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Tn*5* transposase loops DNA in the absence of Tn*5* transposon end sequences

Christian D. Adams,[1] Bernhard Schnurr,[2,3] Dunja Skoko,[2,4] John F. Marko[2,5,6] and William S. Reznikoff[1]*

[1]*Department of Biochemistry, University of Wisconsin at Madison, 433 Babcock Drive, WI 53706, USA.*
[2]*Department of Physics, University of Illinois at Chicago, 845 West Taylor Street, Chicago, IL 60607, USA.*
[3]*Skirball Institute of Biomolecular Medicine and Department of Pathology, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA.*
[4]*Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA.*
Departments of [5]*Biochemistry, Molecular Biology and Cell Biology and* [6]*Physics and Astronomy, Northwestern University, 2205 Tech Drive, Evanston, IL 60208, USA.*

Summary

Transposases mediate transposition first by binding specific DNA end sequences that define a transposable element and then by organizing protein and DNA into a highly structured and stable nucleoprotein 'synaptic' complex. Synaptic complex assembly is a central checkpoint in many transposition mechanisms. The Tn*5* synaptic complex contains two Tn*5* transposase subunits and two Tn*5* transposon end sequences, exhibits extensive protein–end sequence DNA contacts and is the node of a DNA loop. Using single-molecule and bulk biochemical approaches, we found that Tn*5* transposase assembles a stable nucleoprotein complex in the absence of Tn*5* transposon end sequences. Surprisingly, this end sequence-independent complex has structural similarities to the synaptic complex. This complex is the node of a DNA loop; transposase dimerization and DNA specificity mutants affect its assembly; and it likely has the same number of proteins and DNA molecules as the synaptic complex. Furthermore, our results indicate that Tn*5* transposase preferentially binds and loops a subset of non-Tn*5* end sequences. Assembly of end sequence-independent nucleoprotein complexes likely plays a role in the *in vivo* downregulation of transposition and the *cis*-transposition bias of many bacterial transposases.

Accepted 4 October, 2006. *For correspondence. E-mail reznikoff@biochem.wisc.edu; Tel. (+1) 608 262 3608; Fax (+1) 608 265 2603.

Introduction

The assembly of a higher-order nucleoprotein 'synaptic' complex by transposase (Tnp) proteins is a central checkpoint in the mechanisms of many DNA transposable elements (Surette *et al*., 1987; Sakai *et al*., 1995; Bhasin *et al*., 2000). The interwoven architecture (Aldaz *et al*., 1996) of the synaptic complex ensures that Tnp cleaves and moves only DNA defined by the end sequences (ESs) of the transposable element. Additionally, most, if not all, Tnps assemble synaptic complexes such that the initial DNA binding event at each ES is performed by one Tnp subunit (*cis* binding) while the DNA cleavage event at each ES is performed by the Tnp subunit bound initially to the other ES (*trans* catalysis; Sakai *et al*., 1995; Williams *et al*., 1999; Naumann and Reznikoff, 2000; Swanson, 2001). Even with this checkpoint, it seems that the potential for illegitimate (ES-independent) DNA binding is significant given the number of competing non-ES DNA sites in any given genome.

We used single-molecule and bulk-phase experiments developed using the Tn*5* transposition system to investigate ES-independent DNA binding. Our use of the Tn*5* transposition system was motivated by several factors. First, in the presence of Mg^{2+}, Tn*5* Tnp (hereafter referred to as Tnp) can transpose any DNA defined by two inverted Tn*5* ESs (Goryshin and Reznikoff, 1998) with no additional required cofactors or specific DNA topologies (Bhasin *et al*., 2000). Furthermore, extensive genetic and structural data (Davies *et al*., 2000; Reznikoff, 2002) allow us to place the single-molecule and bulk-phase studies in a structure-function context.

Tn*5* is a prokaryotic transposable element defined by inverted, 19 bp ESs (Johnson and Reznikoff, 1983; Zhou *et al*., 1997). Tnp is encoded by the Tn*5* transposon and is the protein responsible for the movement of the Tn*5* mobile genetic element (Reznikoff, 2003). Tnp binds the 19 bp ESs and assembles a synaptic complex that contains two Tnps and two ESs (Bhasin *et al*., 2000; Davies *et al*., 2000). The proposed multistep mechanism of Tn*5* transposition is shown in Fig. 1A.

We previously reported that ES-independent DNA binding affects the reactivity of Tnp and that Tnp can transfer from non-ES DNA to the Tn*5* ES (Steiniger *et al*.,

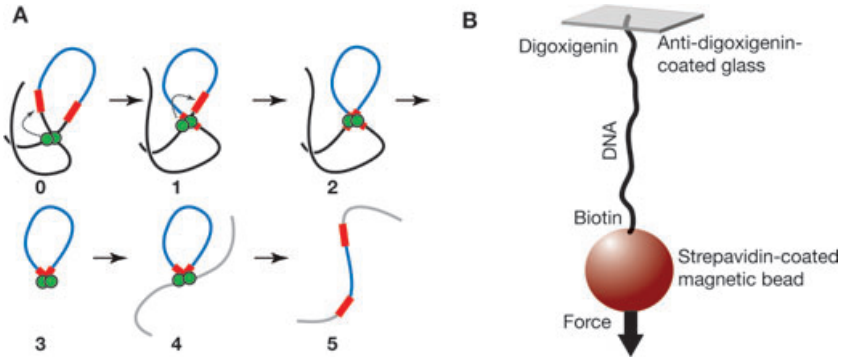

Fig. 1. 'Cut-and-paste' transposition mechanism and experimental design.
A. The Tn*5* transposase (green) moves the Tn*5* Tn (blue) using a cut-and-paste mechanism. In step 0, Tnp has assembled a dimerization-dependent, ES-independent DNA loop. Our current model posits that over short distances, Tnp can locate the ES (red) in step 1 via looping using a direct transfer search mechanism. Tnp assembles a higher-order nucleoprotein complex called the synaptic complex in step 2. The Tn*5* synaptic complex contains two Tnps and two ESs (Bhasin *et al.*, 2000). Assembly of the synaptic complex is required for Tnp to cleave the Tn DNA from the donor backbone DNA (dbb; black) in a series of Mg^{2+}-dependent reactions (Goryshin and Reznikoff, 1998; Reznikoff, 2003). In step 3, the Tnp–Tn complex diffuses from the dbb DNA and in step 4 captures target DNA. In step 5 Tnp inserts the Tn DNA into target DNA, resulting in the movement of the Tn from its original location in the genome to a new location.
B. Single-DNA-molecule 'tethers' were constructed in flow chambers and assayed by using a microscope equipped with permanent magnets. The DNA substrates contained zero. All DNAs were modified on one end with digoxigenin and the other with biotin. Each molecule was attached to a strepavidin-coated 2.8 μm-diameter magnetic beads and an anti-digoxigenin functionalized glass slide. The single-DNA tethers were stretched between the glass slide and magnetic beads by changing the magnetic field gradient applied to the magnetic bead.

