standard deviation of 13%. In controls in which no exchange was expected to occur, the final curve was identical to the initial ssDNA one. Finally, controls performed with dsDNA homologous to a fraction of the ssDNA only yielded a fraction of dsDNA in the final product reduced from the above figure, in proportion of the homology ($69 \pm 10\%$ of the homologous region in average).

In the (dsDNA + ssDNA-RecA in solution) assay, exerting torsional constraints allowed for another type of characterization, namely the observation of a plateau on the extension versus supercoiling plot, reminiscent of the D-loops obtained by hybridization with naked short ssDNA fragments (Strick *et al*, 1998). In our construct, the two initial dsDNA strands are attached at both ends, thus a D-loop is indeed expected after strand exchange and RecA removal. The width of the plateau corresponds to exchange on $70 \pm 10\%$ of the homologous region, consistent with the efficiency of the reaction in the (ssDNA-RecA + dsDNA in solution) assay.

Evidence of accelerated RecA depolymerization dynamics

In both (ssDNA-RecA + dsDNA in solution) and (dsDNA + ssDNA-RecA in solution) assays, the evolution of the length of the molecule in the tweezers during strand exchange presented unexpected features. When a nucleoprotein filament, held in the tweezers, was incubated at high force (10 pN) with homologous dsDNA (Figure 3A, blue curve), its length decreased to about 4.5 μ m, close to the length of a naked dsDNA with the same number of bp under the same force (4 μ m, see Figure 2A, green curve). This strongly suggests that RecA neither remains nor repolymerizes extensively, on the exchanged duplex, since then the length would remain comparable with that of a nucleoprotein filament. The condensation at 0.1 pN was even more striking, decreasing from 5 to 1 μ m or less (see Figure 3B). Again, this can be understood if RecA irreversibly leaves the exchanged duplex shortly after strand exchange, but is hardly compatible with the behavior expected if even a relatively low fraction of the ssDNA converted into dsDNA were still covered with RecA.

The complementary view of the process offered by the (dsDNA + ssDNA-RecA in solution) assay confirms that RecA does not remain stably bound to the heteroduplex after strand exchange in the presence of ATP: we always observed a length decrease (Figure 4, purple and green curves), whereas persistence of RecA on the invaded dsDNA would lead to a length increase, as was indeed observed with ATP γ S (Figure 4, red curve). The length increase in this latter experiment was 780 ± 100 nm, consistent with the insertion of 3.5 kb dsDNA into a nucleoprotein filament (assuming that

the physical properties of the three-strands nucleoprotein filament are close to those of the two-strands one). Thus, our data are fully consistent with extensive RecA coverage of the invaded molecule when operating with ATP γ S as a cofactor, but inconsistent with such RecA coverage in the presence of ATP. To be somewhat more quantitative, the largest positive excursion (temporary length increase) observed in our experiments with ATP was of order 150 nm, the lengthening expected for a synapsis covering at most 1 kb. We thus suggest that this value provides a rough upper bound to the size of the synapsis at any time during the strand exchange in our conditions.

It is now well acknowledged that ssDNA-RecA and dsDNA-RecA nucleoprotein filaments are dynamic entities (Fulconis *et al*, 2004; Bugreeva *et al*, 2005). Indeed, when we keep the ssDNA-RecA filament under low force (0.1 pN, Supplementary Table I, line 4), even in the absence of homologous dsDNA, its length decreased slowly but continuously. In contrast, at a higher force, for example 10 pN, the length of the molecule increased, although much less than with nucleoprotein filament (data not shown). We attribute these features to continuous RecA polymerization and depolymerization: upon depolymerization, ssDNA secondary structures, which are favored at low forces and in the presence of magnesium, develop and prevent reassociation of RecA, leading to a progressive length decrease of the molecule. At high force, in contrast, secondary structures are unfavorable, and the equilibrium is displaced towards net polymerization, leading to a slight size increase. When homologous dsDNA was added (ssDNA-RecA + dsDNA in solution assay), the condensation of the molecule was strongly accelerated, a feature we attribute to an increase of the net depolymerization rate of RecA. This interpretation is also compatible with earlier investigations of the exchange between wild type RecA and its mutant K72R, indicating that the presence of homologous dsDNA activates the exchange between free and bound RecA in a nucleoprotein filament (Shan and Cox, 1997).

Several further experimental controls (Supplementary data) support the existence of fast RecA depolymerization from the exchanged strand, stimulated by the presence of homologous dsDNA.

