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Pulling Apart Catalytically Active Tn*5* Synaptic Complexes Using Magnetic Tweezers

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The Tn*5* transposase is an example of a class of proteins that move DNA sequences (transposons) *via* a process called transposition. DNA transposition is a widespread genetic mobility mechanism that has profoundly affected the genomes of nearly all organisms. We have used single-DNA micromanipulation experiments to study the process by which Tn*5* DNA transposons are identified and processed by their transposase protein. We have determined that the energy barrier to disassemble catalytically active synaptic complexes is 16 kcal mol^{-1}. However, we have found that the looping organization of DNA segments by transposase is less sequence-driven than previously thought. Loops anchored at some non-transposon end sequences display a disassembly energy barrier of 14 kcal mol^{-1}, nearly as stable as the synapses formed at known transposon end sequences. However, these non-transposon end sequence independent complexes do not mediate DNA cleavage. Therefore, the sequence-sensitivity for DNA binding and looping by Tn*5* transposase is significantly less than that required for DNA cleavage. These results have implications for the *in vivo* down regulation of transposition and the *cis*-transposition bias of transposase.

Keywords: transposition; transposase; Tn*5*; protein-nucleic acid interactions; single molecule

Introduction

The movement of genetic material from place to place in the genome (transposition) mediated by transposase proteins (Tnp) has profoundly impacted the composition of genomes of many organisms.[1] A key initial step in transposition is organization of Tnp and transposon DNA into an ordered, catalytically competent, and highly stable[2,3] nucleoprotein "synaptic complex". Synaptic complex assembly is required for DNA cleavage to occur. Tnps in a synaptic complex cut the transposon from "donor" DNA and free it to be inserted elsewhere in the genome. The formation of synaptic complex-like nucleoprotein complexes has generally been thought to occur only at transposon end sequences (ESs), restricting synapsis to occur between transposon ends. This specificity has been proposed to be a checkpoint to transposition, ensuring that Tnps cut out only transposon DNA.

Tnps process DNA by coordinating divalent metal ions with a highly conserved acidic amino acid triad[4–6] (the DDE motif). Interestingly, the same DDE chemistry is used by other DNA-cleaving proteins, e.g. HIV-1 integrase, and the RAG1 protein of V(D)J recombination.[7,8] Because only divalent metals are required for reactivity (these proteins are ATP-independent), the driving force for these reactions is the increasing stability of the synaptic complex as the proteins and associated DNA proceed through transposition. Also, since Tnps are not enzymes in a classical sense (i.e. they do not "turn over", cutting and inserting transposon DNA only once) increasingly high DNA binding affinities characterize their reaction mechanisms.

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Abbreviations used: dbb, donor backbone; ES, end sequence; pN, picoNewton; SD, standard deviation; Tnp, transposase; Tn, transposon.

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Concomitant with this is evidence that Tnp synapses are highly stable: Surette *et al.*[3] showed the Mu Tnp synaptic complex to be resistant to heat and chemical treatment; and comparable observations have been made with the Tn*10* Tnp synaptic complex.[2] Tn*5* Tnp synaptic complexes were reported to be catalytically active more than one year after assembly.[9]

To investigate the mechanisms of synapsis and cleavage, we carried out single-DNA micromanipulations on DNAs containing Tn*5* ESs. Synaptic complex assembly and disassembly by Tn*5* Tnp is monitored *via* DNA pulling using magnetic tweezers. Our use of the Tn*5* transposition system was motivated by several factors. In the presence of Mg^{2+}, Tn*5* Tnp can move any DNA defined by two inverted Tn*5* ESs[10] with no additional required cofactors or specific DNA topologies[11] (Figure 1(a)). Furthermore, extensive genetic and structural data[12,13] allow us to place the single molecule studies in structure–function context.