2006a). However, structural characteristics of the ES-independent Tnp–DNA complex, the role of ES-independent DNA binding in the Tn*5* transposition reaction and the Tnp contacts involved in the ES-independent DNA binding reaction remained unknown.

In this article, we report that Tnp loops DNA in the absence of the Tn*5* ES. These ES-independent nucleoprotein complexes have structural similarities to the synaptic complex. ES-independent DNA binding is reduced by dimerization-defective Tnp mutants and is affected by Tnp mutants that impair Tnp-ES binding. Analysis of the ES-independent looping reaction suggests Tnp preferentially binds a subset of non-ES, 'non-canonical' Tn*5* sequences. Assembly of ES-independent nucleoprotein complexes is a previously unidentified mechanism for the well-known *cis* bias of many bacterial transposition systems to mobilize DNA located at or near the Tnp gene (Johnson *et al.*, 1982; Morisato *et al.*, 1983; Derbyshire *et al.*, 1990), and for the *in vivo* downregulation of transposition (Reznikoff, 2003). The data are also consistent with loop-dependent direct transfer as a potential search mechanism utilized by Tnp to locate its ES over short distances.

Results

Tnp assembles ES-independent nucleoprotein complexes

To investigate the gross structural properties of ES-independent nucleoprotein complexes assembled by Tnp, we incubated Tnp (400 nM; all experiments use a Tnp variant with E54K, M56A and L372P amino acid substitutions; Wiegand and Reznikoff, 1992; Weinreich *et al.*, 1994a; Zhou and Reznikoff, 1997) with 30-mer DNA oligonucleotides (40 nM) containing 19 bp ES or non-ES DNA flanked by 5 bp transposon DNA and 6 bp donor backbone under a variety of salt concentrations and temperatures. The non-ES DNA substrate called R1 (Fig. 2B) was generated by replacing 14 of the 19 ES bases with a randomly generated sequence.

Using a gel-shift assay, we detected Tnp binding to both the ES and non-ES oligonucleotides in a pH 7.5 HEPES binding buffer containing 100 mM NaCl and 20 ng μl^{-1} salmon testes DNA at 25°C (Fig. 2A, lanes 3 and 4). Lane 3 shows robust Tnp binding to the ES resulting in the assembly of the well-characterized paired ends complex (PEC; Bhasin *et al.*, 2000; Davies *et al.*, 2000). The PEC is a catalytically functional Tn*5* nucleoprotein complex consisting of a dimer of Tnps bound to two ESs.

Lane 4 shows Tnp binding to the non-ES DNA oligonucleotide R1 resulting in the assembly of an ES-independent nucleoprotein complex. Assembly of an ES-independent nucleoprotein complex has not been previously reported for the Tn*5* system. Qualitative inspection of the DNA shift in Fig. 2A shows that far less ES-independent nucleoprotein complex assembles than PEC assembles; moreover, ES-independent nucleoprotein complexes are not observed at 37°C and readily disassemble in the presence of divalent metals (data not shown). We note that ES-independent nucleoprotein com-