- (i) When ATP was replaced by ATP γ S, no condensation was observed, in keeping with its known inhibition of RecA depolymerization.
- (ii) The condensation rate was faster with a 14 kb homology than with a 3.5 kb one.
- (iii) The presence of heterologous DNA tended to increase the nucleoprotein filament dynamics, although much less than homologous one (shortening under low force, Supplementary Table I and lengthening under high force, Figure 3A, purple). This might be attributed to the existence of short, partially homologous sections on the globally heterologous DNA, which probably lead to transient, unstable synapses with ss/dsDNA junctions that favor the exchange of RecA.
- (iv) Finally, the condensation was slightly faster when the 14 kb homologous dsDNA had been sonicated. The shortening rate thus seems accelerated in conditions in which the presence of several synapses is more probable.

Fast depolymerization is indeed a key assumption of several variants of the monomer redistribution model

(Bianco *et al*, 1998 and PR Bianco and SC Kowalczykowski, personal communications), in which it is instrumental for the passage of discontinuities and heterologies. Our present experiments provide no direct evidence about the passage of heterologies, since we worked only with homologous (or fully heterologous) strands, but the fast depolymerization we observed is in keeping with this family of models. The facilitated rotation model neither requires nor precludes extended coverage of the dsDNA by RecA, so this aspect of our data is 'neutral' with regard to this model. In contrast, it seems to contradict the 'hydrolysis wave model', which suggests that RecA depolymerization is immediately followed by repolymerization on the same dsDNA.

Generation of torsional strain

We have directly observed in the (dsDNA + ssDNA-RecA in solution) assay that strand invasion can occur only if the dsDNA is negatively supercoiled. This is a very direct verification that the dsDNA has to be untwisted in order to be incorporated into the synapsis, and that the nucleoprotein filament alone is not able to invade a torsionally blocked dsDNA if it is not helped by some mechanism releasing torsional stress. More surprisingly, our results on the (ssDNA-RecA + dsDNA in solution) assay also suggest that some torsional stress can be generated along a nucleoprotein filament with free rotation at its two ends, as we shall now discuss. When an ssDNA nucleoprotein filament was incubated with homologous dsDNA under a 0.1 pN stretching force, its length decreased to less than 0.5 μ m. This length would be compatible with that of a naked ssDNA. Yet, when the molecule was rinsed immediately after this incubation at 0.1 pN, force versus length curves demonstrated the presence of a dsDNA/ssDNA hybrid, with a majority of dsDNA. If this hybrid was linear and unconstrained, it would have a length of at least 1.5 μ m. Thus, the dsDNA portions of the hybrid molecule did not have their natural end-to-end distance at the end of incubation. A simple explanation would be that they were writhed. This might seem surprising, since we start with a ssDNA that can freely rotate at its anchoring points. However, in the recombination experiment, we have at least two DNA partners in interaction. Thus, although the hybrid complex is not able to transmit external torsional stress associated with the tweezers, it could generate torsional stress internally, for example, if RecA depolymerization would occur along a portion of the synapsis flanked by two sections in which the invaded dsDNA is still engaged in a nucleoprotein filament. This is indeed the mechanism proposed in Bianco *et al* (1998) to explain the bypass of heterologies, without requiring translocational 'motor' activity. At low force, rotational constraint can be relaxed by the formation of plectonemes on the dsDNA, exchanging twist for writhe at the expense of length. Thus, in our experiments, during the first 0.1 pN stage and upon RecA depolymerization, both the ssDNA portions of the hybrid molecule and the exchanged one may exert a pulling force and decrease the hybrid molecule length towards zero, if they are flanked by RecA-covered regions. For the ssDNA part, this force would be associated with secondary structures; for the dsDNA part, it should be associated with plectonemes. We expect that repetitive stretching sequences, by progressively destroying plectonemes and secondary structures, favor progressive rearrangement of the fiber towards a simpler (and thus less

constrained) conformation. Indeed, upon cycling, the behavior of the molecule resembled more and more that of a naked, rotationally free dsDNA (with almost instantaneous return to a finite length around 2 μm upon force decrease, see Supplementary Figures 2 and 3B, red curve). The presence of supercoiling and secondary structures is also supported by experiments performed at high salt concentration, and in the presence of topoisomerases (see Supplementary Figure 4).