Here, we assemble and manipulate Tn*5* synaptic complexes on single transposons. We show that synaptic complexes assembled by Tn*5* Tnp between two Tn*5* ESs on a single molecule of DNA are catalytically competent while ES-independent loops assembled by Tn*5* Tnp on similar single molecules lacking the Tn*5* ES do not provoke DNA cleavage. Our single-molecule assay allows us to separately dissect synaptic complexes from ES-independent

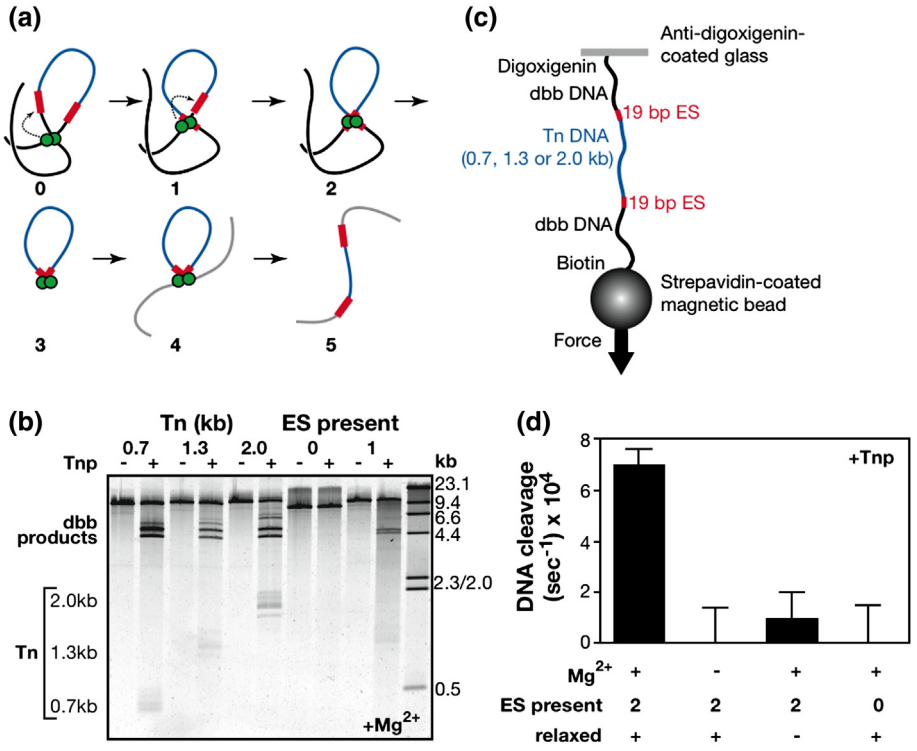

Figure 1. "Cut-and-paste" transposition mechanism, experimental design and formation of catalytically competent synaptic complexes in bulk and on single DNA molecules. (a) The Tn*5* transposase (green) moves the Tn*5* Tn (blue) using a cut-and-paste mechanism. In step **0**, Tnp has assembled a dimerization-dependent, ES-independent DNA loop. Our current model posits that Tnp then locates 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.[11] 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.[10] 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 (this Figure also appears elsewhere[20]). (b) Substrates containing the ES are catalytically competent to be cut by Tnp in bulk. DNA substrates were incubated with 10 mM Mg^{2+} and + or - Tnp. Both the dbb (4.9 kb and 4.4 kb) and transposon products are observed in the +Tnp lanes for the 0.7, 1.3 and 2.0 kb substrates. No products are observed with the zero ES substrate, dbb products are observed with the one ES substrate resulting from intermolecular reactions. (c) Single DNA molecule "tethers" were constructed in flow chambers and assayed by using a microscope equipped with permanent magnets. The DNA substrates contained zero, one or two ESs (red) with 0.7, 1.3 and 2.0 kb inserted between the two ES. All five substrates were modified with digoxigenin and biotin and attached to a strepavidin coated 2.8 μm magnetic bead and an anti-digoxigenin functionalized glass slide. Single DNA "tethers" were stretched between the glass slide and magnetic beads by changing the magnetic field gradient applied to the magnetic bead. (d) Catalytically active synaptic complexes can be assembled on single DNA molecules. Single DNA molecules were assayed for Tnp catalysis by monitoring the rate of DNA cleavage (disappearance of the tethered bead) under several experimental conditions. All molecules were incubated with ~50 nM Tnp, DNA cleavage is observed only when the single DNA molecules with two ESs are incubated in the presence of Mg^{2+} and the DNA is relaxed.

loops. We used magnetic tweezers to disrupt the two types of DNA loops to measure the mechanical stability of the loops. We measured the stability of catalytically active synaptic complexes and found that they are very stable; the energy barrier to disrupt the Tn*5* synaptic complex is 16 kcal mol^{-1}. Interestingly, we also found that some ES-independent loops, while highly stable, are less so than synaptic complexes. However, only synaptic complexes cleave DNA. Thus ES-independent DNA looping by Tnp involves a lower level of sequence discrimination than catalysis, consistent with proposals based on genetic experiments with IS*903*,[14,15] Tn*10*[16] and Tn*5*.[17]