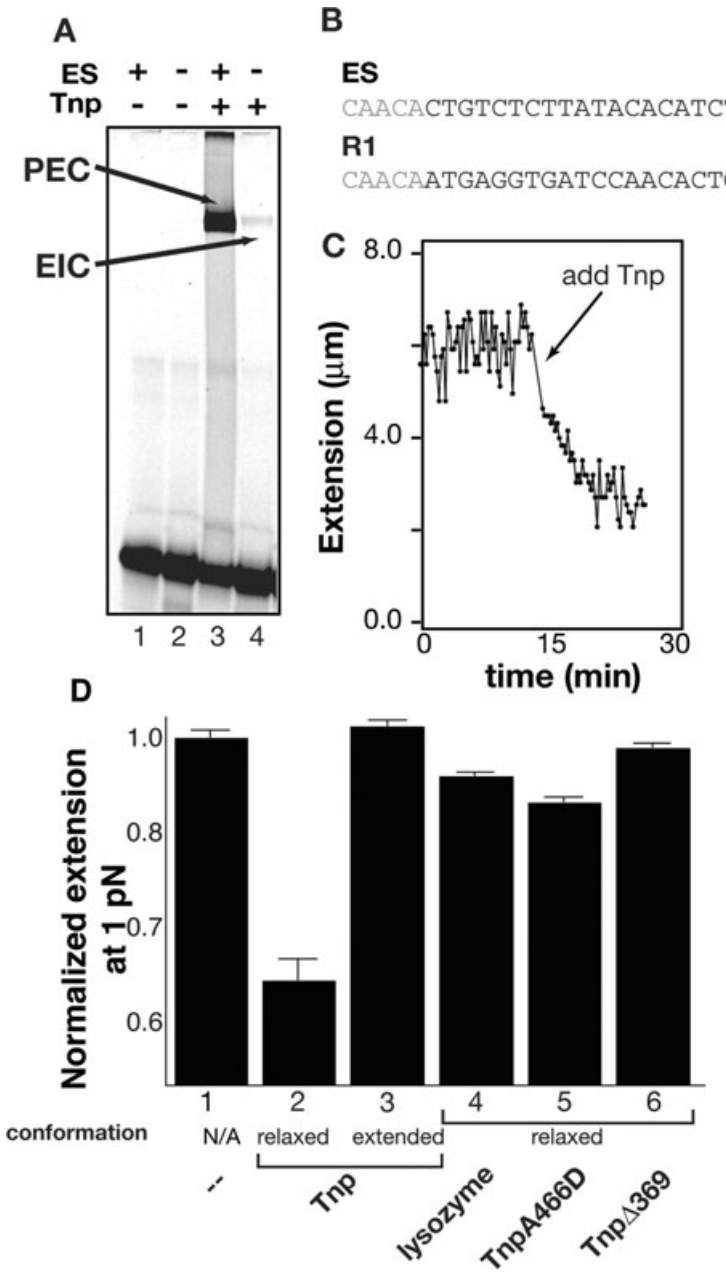

Fig. 2. Tnp binds non-ES DNA.
A. Tnp (400 nM) was incubated with 30-mer oligonucleotides containing the ES (lane 3) or without the ES (lane 4). The ES-dependent paired ends complex (PEC) is in lane 3; an ES-independent (EIC) complex is in lane 4. The PEC and EIC have the same mobility, suggesting the PEC and EIC contain the same number of Tnps and DNAs.
B. The sequence of the oligonucleotides used in (A).
C. A representative extension-time trace of a relaxed Tnp-free single 48.5 kb λ-DNA molecule shows Brownian fluctuations around 6 μm before addition of Tnp. Addition of Tnp (50 nM) results in a condensation of the molecule decreasing the extension to around 2.5 μm over a time-course of about 10 min.
D. Proteins were incubated with non-ES DNA for 20 min with the DNA either relaxed (loops) or extended (no loops). After incubation, extension at 1 pN was measured and normalized to extension at 1 pN for the Tnp-free 0 ES DNA (bar 1; $n = 3$). Tnp (50 nM) condenses relaxed 0 ES DNA (bar 2; $n = 26$), but not extended 0 ES DNA (bar 3; $n = 3$). Unlike Tnp, lysozyme (500 nM) does not condense relaxed 0 ES DNA (bar 4; $n = 3$) to nearly the same level as 10 times less Tnp. Tnp condensation requires dimerization, as TnpA466D (50 nM) and TnpΔ369 (50 nM), two Tnp dimerization-defective mutants, also do not condense relaxed non-ES λ-DNA (bars 5; $n = 3$; and 6; $n = 3$) to the same level as an equal concentration of Tnp.

plexes are not non-specific complexes, but clearly exhibit some level of specificity in binding, as they assemble in the presence of an excess of competing salmon testes DNA.

The mobility of the ES-independent nucleoprotein complex is roughly the same as the mobility of the PEC, so we estimate that the molecular weight and stoichiometry of the ES-independent nucleoprotein complex is similar to the PEC. Thus, the ES-independent nucleoprotein complex likely consists of two Tnp subunits and two DNA segments.

Tnp binds to and condenses single molecules of non-ES DNA

To more accurately define Tnp/ES-independent DNA interactions observed by gel-shift assay, we studied Tnp binding to several non-ES DNA molecules using a single-DNA-molecule Tnp binding assay developed and reported in this article as well as in Steiniger *et al*. (2006b) and C.D. Adams, B. Schnurr, J.F. Marko and W.S. Reznikoff (submitted). Using this assay, we analysed Tnp binding to three non-ES DNA molecules (λ-DNA, 0 ES and 0 ES′).

Linear forms of the three DNA substrates were prepared for use as single-DNA-molecule 'tethers' by ligation of biotin- and digoxigenin-labelled DNA fragments to the ends of the molecules (Strick *et al*., 1996). The modified DNAs were then incubated with strepavidin-coated magnetic beads and anti-digoxigenin-functionalized glass flow cells to create a single-DNA-molecule tether with a magnetic bead 'handle' (Skoko *et al*., 2004; Fig. 1B). Varying the magnetic field gradient controlled the force applied on the DNA tethers by pulling on the magnetic bead handle. Application of force alters the conformation of the DNA tether and yields force-extension properties of DNA that are well understood (Bustamante *et al*., 1994; Marko and Siggia, 1995). When fully 'stretched out' (> 5 pN), contour lengths of the molecules we studied are: λ-DNA (48.5 kb, 16.4 μm), 0 ES (8.6 kb, 2.9 μm) and 0 ES′ (10.6 kb, 3.6 μm).

In general, we define an 'extended' molecule as a tether that is under sufficient tension (> 1 pN) such that the spontaneous formation of loops is exceedingly rare (Sankararaman and Marko, 2005; Skoko *et al*., 2005; Yan *et al*., 2005) while a 'relaxed' molecule is a tether that is under a low enough tension (< 0.1 pN) that DNA loops (formed via DNA 'crossings') can spontaneously form (although rarely) via thermal fluctuations. The magnetic field gradient can be dynamically controlled so that in any one experiment Tnp can be incubated with a single DNA tether that is extended (no loops) or relaxed (loops).

First we studied Tnp binding to single relaxed molecules of λ-DNA in buffer conditions nearly the same as those used in the bulk gel-shift assay (salmon testes DNA was omitted from the single-DNA-molecule binding reactions). Figure 2C shows an extension-time trace of relaxed λ-DNA. When relaxed, λ-DNA has an extension of ~6 μm. The 'up' and 'down' movements in the trace are Brownian motion. In this force regime, DNA undergoes random coil fluctuations that bring distant segments together (Sankararaman and Marko, 2005). After addition of Tnp (50 nM), the extension of the λ-DNA molecule decreased to 2.5 μm as Tnp condensed the λ-DNA over 10 min (Fig. 2C).

To further characterize the Tnp-mediated DNA condensation reaction, we assayed Tnp (50 nM) binding to the 0 ES DNA tether. After incubation (20 min, 25°C), 1 pN of force was applied to the non-ES tethers and extension was measured and normalized to Tnp-free non-ES DNA (Fig. 2D, bar 1).

Transposase condenses relaxed 0 ES′ DNA (bar 2) so that at 1 pN, its extension is only 0.65 ± 0.1 (SD, $n = 26$) that of Tnp-free 0 ES DNA. Condensation is not detected when Tnp is reacted with extended DNA tethers (bar 3). After performing these measurements, the sample cell was flushed with Tnp-free buffer and the force applied to these molecules was reduced so that the tethers could adopt a relaxed conformation. After 20 min incubation, 1 pN of force was reapplied to these molecules. The extension of these tethers was roughly the same as that reported for Tnp incubation with relaxed DNA (bar 2). Thus, Tnp binds both extended and relaxed DNA but only condenses relaxed DNA. This observation suggests a role for DNA loops in the Tnp condensation reaction.

To demonstrate that the Tnp condensation effects are not due solely to the electropositive character of Tnp [isoelectric point (pI) ~ 10], we reacted lysozyme (pI ~ 10; a protein that is not known to bind DNA) with relaxed 0 ES DNA exactly as done previously with Tnp. Lysozyme (500 nM; 10 times the Tnp concentration) results in an extension of 0 ES DNA to 0.95 ± 0.01 (SD, $n = 3$) that of Tnp-free 0 ES DNA (bar 4).