Topological link between the newly formed heteroduplex and the exchanged ssDNA

The extensive formation of D-loops during the (dsDNA + ssDNA-RecA in solution) assay is reminiscent of the behavior obtained with oligonucleotides, but it also presents striking differences with these earlier experiments. In particular, the presence of a reversible plateau when the exchange was performed with RecA, suggests that the invading ssDNA and the dsDNA in the tweezers are topologically linked. This property was not recovered when exchange was catalyzed by SSB, so it seems a specific property of RecA. Remembering that in our experiments, the dsDNA is held at both ends by its two strands, and that its invasion by the nucleoprotein filament implies a 'spooling' process (i.e. rotation of at least one extremity of the nucleoprotein filament around the duplex), this behavior could be explained by a multisteps process, in which strand exchange starts at one location along the dsDNA, and after some time (during which the nucleoprotein filament has rotated at least once around the dsDNA), a second strand exchange process initiates elsewhere along the molecule. This process would not only explain our results, but it would also lead to a situation similar to this invoked by Bianco *et al* (1998) to explain heterology bypass (presence of a RecA-free zone flanked by two sections of triplex nucleoprotein filament), without requiring RecA repolymerization on the duplex.

The 'facilitated rotation' model also involves sequences of sections of the nucleoprotein filament in which the dsDNA is spooled, and sections in which it is not, which could lead to topological links in our (dsDNA + ssDNA-RecA in solution) assay. However, in contrast with the Bianco-Kowalczykowski model, it assumes that the sections in which dsDNA is not spooled are also covered with RecA (since the generation of torsional stress is supposed to be generated by a translocation of the unspooled dsDNA sections around the nucleoprotein filament). Considering the difference in rise between dsDNA and nucleoprotein filaments, we do not see how such alternation could occur along a nucleoprotein filament fully covered with RecA and interacting with a fully homologous dsDNA (this is a consequence of the 'rise paradox' discussed in Klapstein *et al*, 2004).

Final comments and perspectives

We studied for the first time RecA-mediated homologous recombination at the single-molecule level. In a first series of experiments, ssDNA held in the tweezer was incubated with RecA in the presence of ATP to yield a nucleoprotein filament, and homologous dsDNA was introduced in solution. The length of the molecule decreased during incubation, a behavior interpreted as a consequence of strand-exchange associated with the generation of torsional strain along the molecule. This interpretation is consistent with the assumptions explaining the crossing of nucleoprotein filament

discontinuities and heterologies in the 'monomer redistribution' and 'facilitated rotation' models. After extensive rinsing of RecA, the reaction product was characterized by measuring the force versus length response of the molecule. The latter could be accurately fitted by a linear combination of the behaviors of ssDNA and dsDNA, providing a measure of the fraction of dsDNA in the product, 76% on average.

An experiment dual from the above was also performed, holding a naked, un-nicked dsDNA molecule in the tweezer, and injecting into the channel preassembled nucleoprotein filaments homologous to a part of the dsDNA. Again, a length decrease and strong fluctuations were observed during incubation, except when the nucleoprotein filaments had been incubated with ATP γ S instead of ATP. The final product was characterized by performing a length versus rotation supercoiling assay after extensive RecA rinsing, and comparing it with that of the initial dsDNA. On average, the exchanged area covered about 70% of the homologous zone, consistent with the first series of experiments.

Both experiments imply a rapid depolymerization of RecA from the dsDNA after strand exchange, and allowed to put an upper bound to the length of dsDNA covered by the synapsis (three strands nucleoprotein filament section), around 1 kb. Our results are fully consistent with those of Shan and Cox (1997), showing a catalytic effect of synapses on RecA depolymerization. This feature would provide a kinetic interpretation for the irreversibility of strand exchange: if, as suggested here, RecA depolymerizes more rapidly from the synapsis than it can repolymerize on the exchanged ssDNA, this may leave the cell enough time to make strand exchange irreversible by the action of other enzymes (such as resolvases).

Releasing RecA from the heteroduplex just after strand exchange is also interesting from a 'protein economy' point of view, considering that during meiosis or DNA repair following strong genotoxic stress, the cell has a very limited time to achieve multiple strand exchange operations: a mechanism requiring a long sequestration of the protein onto the exchanged filament or onto the released ssDNA would not be favourable.

Fast RecA depolymerization is also an appealing feature on a mechanical-energetic ground: *in vivo*, intact dsDNA is in general not free to rotate, so due to the different pitch of dsDNA in the naked form and inside a nucleoprotein filament, strand invasion induces torsional stress on the invaded duplex, that would increase and ultimately block the process, if RecA were to remain on the exchanged heteroduplex. This would make strand exchange totally dependent on an intense topoisomerase activity. Depolymerizing RecA just after strand exchange maintains strain generation at a minimal value. Indeed, if strand exchange is performed thanks to a limited and roughly constant length of synapsis traveling on the dsDNA, topoisomerases are required only during the establishment of the synapsis, and strand exchange can then proceed in a steady-state regarding torsional strain.