Results

Linear DNA substrates of sizes from 8 kb to 12 kb were constructed. These substrates contained DNA sequences of 0.7, 1.3 or 2.0 kb flanked by two inverted Tn*5* ESs and two control DNAs with either none or one Tn*5* ES. First, we checked using bulk biochemical experiments that these DNAs, which were to be used subsequently in the single-DNA experiments, were properly cleaved by Tnp *in vitro* in the presence of 10 mM Mg^{2+}. For the two ES DNAs, Tnp cut DNA at the ends of the ESs (Figure 1(b)), resulting in release of donor backbone (dbb) DNA and transposon (Tn) DNA of the expected lengths for the 0.7, 1.3 or 2.0 kb DNAs containing two ESs. No products were observed with the zero ES substrate; dbb products were observed with the one ES substrate, consistent with the known ability of Tnp to assemble a synapse between two unconnected segments of DNA (a paired ends complex).[11]

These linear DNAs were prepared for use as "tethers" by ligation of biotin and digoxigenin-labeled 1 kb DNA fragments to the ends.[18] The modified DNAs were then incubated with strepavidin-coated magnetic beads and antidigoxigenin-functionalized glass flow cells[19] (Figure 1(c)). Once the single DNA molecule tethers were constructed, variation of the magnetic field gradient was used to control the force applied to the DNA molecule. Application of force was used to change the conformation of the DNA molecule; increasing applied force extends the tether while decreasing applied force relaxes the tether. Applied force was also used to disrupt protein–DNA and protein–protein interactions. DNA extension was monitored as a function of both time and force.

Catalytic activity analyses were performed on single DNA molecule tethers to determine whether catalytically functional complexes could be assembled on single tethered DNA molecules. Single molecule substrates in relaxed (low force <0.04 pN) or extended (high force >1 pN) states, either containing or lacking Tn*5* ESs, were incubated with Tnp. Tnp binds to both extended and relaxed DNA;[20] however, loop formation is possible only with relaxed DNA.[21,22] Thus, assaying DNA cleavage activity on single DNA molecules that are either extended (no looping) or relaxed (looping) provides a means to determine whether observed cleavage events take place via synaptic complex formation (i.e. looping) and not some other, non-looping mediated process (e.g. tether attachment point failure or non-specific nicking).

After reacting Tnp with relaxed or extended DNA tethers, which contained or lacked the ESs, 10 mM Mg^{2+} was added to trigger catalysis. Catalysis (DNA cleavage) was assayed as the observation of disappearance of the bead attached to the end of the single DNA molecule tether. The disappearance of the bead is caused by Tnp-induced, Mg^{2+}-dependent cleavage of the single DNA molecule substrate in a synaptic complex. Cleavage occurs only in the presence of Mg^{2+} on relaxed (looped) single DNA molecules containing two Tn*5* ESs (Figure 1(d)) showing that catalytically active Tn*5* synaptic complexes can be assembled on single tethered molecules of DNA containing two inverted Tn*5* ESs. We note that beads can also disappear because of other factors (i.e. failure of the attachment points on either the magnetic bead or the glass surface). However, in this assay, only one DNA breakage event was observed in all control experiments (-Mg^{2+}, extended tether and no ES; Figure 1(d)) over a combined time of 6.8 h while five breakage events were observed in the presence of Mg^{2+} over a time of 2.0 h. Thus, we conclude that most tether breakage events observed in the presence of Tnp and Mg^{2+} are due to Tnp-induced DNA cleavage and not some other tether-breakage process.

To measure synaptic complex stability, single DNA molecule tethers in a relaxed conformation were incubated with Tnp in the absence of Mg^{2+}. Tnp binds and condenses DNA in a dimerization-dependent reaction requiring relaxed DNA.[20] Applied force was used to disrupt the nucleoprotein complexes that assembled during incubation. Representative traces of extension *versus* time data (Figure 2(a)–(e)) show that discrete force-driven opening events (steps) are observed following the imposition of applied force on the condensed DNAs. These opening events correspond to the disruption of Tnp-mediated DNA loops. In all experiments described here, single DNA tethers are subjected to extensive calibration in the absence of protein. In these calibration steps in the absence of protein, step-opening events are never observed at constant force. Thus, the steps are a protein-dependent process.