Tnp-mediated DNA condensation is Tnp dimerization dependent

Synapsis requires Tnps that are competent for dimerization. This interaction depends on protein–protein interactions in the carboxy-terminus of Tnp (Steiniger-White and Reznikoff, 2000). To determine whether Tnp ES-independent DNA condensation also involves carboxy-terminal dimerization, two Tnp dimerization-deficient proteins (50 nM) were reacted with relaxed non-ES (λ-DNA) molecules. TnpA466D contains a point mutation that prevents dimerization (Steiniger-White and Reznikoff, 2000); TnpΔ369 is missing the carboxy-terminal 110 amino acids of the full-length protein (York and Reznikoff, 1996). Both Tnps are deficient for dimerization yet both contain the amino-terminal ES DNA specificity binding region. Dimerization-deficient Tnps do not condense the non-ES DNA (Fig. 2D, bars 5 and 6). Incubation of TnpA466D with the non-ES DNA results in an extension of 0.92 ± 0.01 (SD, $n = 3$) of Tnp-free DNA and incubation of TnpΔ369 with the non-ES DNA results in an extension of 0.99 ± 0.01 (SD, $n = 3$) of Tnp-free non-ES DNA.

ES-independent DNA binding involves Tnp contacts essential for ES identification

Previously the Tn*5* transposition mechanism was posited to exhibit two separate DNA-binding processes (reviewed in Reznikoff, 2003). The first process is involved in the identification of the transposon ESs (Fig. 1A, steps 0–2), while the second is the post-excision target DNA capture process (Fig. 1A, step 4). Examination of the co-crystal structure of Tnp bound to ES DNA (Davies *et al*., 2000) shows that the ES DNA specificity binding region of Tnp is separate from the proposed target binding groove (Davies *et al*., 2000; Rice and Baker, 2001).

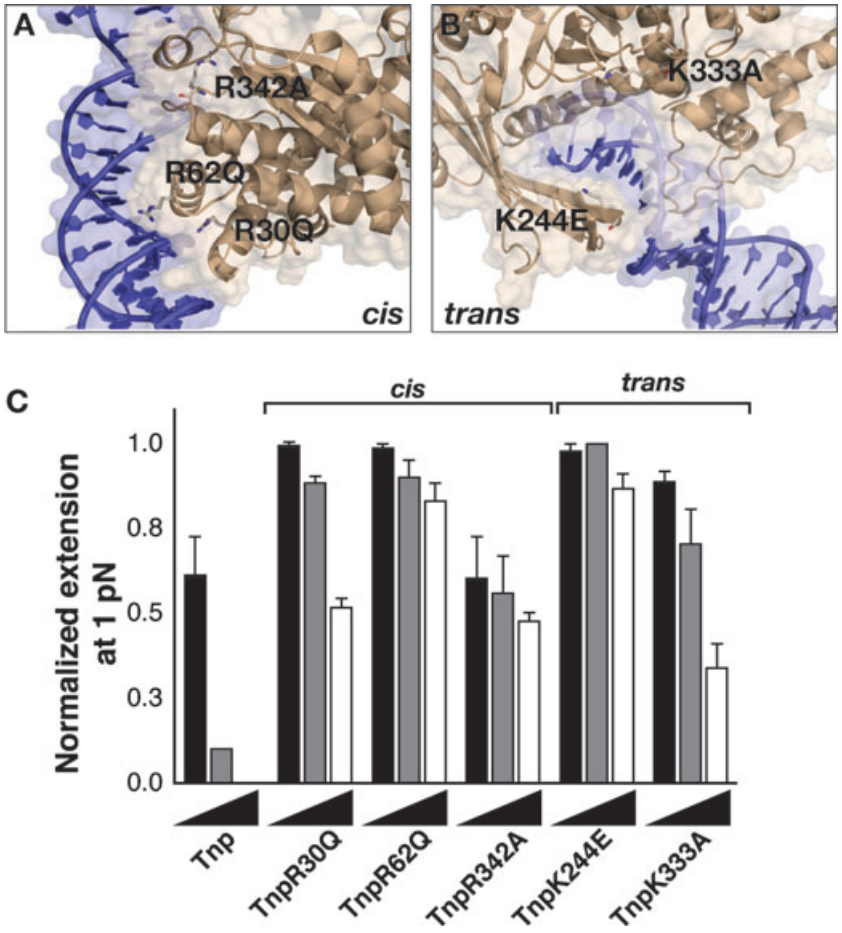

Fig. 3. ES-specific DNA-binding domains are required for ES-independent DNA condensation.
A and B. The co-crystal structure of Tnp bound to ES DNA (Davies *et al*., 2000) shows extensive (A) *cis* and (B) *trans* Tnp–DNA contacts. *Cis* DNA contacts are defined as interactions between the ES and the amino-terminus of Tnp while *trans* contacts on the same ES are contributed primarily by the active site of the other Tnp subunit. Amino acid substitutions in the *cis* DNA binding region [R30Q, R62Q (Twining *et al*., 2001) and R342A (R. Sterling and W.S. Reznikoff, unpublished)] and *trans* DNA binding regions [K244E (Steiniger *et al*., 2006b) and K333A (Naumann and Reznikoff, 2002)] prevent or severely inhibit synaptic complex assembly.
C. Single-DNA-molecule condensation analysis of DNA binding mutants incubated with 0 ES tethers shows that residues required for synapsis in the *cis* and *trans* DNA binding regions are also required for efficient ES-independent DNA condensation. Tnp variants were titrated from 50 (black) to 200 (white) nM ($n = 3$ for all measurements).

We have used point mutations in the ES DNA-specific binding contact region to determine whether these ES DNA-specific binding contacts are involved in the ES-independent DNA binding and condensation reaction. Specifically, we used a series of mutant Tnps that have been identified and characterized in previous studies (Twining *et al*., 2001; Naumann and Reznikoff, 2002; R. Sterling and W.S. Reznikoff, unpublished; Steiniger *et al*., 2006b). These studies targeted both the *cis* and *trans* DNA binding regions in Tnp. *Cis* interactions are between the ES and the amino-terminal DNA binding region of the protein (Fig. 3A) while the *trans* interactions are between the ES and the Tnp subunit that is responsible for catalysis (Fig. 3B). Point mutations in both the *cis* [R30Q, R62Q (Twining *et al*., 2001) and R342A (R. Sterling and W.S. Reznikoff)] and *trans* [K244E (Steiniger *et al*., 2006b) and K333A (Naumann and Reznikoff, 2002)] contacts inhibit or greatly diminish transposition efficiency *in vivo* and synapsis *in vitro*. No mutants defective in target binding have been isolated.