Finally, our results sheds new light on the suggestion made (Bianco *et al*, 1998) that negative supercoiling induced by the release of naked dsDNA from a nucleoprotein filament may be instrumental in crossing filament discontinuities and heterologies. Our results present several features consistent with this model, in particular the generation of torsional strain along a ssDNA nucleoprotein filament during strand

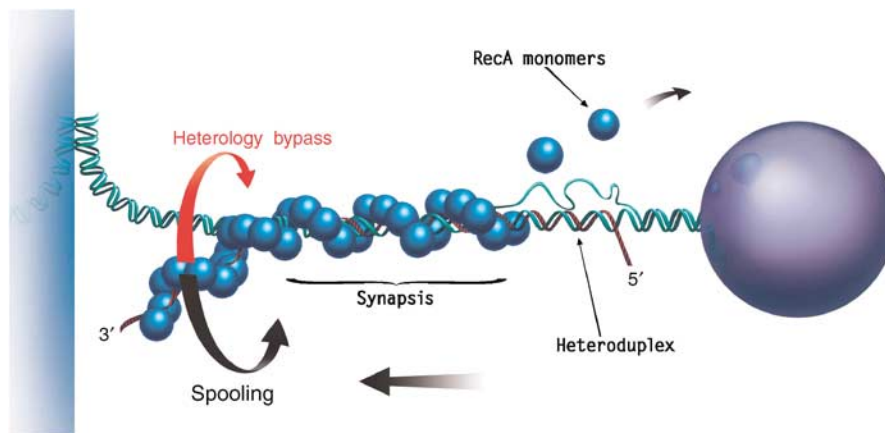


Figure 6 Proposed mechanism for depolymerization-induced strand exchange. dsDNA is invaded by spooling (black arrow) on a synapsis progressing 5′–3′ on the ssDNA nucleoprotein filament. Depolymerization of RecA at the rear of the synapsis releases naked dsDNA and an exchanged ssDNA, which can, depending on the environment, develop secondary structures or be covered by SSB or RecA (not represented here). The red arrow represent a behavior suggested by Bianco *et al* (1998), for the passage of heterologies: Upon arrest of synapsis progression by the heterology, the synapsis rotation should be reversed by the release of torsional stress stored on DNA, induced by continuing RecA depolymerization no more compensated by strand invasion. This results in an untwisting of the heterologous zone if the invading and invaded DNAs are topologically linked downstream (not represented).

exchange and the formation of topological links between the invaded and invading DNA in the (dsDNA + ssDNA-RecA in solution) assay. A dynamic synapsis traveling along the dsDNA and followed by fast RecA depolymerization would indeed put the exchanging pair in the situation proposed by Bianco *et al* for heterology bypass.

Since the heteroduplex twist is lower when it is covered by RecA than when it is naked, strand invasion creates downstream of the synapsis positive torsion on the invaded duplex (Figure 6). During steady-state progression of the synapsis, this is compensated by RecA depolymerization (and possibly extra action of topoisomerases *in vivo*). However, if the spooling is halted, for example, by a heterologous zone, the synapsis will shrink. Negative supercoiling will then accumulate upon RecA depolymerization and be transmitted upfront by clockwise rotation, without being absorbed any more by strand invasion (Figure 6, red arrow). This mechanism is consistent with that proposed in Bianco *et al* (1998) and with the generation of torsional stress separating strands of heterologous inserts (Jwang and Radding, 1992). It would also resolve the paradox of requiring counterclockwise rotation of the synapsis for duplex invasion (Howard-Flanders *et al*, 1984; Reddy *et al*, 1995), and clockwise rotation for heterology bypass (Morel *et al*, 1994). More generally, the rich and partly unexpected behavior uncovered here suggests that homologous recombination *in vivo* should involve a subtle ‘tuning’ of torsional stress, depending on the combined action of strand invasion, RecA depolymerization and topoisomerases.

Materials and methods

Sample preparation and experimental setup

The DNA manipulated in the magnetic tweezers is an ~14 kb fragment obtained by PCR from phage λ DNA, ligated at one end to a multidigoxigenin labeled fragment (600 bp) and at the other end to a multibiotin labeled fragment (600 bp) (Fulconis *et al*, 2004). The DNA is then bound to a large excess of streptavidin-coated 2.8 μ m magnetic beads (Dynal) in Binding Buffer. To trap ssDNA in the tweezers, a denaturation step is added before binding to the