Catalytically active synaptic complexes are DNA loops with two Tnps and two ESs at the node of the loop. We observed DNA loops of a size matching the transposon length when performing the disruption experiment on single molecules containing two inverted Tn*5* ESs (Figure 2). With the tether containing the 0.7 kb Tn, opening events of the expected size (approximately 0.2 μm, correspomding to 0.7 kb) were observed (Figure 2(c)). With this substrate, however, synaptic complex opening events cannot be distinguished from ES-independent opening events.[20] When the length between the ESs is increased to 1.3 kb and 2.0 kb, opening events

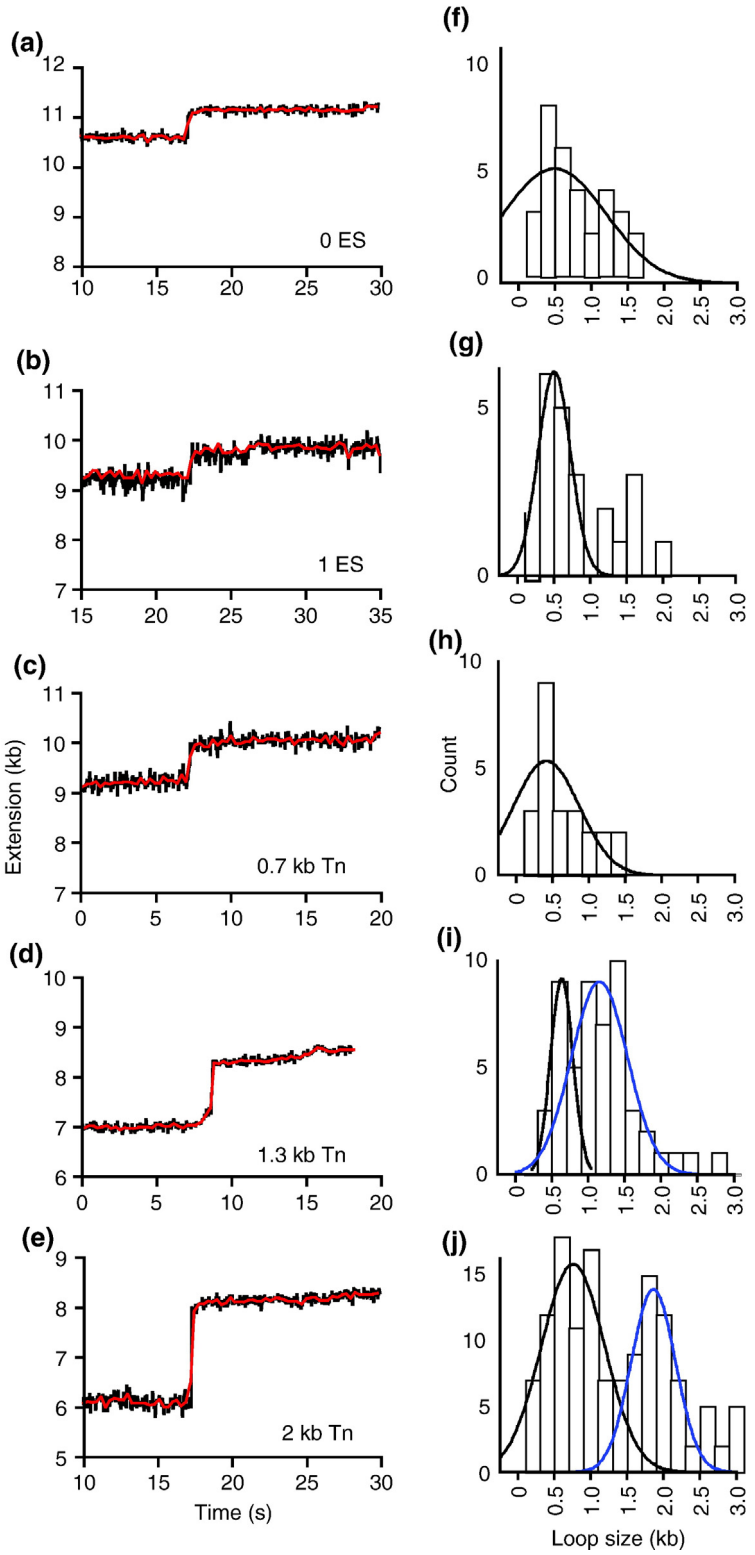

Figure 2. Synaptic complex opening events and ES-independent looping events can be observed on single DNA molecules. (a)–(e)) A representative plot of extension (kb) *versus* time at a constant force shows discrete opening events that correspond to Tnp mediated DNA loops. ES-independent loop opening events are observed with the zero (a) and one (b) ES substrates. Synaptic complex opening events are observed when two ESs are present, as loop opening events corresponding to 0.7 kb (c), 1.3 kb (d) and 2.0 kb (e) occur on each of these three substrates. For these representative plots, a force of 2 pN was used in (b), 5 pN was used in (c), 10 pN was used in (a), (d) and (e). Opening events on these substrates were observed at many forces and these plots are shown as representative opening events. (f)–(j)) A plot of frequency of loop size shows that ES-independent loops are observed on all substrates and have a size of about 0.5(±0.1) kb. When the ESs are present, a second distribution becomes clear for the 1.3 kb (i) and 2.0 kb (j) substrates. Both the ES-independent loop (black) and synaptic complex (blue) loop distributions were fit to separate Gaussian distributions.

of expected sizes (1.3 kb or 2.0 kb) corresponding to disruption of the synaptic complex were observed (Figure 2(d) and (e)) and were easily distinguished from ES-independent looping events. We also observed loop opening events on single DNA molecules with zero or one ES (Figure 2(a) and (b)). These opening events are ES-independent DNA loops.[20]

By observing many individual loop opening events in several experiments on each of the five DNAs, we were able to differentiate between disruption of synaptic complexes and the other ES-independent loops on the basis of loop (step) size. Figure 2(f) and (g) show the frequency of loop sizes observed with the zero and one ES single DNA substrates. This distribution was fit to a Gaussian