These ES DNA specificity mutant Tnps (titrated from 50 to 200 nM) were separately incubated with relaxed, 0 ES DNA for 20 min at 25°C and then extended with 1 pN of tension to measure extension and assay condensation using the same protocol described above. The ES DNA binding mutants (both *cis* and *trans*) are defective for DNA condensation when compared with Tnp. Tnp binding to 0 ES DNA results in an almost complete loss of extension at concentrations above 100 nM (Fig. 3C). However, this 'completely condensed' state was not observed for any of the DNA binding mutants tested in this assay. Of the *cis* DNA specificity binding mutants, TnpR30Q and TnpR62Q exhibited very little DNA condensation activity and TnpR342A condensed the 0 ES DNA, but much less robustly than as observed with Tnp. Of the *trans* DNA binding mutants, TnpK244E had no appreciable DNA condensation activity at any concentration while TnpK333A ES-independent DNA binding activity was comparable to that observed with TnpR30Q. Thus, mutations in the region of Tnp required for transposon ES identification diminish or eliminate the ES-independent DNA condensation reaction, suggesting that ES-independent DNA binding is a transposon ES binding-related process, not a target capture-related process.

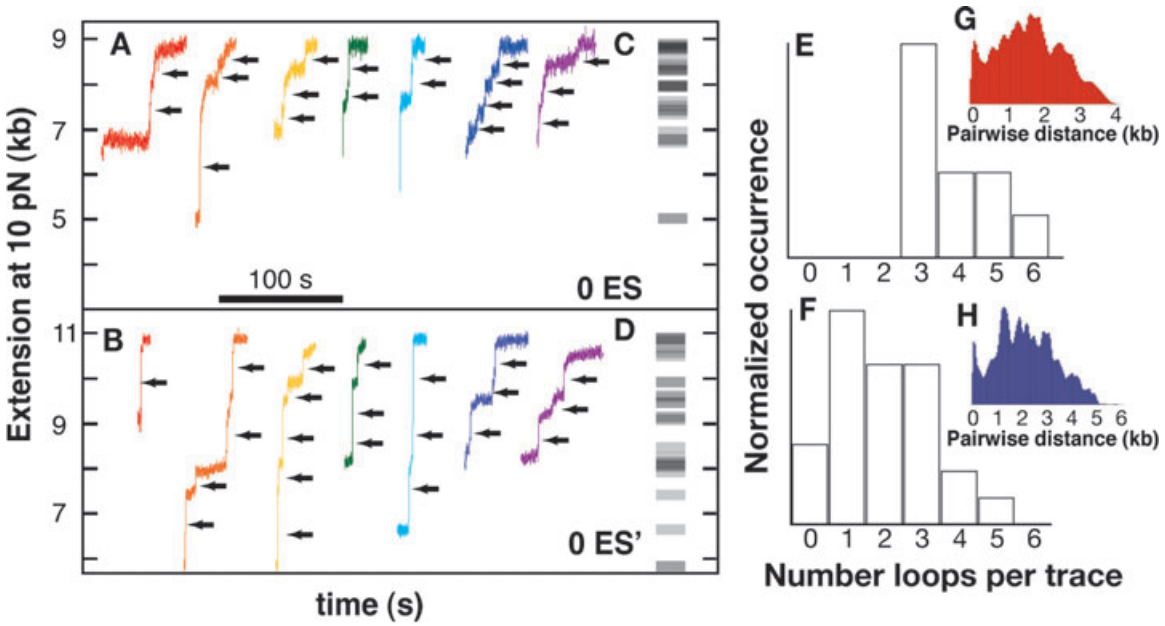

Fig. 4. Tnp binds a subset of non-canonical DNA sequences.
A–D. Seven representative extension-time traces observed under constant force (10 pN) after Tnp-induced condensation of relaxed (A) 0 ES and (B) 0 ES′ DNA show a pattern of opening within each 'family' of traces. Steps are indicated with black arrows. The plateaus observed in the traces of (C) 0 ES and (D) 0 ES′ DNAs were designated with lines. The lines corresponding to each plateau were then overlaid to generate a pattern where plateaus observed in multiple traces are seen as darker lines.
E–H. A plot of frequency of loops per extension-time trace on the (E) 0 ES ($n = 10$) and (F) 0 ES′ ($n = 26$) DNAs shows that the number of loops assembled per non-ES DNA is far less than expected given completely random looping. Additionally, the number of loops observed per 0 ES DNA trace does not follow a normal distribution, suggesting a sequence-directed binding and looping reaction. A histogram of the pairwise distance for (G) 0 ES and (H) 0 ES′ shows peaks corresponding to the observed loops in some of the traces in (A) and (B).

Tnp binding increases DNA persistence length

The decrease in extension of the non-ES DNA observed upon addition of Tnp could be due to Tnp-induced alterations in the DNA flexibility (bending/stiffening) or to other factors, such as Tnp-mediated DNA looping or wrapping of DNA around Tnp. To estimate the contribution of changes in DNA flexibility from Tnp binding, we calculated the persistence length (P) of the DNA before and after Tnp binding. P is a measurement of the stiffness of a DNA polymer, and may be readily determined from force-extension measurements (Marko and Siggia, 1995). Protein-induced changes in P are caused by bending (decrease P) or stiffening (increase P) of the DNA polymer. In buffer conditions used in this study (100 mM monovalent ion), P for protein-free DNA is about 150 bp (50 nm; Hagerman, 1988). A linear fit of the reciprocal of the square root of force to extension data using the worm-like chain model (Marko and Siggia, 1995) allowed us to estimate P for the single DNAs studied in these experiments. We measure P for Tnp-free non-ES DNA to be 141 $\pm$ 1 bp. Binding of Tnp to DNA increases P (stiffens the DNA) to 170 $\pm$ 20 bp. We note that if Tnp bent DNA one would expect a decrease in P determined in this manner (Yan and Marko, 2003). Thus, Tnp-mediated condensation is not due to DNA bending.