beads, by heating at 96°C for 90 s and quenching in iced water. The DNA-beads solution is then introduced by a controlled flow into a poly-dimethylsiloxane microfluidic channel placed on an antidigoxigenin-coated glass coverslip. After 30 min incubation, most of the unbound beads are washed out of the channel with Binding Buffer. A pair of magnets placed above the channel pulls on the magnetic beads and provides a vertical stretching force on the DNA, tunable in the range from 0.001 to 15 pN. The extension of a DNA molecule is given by the distance between the bead and the glass surface, measured by real-time analysis of the bead’s image recorded at 120 Hz through the $\times 100$ objective of an inverted microscope. The force is calibrated by real-time analysis of the beads’ x - y Brownian motion (Strick *et al*, 1996). When both strands of an un-nicked dsDNA are linked to the surface and the bead, rotation of the magnets twists or untwists the molecule. Thus, the force and the degree of supercoiling are independently controlled. Force versus extension and extension versus supercoiling plots are obtained by averaging the DNA length measured in real time over ~5 s per data point.

(ssDNA-RecA + dsDNA in solution) assay

RecA protein (NEB) was supplied at a concentration of 6 μ M in Binding Buffer + 2 mM Mg^{2+} (15 mM Tris-HCl at pH 7.5, 25 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.05% Tween-20), together with ATP 2 mM. Alternatively, ATP γ S was supplied at 1 mM. After checking that RecA has fully polymerized on the ssDNA, dsDNA was added in Binding Buffer + 12 mM Mg^{2+} along with 6 μ M RecA and 2 mM ATP. For reactions performed with ssDNA covered with ATP γ S, only dsDNA was introduced in the channel without RecA or cofactor, since depolymerization does not occur spontaneously in this case.

We use three following homologous dsDNAs:

- (i) 14 kb DNA obtained by PCR, identical to the tweezers DNA except for the polybiotin and polydigoxigenin fragments at its extremities;
- (ii) 3.5 kb DNA produced by PCR, identical to a central region of the 14 kb fragment (nucleotides 26944–30562 of phage λ); and
- (iii) 48.5 kb long λ DNA, sonicated for 9 min to produce molecules distributed around 200 bp (checked by gel electrophoresis). 30% of these molecules are identical to the 14 kb part of the tweezers DNA, the others are heterologous.

The 1 kb DNA ladder from NEB was used as heterologous control. Usual concentrations of dsDNA during incubation were 30 ng/ μ l for 14 kb DNA and 3.5 kb DNA; 100 ng/ μ l for sonicated λ (30 ng/ μ l of homologous fragments), and 100 ng/ μ l for heterologous DNA. When SSB protein is added, the concentration is 0.25 μ M (Sigma). The molecule was stretched in the 0.1–10 pN range during incubation for typically 1 h. We then extensively rinse the channel,

in order to eliminate RecA and study the final exchange product. If the reaction is conducted with ATP, rinsing for 10 min in Binding Buffer without RecA or ATP is sufficient to ensure complete RecA depolymerization. If ATP γ S is used, it is necessary to flow a 500 mM NaCl + 25 mM EDTA buffer for > 1 h to depolymerize RecA.

(dsDNA + ssDNA-RecA in solution) assay

Nucleoprotein filaments were prepared as follows. A 3.5 kb fragment with a single biotin label on one strand was prepared by PCR (one of the two primers is provided with a 5' biotin label). Three hundred and fifty nanograms of DNA were bound to 10 μ l of streptavidin-coated MyOne magnetic beads (Dyna, used as provided) suspended in a TE (pH 7.5) + 2 M NaCl buffer (1 h incubation). The beads were then suspended in 10 mM Tris-HCl (pH 7.5) and the mixture was heated (1 min at 96°C) and quenched in iced water. The supernatant, containing the ssDNA, was incubated at 37°C with RecA and ATP in Binding Buffer + 2 mM Mg²⁺ for 5 min. The estimated concentrations are RecA, 4 μ M; ssDNA, 17 μ M; ATP, 2 mM. ssDNA was in excess, so as to leave as little free RecA as possible in the solution. After incubation, and immediately before injection into the channel, the ATP concentration was raised to 8 mM so that enough ATP is provided during the reaction. Indeed, ATP is significantly hydrolyzed by RecA when

there is ssDNA in solution (which was not the case in the ssDNA-RecA + dsDNA experiment). The length of the molecule was recorded during incubation, with or without supercoiling the molecule (see Results section). After incubation (90 min except stated otherwise), RecA was rinsed from the molecule(s) in the tweezers by flowing a 15 mM Tris-HCl at pH 7.5, 500 mM NaCl, 1 mM DTT, 25 mM EDTA, 0.05% Tween-20 solution in the cell for 10 min, and the final molecule was characterized by its size evolution as a function of supercoiling under a 0.3 pN force.

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