function and a mean loop size of 0.5(±0.1) kb (standard deviation (SD), $n=23$ for no ES, $n=21$ for one ES) was calculated for the zero and one ES substrates.

When the same frequency analysis was performed on the 0.7 kb transposon substrate (Figure 2(h)), a loop size of 0.5(±0.1) kb (SD, $n=24$) was found. However, given the observation that Tnp assembles both specific and ES-independent DNA loops, it is not clear which of the observed events is the result of disruption of synaptic complexes.

Delineation between synaptic complex disruption and ES-independent loop opening events is clearer in the data for the 1.3 kb transposon substrate (Figure 2(i)) and is complete with the 2.0 kb transposon substrate (Figure 2(j)). Gaussian fits to these data show a mean ES-independent loop size of 0.6(±0.2)kb (SD, $n=17$) and 0.7(±0.4) kb (SD, $n=81$), respectively, and a synaptic complex loop size of 1.2(±0.4) kb (SD, $n=32$) and 1.9(±0.3) kb, respectively (SD, $n=43$). Thus, both synaptic complexes and ES-independent loops can be observed and differentiated on single molecule substrates containing Tn*5* ESs separated by 1.3 kb or 2.0 kb.

Because of the clear separation between the ES and ES-independent opening events observed on the 2.0 kb transposon substrate, these events were further analyzed to characterize the stability of the synaptic complex. To determine the stability of the synaptic complex, the rate of disruption of the synaptic complex was measured as a function of force. Using Kramers' theory for thermal barrier crossing[23] in a form suitable for single-molecule applications derived by Ritchie & Evans,[24] we can determine the activation energy barrier opposing synaptic complex disassembly. The rate of complex disassembly, r_{dis}, is related to force by:

$$r_{dis} = r_0 e^{(\varepsilon_{b,0} - f\Delta x/k_B T)}$$

where r_0 is the estimated "attempt frequency" ($\sim 1\times10^7$ s^{-1}), i.e. the rate at which the synapsed Tnps would move if there were no interactions between the subunits and the DNA. Our estimate of r_0 is similar to estimates made in other, similar studies.[25,26] $\varepsilon_{b,0}$ is the activation energy barrier to synaptic complex disassembly, Δx is the deformation distance of the complex at which the energy barrier peak occurs, f is known applied force, k_B is the Boltzmann constant and T is the absolute temperature ($k_BT = 4.1\times10^{-21}$ J at 25 °C). Figure 3(a) shows a sketch of how the exponent is expected to behave in the absence (black) and presence (red) of force; applied force acts to reduce the barrier.