Tnp assembles ES-independent DNA loops

Several lines of evidence suggested to us that Tnp-mediated ES-independent DNA condensation occurs via the formation of DNA loops. The evidence, which is elaborated on in the *Discussion*, is that Tnp condenses only relaxed DNA, that condensation takes place on a timescale (~10 min) consistent with DNA looping and that Tnp does not bend non-ES DNA upon condensation.

To directly determine whether Tnp assembles ES-independent loops in DNA, we condensed single 0 ES or 0 ES′ tethers with Tnp (50 nM) in many separate trials. We then applied force (10 pN) to the Tnp-condensed tethers and monitored the tether's extension as a function of time (Fig. 4A and B). The decondensation process is characterized by short regions of constant extension (plateaus) punctuated by discrete opening events (steps). Steps observed on seven representative extension-times traces with 0 ES (Fig. 4A) and with 0 ES′ (Fig. 4B) are marked with arrows. This 'plateau/step' signal has been observed in similar condensation/extension experiments

with other sequence-directed DNA looping systems (e.g. BspMI; Yan *et al*., 2004).

Tnp binds a subset of non-canonical DNA sequences

In the condensation reactions described in the preceding sections, we always observed that Tnp (50 nM) never completely condensed the DNA to 'zero extension'. Zero extension is the result expected for DNA looping proteins that have no sequence specificity (Skoko *et al*., 2005). In our experiments, 50 nM Tnp reproducibly condensed DNA by only about 35%, indicating that much of the DNA is not condensed (Fig. 2D, bar 2). In the series of 26 such experiments carried out, the degree of condensation varied from 24% to 46%. Similarly, the Tnp-mediated condensation of λ-DNA (Fig. 2C) did not completely condense the DNA but stopped at ~2.5 μm. These observations suggest that Tnp is not binding to non-ES DNA completely randomly but rather that it binds to a subset of non-random, 'non-canonical' sequences.

To test the possibility that Tnp ES-independent binding is sequence directed, we analysed a series of condensation/extension experiments asking whether the loop opening pattern was the same for the same DNA sequences (suggesting Tnp bound the same sites when stabilizing the ES-independent loops) and different for the 0 ES and 0 ES′ DNA sequences. We observed that within each family of traces (i.e. all traces collected for 0 ES or 0 ES′ DNA) the individual molecules exhibited somewhat similar opening patterns, with steps of the same size often opening in the same order and plateaus occurring at the same locations in the plot (see Fig. 4A for 0 ES and Fig. 4B for 0 ES′).

To facilitate comparison between separate traces, plateaus in individual extension-times traces were marked with lines. These lines were then overlaid for each family of traces (Fig. 4C and D). In this analysis, plateaus observed in multiple traces are darker than those observed in few traces. For the 0 ES and 0 ES′ tethers, several plateaus are clearly more often observed than other plateaus. Additionally, the pattern of plateaus is different for the two tethers. Thus, Tnp generally assembles loops of the same size on each individual tether (i.e. 0 ES or 0 ES′). However, loop opening patterns are different between 0 ES and 0 ES′, suggesting that Tnp binding and looping is dependent on the sequence of the tether.

To determine the degree of randomness of ES-independent DNA binding and looping, we analysed the number, distribution and size of loops observed on these two tethers. A histogram of the number of loops (greater than 150 bp) observed per extension-time trace on the 0 ES (Fig. 4E) and 0 ES′ (Fig. 4F) DNAs shows that Tnp assembles far fewer loops than would be predicted given entirely random DNA binding and looping [~0.5 kb is the most likely size for a spontaneously formed DNA loop under these conditions (Sankararaman and Marko, 2005); thus 16–20 loops should be observed per 8.6 kb (0 ES) or 10.6 kb (0 ES′) tether]. For 0 ES DNA, most extension-time traces contained three loops, with no more than six loops observed for any individual tether. For 0 ES′ DNA, most extension-time traces contained one to three loops, with some traces containing zero loops; no more than five loops were observed for any individual tether. Furthermore, the number of loops per trace is different between the two DNA sequences. More loops per trace are observed in the 0 ES substrate than the 0 ES′ substrate, indicating 0 ES′ has a greater number of favourable non-canonical DNA sequences for Tnp binding. The data suggest that non-random loops are being assembled between non-canonical Tn*5* binding sites in the 0 ES and 0 ES′ DNAs.

To quantitatively measure the opening pattern in these traces, the distance between individual extension measurement and every other extension measurement in the extension array was calculated (pairwise distance). The histogram of the pairwise distances for these traces shows that the distances between plateaus are not randomly distributed but cluster around with well-defined values. Moreover, the histograms for the 0 ES (Fig. 4G) and 0 ES′ (Fig. 4H) substrates are obviously different, further underscoring the degree of non-randomness in DNA binding on these two unrelated non-ES DNAs. The zero-length loops and do not represent physical loops.

Discussion

We have used single-DNA-molecule micromanipulations to study DNA binding and looping in the Tn*5* transposition system. We found that Tnp binds and loops DNA in the absence of Tn*5* transposon ESs. In characterizing these ES-independent nucleoprotein complexes, we found that they are very similar to the ES-dependent synaptic complex. ES-independent nucleoprotein complexes likely contain the same number of Tnps and DNA segments. ES-independent DNA complex assembly is inhibited by dimerization-deficient Tnps, the same as has been reported for the synaptic complex (Steiniger-White and Reznikoff, 2000). We confirmed that the ES-independent DNA-binding process is related to transposon ES identification (and not target capture) as ES DNA binding-defective mutants of Tnp affect the ES-independent DNA-binding process. Finally, like the synaptic complex, ES-independent DNA complexes are the nodes of DNA loops. The characteristics of the ES-independent DNA looping reaction and the role of ES-independent DNA binding and looping in the Tn*5* transposition mechanism are discussed here.

Evidence for DNA looping without Tn5 transposon ESs

Several lines of evidence suggested to us that Tnp-mediated condensation is due to Tnp stabilization of DNA loops. First, spontaneous DNA looping occurs only at low forces (< 1 pN; Sankararaman and Marko, 2005; Skoko *et al*., 2005; Yan *et al*., 2005), a force-extension property of DNA that is consistent with our observation that relaxed DNA is required for Tnp-mediated DNA condensation (Fig. 2D). The slow time-course (compared with DNA bending proteins; Skoko *et al*., 2004) of this condensation also suggested a role for DNA loop formation. Spontaneous formation of DNA loops via DNA 'crossings' is strongly suppressed by even very low forces (< 0.1 pN; Sankararaman and Marko, 2005; Skoko *et al*., 2005; Yan *et al*., 2005). Therefore, Tnp stabilization of these rarely formed DNA crossings is expected to be a slower process than if the condensation were due only to Tnp-mediated DNA bending. DNA bending was also eliminated as a mechanism for Tnp-mediated ES-independent DNA condensation when we calculated the apparent persistence length and found Tnp does not bend DNA.

Direct evidence for DNA looping was obtained when we extended Tnp-condensed non-ES DNA tethers with applied force (10 pN) and observed plateaus of constant extension punctuated with discrete steps in the extension-time trace. This plateau/step signal has been used in other known DNA looping systems (e.g. BspMI; Yan *et al*., 2004) to assay DNA looping. Also, in protein–DNA systems where DNA looping was not previously known to play a role (e.g. Fis; Skoko *et al*., 2005), the plateau/step signal has been used to study DNA looping. Finally, in Tn*5* Tnp synapsis studies (C.D. Adams, B. Schnurr, J.F. Marko and W.S. Reznikoff, submitted) using tethers containing single transposons of various lengths, we observed that the step size correlates with known transposon length. The evidence presented here strongly suggests that the Tnp-mediated condensation of non-ES DNA occurs via the formation of DNA loops.

Finally, like observations with full-length Tn*5* Tnp used in these studies, shorting of DNA as a result of DNA looping between specific ESs was detected by tethered particle motion using the amino-terminal DNA-binding domain of the IS*911* Tnp (Pouget *et al*., 2006). However, no ES-independent decrease in DNA extension was observed for IS*911*, possibly because the carboxy-terminal, catalytic domain was removed from the protein used in these experiments.

Sequence bias during ES-independent DNA binding and looping?

We always observed Tnp-mediated ES-independent looping to only partly condense the DNA (Fig. 2). Also, the number of loops we observed on the DNA substrates was far less than the number of loops possible given random looping (Fig. 4). Furthermore, we observed patterns of loop opening that were similar for the same DNA sequences and different for unrelated DNA sequences (Fig. 4). These data suggested that only a subset of all DNA sequences could participate in Tnp-mediated ES-independent DNA looping. Tnp's selective binding to a family of 'non-canonical' DNA sequences contrasts strongly with the complete (100%) DNA compaction generated by the non-sequence-specific loop-forming protein Fis (Skoko *et al*., 2005). Fis will condense DNA tethers to zero extension as Fis proteins presumably can stabilize DNA loops on all or nearly all sequences in the DNA tether. This property was never observed with Tnp, even when incubated with non-ES DNA overnight. It is not completely surprising that some level of sequence bias is also observed in non-ES binding and looping as Tnp preferentially binds the specific Tn*5* transposon ES over non-ES DNA (Steiniger *et al*., 2006b).

We note that a Tnp variant that alters DNA specificity (E54K; Zhou *et al*., 1997), enhances *trans*-transposition activity (L372P; Weinreich *et al*., 1994a) and prevents the expression of a Tnp inhibitor protein (M56A; Wiegand and Reznikoff, 1992) was used in these studies. The altered or enhanced activities of this Tnp are quite relevant and related to the activities of the wild-type Tnp. The E54K mutation does alter DNA specificity and likely plays a role in the ES-independent DNA binding reaction. That role, however, is likely to favour one set of non-ES sequences over another, not to change the overall effect of ES-independent DNA binding. The other two mutations (M56A and L372P) do not affect DNA binding. The L372P mutation enhances *trans* activity by favouring a conformation of the protein that enhances interactions between the amino-terminus and DNA; this is a conformation that the wild-type protein is proposed to adopt, but more rarely than with this Tnp variant. The M56A mutation blocks expression of the alternate inhibitor protein and to our knowledge does not affect the ES DNA binding (or any other) activity of Tnp.

Sequence requirements for catalysis: comparison with other transposition and recombination systems

Despite the similarity between the catalytic synaptic complex and the ES-independent nucleoprotein complex, DNA cleavage or nicking activities related to ES-independent DNA looping has never been observed, on single tethers or in *in vitro* transposition assays using the 0 ES substrate (C.D. Adams, B. Schnurr, J.F. Marko and W.S. Reznikoff, submitted). This is a distinct result when compared with other instances of non-canonical, non-specific and pseudo-specific binding reported in the Tn*5*, Tn*10* and V(D)J recombination systems. In glycerol,

the Tn*10* Tnp was observed to bind one Tn*10* ES and one 'pseudo-end', resulting in 'non-canonical transposon-promoted rearrangements' (Chalmers and Kleckner, 1996). Non-specific DNA can also be incorporated into Tn*10* PECs (Sakai *et al*., 1995). In fact, a 'cryptic end' that supports transposition has been described for the Tn*5* transposition system (Goryshin and Reznikoff, 1998). Furthermore, the RAG 1/2 recombination proteins of the V(D)J recombination system are thought to bind and nick DNA at 'pseudo-RSSs' (Tycko and Sklar, 1990; Roth, 2003; Lee *et al*., 2004). While some level of DNA chemistry is supported upon binding illegitimate DNA in these instances, all of these examples are of nucleoprotein complexes that consist of one ES and one non-ES DNA.

It is possible that the additional sequence-specific contact imparted by one correct ES in the synaptic complex is all that is required to support some level catalysis. Alternatively, a DNA sequence-dependent conformational change in the Tnp structure could be a checkpoint for transposition, standing guard between synapsis and catalysis on legitimate ES DNA sequences and blocking catalysis on illegitimate DNA sequences. For example, the β-loop region of Tnp was recently shown to be involved in conformational changes affecting reactivity (Steiniger *et al*., 2006b). Because of proximity to specific DNA sequences it may play a role in differentiating between the legitimate and illegitimate DNA sequences.

*Possible functional roles of the ES-independent DNA loops in Tn*5 *transposition*

There are several potential roles for the assembly of nucleoprotein complexes and DNA looping by Tnp on non-ES DNA sequences. First, Tnp-mediated ES-independent DNA binding and looping likely competes with ES binding and looping (synapsis), thereby keeping the transposition activity of Tnp quite low. Transposition is an inherently damaging process for a genome and transposition systems have many mechanisms to minimize transposition. For Tn*5*, the *in vivo* transposition frequency is on the order of one event per 10^5 cells despite abundant cellular Tnp (Reznikoff, 2003). Mechanisms to prevent frequent transposition in the cell include a Tn*5* Tnp-specific inhibitor protein, suboptimal ES recognition and occlusion of the amino-terminal DNA-binding domain by the carboxy-terminal dimerization domain (reviewed in Reznikoff, 2003). The robust ES-independent binding activity reported here and in Steiniger *et al*. (2006b) and C.D. Adams, B. Schnurr, J.F. Marko and W.S. Reznikoff (submitted) increases non-productive Tnp–DNA interactions, thereby greatly decreasing the number of Tnps that can mobilize transposon DNA.