Figure 3(b) shows a plot of $\ln(r_{dis}/r_0)$ *versus* force that allows determination of $\varepsilon_{b,0}$ and Δx. These parameters were determined as the slope and intercept of a linear fit of the $\ln(r_{dis}/r_0)$ to force. We find that 16.0(±0.2) kcal mol^{-1} is the activation energy barrier for disassembly of the Tn*5* synaptic complex. This substantial energy is essentially the mechanical work required to disrupt the synaptic complex, and provides a quantitative substantiation of qualitative observations of the high degree of stability of the Tn*5*[9] synaptic complex as well as the Tn*10*[2] and Mu[3] synaptic complexes.

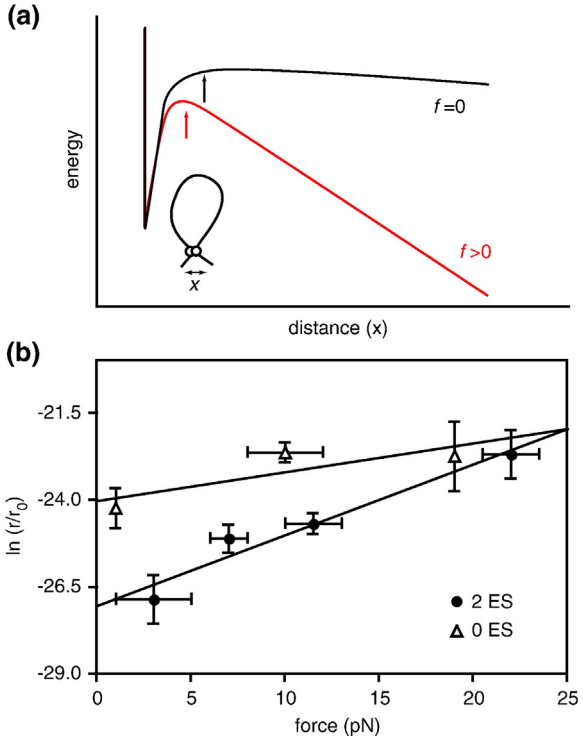

Figure 3. Determining the stability of the synaptic complex. (a) Sketch of energy landscape for synaptic complex disassembly as a function of the complex deformation (x) in the direction of applied force. To open the complex an energy barrier $\varepsilon_{b,o}$ (black arrow) at deformation $x=\Delta x$ must be crossed. When force f is applied, the energy landscape is shifted by an amount $-fx$, reducing the barrier height to $\varepsilon_{b,o} - f\Delta x$ (red arrow). Measuring the rate of synaptic complex opening as a function of force determines both $\varepsilon_{b,o}$ and Δx[23,24] (also see the text). (b) The activation energy barrier to synaptic complex disassembly is 16.0(±0.2) kcal mol^{-1} and the distance the two Tnp subunits must be separated to disrupt the synaptic complex is 8.2(±0.1) Å (●). The activation energy barrier to disrupt the non-specific-DNA loops is 14.2(±0.2) kcal mol^{-1} and ~3 Å must be separated to disrupt the non-specific DNA complexes (△). A plot of the $\ln(r_{dis}/r_0)$ *versus* force gives $\varepsilon_{b,0}$ and Δx from the *y*-intercept and slope, respectively.

Following a similar approach we determined the barrier energy to disassembly of the ES-independent nucleoprotein complexes. The activation energy barrier for disruption of the ES-independent DNA loops is 14.2(±0.2) kcal mol^{-1}, only ~2 kcal mol^{-1} lower than synaptic complexes. The affinity of Tnp for the DNA in the ES-independent loops is surprisingly strong. However, this high affinity likely represents only the most stable of a subset of non-canonical DNA binding sites. It is possible that weaker Tnp binding and looping sites may exist in a given DNA sequence[27] that are insufficiently stable to be analyzed using our pulling assay.