Additionally, the Tnps in many transposition systems have been observed to act *in cis* on the same DNA that encoded the Tnp gene (IS*903* Tn*5* and Tn*10*; Derbyshire *et al*., 1990; Johnson *et al*., 1982; Morisato *et al*., 1983). The current model for Tn*5*'s *cis* preference suggested that the inhibitory interaction between the amino- and carboxyl-termini would lead to a *cis* bias because the amino-terminus of Tnp could bind to the ES before complete translation of the carboxy-terminus (Weinreich *et al*., 1994b). The present work demonstrates an additional mechanism for imposing the *cis* preference: robust ES-independent DNA binding effectively tethers Tnp close to its site of synthesis, preventing Tnp from mobilizing Tn*5* elements in *trans* (Steiniger *et al*., 2006b).

An alternate role of the ES-independent DNA looping activity suggests Tnp may search for the ES using a DNA looping-based direct transfer search mechanism. The direct transfer search mechanism requires that Tnp bind two DNA segments simultaneously to move through the genome while searching for the ES (Halford and Marko, 2004). We showed Tnp could move from non-ES DNA to the ES (Steiniger *et al*., 2006b). We also showed that Tnp does not efficiently disassociate from non-ES DNA (eliminating a 'hopping/jumping' mechanism) and that sliding does not play a role over distances greater than ~0.4 kb (Steiniger *et al*., 2006b). Thus, of the three generally accepted search mechanisms (direct transfer, hopping/jumping and sliding; Halford and Marko, 2004), only direct transfer over short distances remains as a potential search mechanism. However, Tnp moving significant distances in the genome (> 0.4 kb) to locate ESs via direct transfer may be unlikely as ES-independent DNA loops are quite stable against force.

Our report of Tnp-mediated DNA looping in the absence of the Tn*5* ESs suggests that ES-independent DNA binding and looping likely competes with the identification and processing of transposon ESs by Tnp. This competition results in an overall reduction of transposition events and is likely another mechanism for Tn*5*'s *cis* bias.

Experimental procedures

Proteins, molecular constructs and beads

All Tnp proteins contained the E54K/M56A/L372P (Wiegand and Reznikoff, 1992; Weinreich *et al*., 1994a; Zhou and Reznikoff, 1997) changes and were purified as described (Bhasin *et al*., 1999). Ultrapure grade lysozyme was from USB. Plasmids were constructed using standard cloning methods (Sambrook and Russell, 2001). 0 ES is an 8.6 kb pBR322-derived plasmid containing adenovirus DNA and 0 ES′ is a 10.6 kb pUC19-derived plasmid carrying a fragment of λ-DNA. These plasmids were linearized by digestion with SacI and NcoI. Linear λ-DNA was purchased (Promega). The linear fragments of the plasmids were then modified on either end with 1000 bp fragments containing digoxigenin-11-dUTP or biotin-16-dUTP (Roche) using a ligation reaction. The *cos* ends of λ-DNA were modified with digoxigenin and biotin as

described (Skoko *et al*., 2004). All modified DNA substrates were incubated with strepavidin-coated 2.8 μm-diameter magnetic beads (M-280, Dynal Biotech) which had been treated with 4 mg ml^{-1} bovine serum albumin (BSA; NEB) in phosphate-buffered saline (PBS) for 10 min.

Gel-shift assays

Transposase (400 nM) was incubated with DNA binding buffer [100 mM NaCl, HEPES pH 7.5, 20 ng μl^{-1} salmon testes DNA (Sigma)] and fluorescently labelled 30-mer oligonucleotides (40 nM; Integrated DNA Technologies) at 25°C for 60 min. The sequences of the ES-containing oligonucleotide and the non-ES oligonucleotide R1 are shown in Fig. 2B. R1 was generated by replacing 14 of the 19 ES base pairs with randomly generated sequence. Gels containing fluorescently labelled DNA were imaged using a Typhoon 9410 (Amersham Biosciences).

Sample cell preparation

Sample cells were constructed as described (Smith *et al*., 1992; Strick *et al*., 1996; Skoko *et al*., 2004). The 40 μl sample chamber was incubated with 4 mg ml^{-1} BSA (NEB) in PBS for 10 min. The modified DNA plus magnetic beads was then flowed into the chamber in PBS and the digoxigenin end of the molecules was incubated with the anti-digoxigenin cover glass by inverting the flow chamber for 10 min in PBS.

Microscopy

Single-molecule experiments were conducted on a vertical magnetic tweezer apparatus as described (Skoko *et al*., 2004). Extension of the single-molecule tethers was measured by calibrating a library of out-of-focus images of the bead generally as described (Strick *et al*., 1996), resulting in measurements with ~20 nm accuracy in extension and data acquisition rates of 15 Hz. Extension of λ-DNA was measured using an auto focus algorithm (Skoko *et al*., 2004). Forces (± 5%) were determined by measuring the transverse fluctuations of the magnetic bead (Strick *et al*., 1996).

Single-DNA experimental protocol

Single-molecule tethers were located in the sample chamber and force-extension measurements were performed to ensure only one DNA is tethered between the glass side and the magnetic bead. The tether was calibrated for force and 50 nM Tnp was flowed into the sample chamber in DNA binding buffer (20 mM HEPES, pH 7.5, 100 mM K Glu). DNA condensation was assayed by extending the relaxed DNA and measuring the extension at 1 pN of tension. Loop formation was assayed by increasing the applied force and monitoring the resulting extension-time traces. DNA condensation and synapsis experiments were performed at 25 ± 3°C.

Data analysis

Extension-time data were analysed using custom-written software that allowed semi-automated step event analysis. Events were tagged and reduced to several pieces of information (time, force, extension) for further analysis (data reduction with Excel, plotting and fitting using Graphpad Prism). Pairwise distances of extension-time traces were analysed using the Spatstat package (Baddeley and Turner, 2005) in the R Statistical Analysis program (R Development Core Team, 2005).

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