We also calculated the barrier position Δx, which is a measure of the distance that the complex must be deformed to disrupt the interactions stabilizing the Tnp–DNA loop complex. This distance represents the position of the peak of the energy barrier to disassembly along the pulling direction.[24] For the 2.0 kb transposon synaptic complexes, this distance is 8.2(±0.1)Å, which is reasonable given the number of base-pairs contacted by Tnp[13] and by comparing to the nucleosome core, which contacts 147 bp and has a Δx value of 13 Å[26]. This relatively long distance indicates the degree to which the synaptic complex must be deformed in order to trigger its opening; this deformation likely involves of disruption a combination of protein–protein and protein–DNA interactions.

When performed on the ES-independent loops, this analysis gives a Δx value of 3.4(±0.2) Å, showing that while the ES-independent Tnp–DNA complex may be more stable than expected, the deformation distance associated with disruption of these complexes is much less than for the synaptic complex, which is consistent with fewer specific Tnp–DNA contacts being the primary source of stability of these complexes, *versus* the specific interactions in the synaptic complex.[13]

The lifetimes of synaptic complexes and ES-independent nucleoprotein complexes were measured at 10 pN. Lifetimes were measured as the time until a step corresponding to the disruption of a synaptic complex was observed. Synaptic complexes are clearly more stable, as the half-life calculated from a single exponential decay model for the two types of nucleoprotein complexes is 10 s for the synaptic complex and 6 s for the ES-independent nucleoprotein complex. Again, the ES-independent complexes appear weaker, a characteristic expected from fewer base specific contacts.

Discussion

We have reported a single-molecule analysis of the stability of the catalytically active Tn*5* synaptic complex. We measured the energy barriers associated with disruption of the synaptic complex, as well as the corresponding barriers associated with ES-independent nucleoprotein complexes[20] assembled by Tnp. The qualitative stability of the synaptic complexes assembled by various transposases is well known.[2,3,9] However, quantitative measurements of the stability have not been performed. Our measurement of 16 kcal mol^{-1} to disrupt the Tn*5* synaptic complex is consistent with the observations that transposases assemble very stable nucleoprotein complexes in their reaction mechanisms. Disruption of the synaptic complex requires, at the most, release of two Tn*5* 19 bp ESs from two Tnps. The activation energy barrier for a related process, the release of up to 147 bp from the nucleosome core, has been measured using optical tweezers and is in the range of 17.5 to 22 kcal mol^{-1}, depending on DNA sequence and salt conditions of the measurement.[25,26] Thus, on a per base-pair released scale, the activation energy barrier for nucleoprotein complex disruption is greater for the Tn*5* synaptic complex (~0.42 kcal mol^{-1} bp^{-1}) than for the nucleosome (~0.15–0.12 kcal mol^{-1} bp^{-1}).

It is not clear what structural components of the synaptic complex architecture are disrupted as the synaptic complex is pulled apart. Do monomers of Tnp remain bound to DNA (i.e. primarily protein-protein interactions disrupted) or is the protein pulled off the DNA (primarily protein–DNA interactions disrupted) or is the disruption a combination of both interactions?

Some observations suggest that synaptic complex disassembly may be primarily disassembly of protein–protein interactions. In repeated single molecule disruption assays on the same DNA tether with the same Tnp in a reaction buffer emptied of free Tnp (i.e. after replacement of Tnp solution by buffer after binding of Tnp to DNA), loops repeatedly reassembled after disruption, suggesting that Tnp remained bound to the ES and reassembled a synaptic complex (data not shown). Thus, the force-driven disruption events may be primarily due to failure of protein–protein interactions, though both protein–protein and protein–DNA contacts must be disrupted for complete synaptic complex disassembly.

In observing synaptic complex disruption events, we also observed a background ES-independent DNA loop size of 0.5 kb. This size is roughly the peak distribution of the likelihood for spontaneous loop formation.[21] However, experiments on ES-independent DNA looping[20] have suggested Tnp binds a subset of non-ES DNA segments when assembling ES-independent DNA loops. We plan to precisely determine the sequence of hypothetical "non-canonical" Tn*5* Tnp binding sites.

We speculate that Tnp's high affinity for certain non-ES DNA sequences may help to prevent frequent transposition events *in vivo*[27,28] *via* establishment of highly stable, non-ES DNA–Tnp complexes that substantially reduce the amount of active Tnp in the cell, contributing to the down regulation of transposition. We note that non-specific Tnp-DNA interactions in the absence of looped complex formation could also decrease transposition through the binding of free Tnp. However, non-looped ES-independent Tnp–DNA complexes are likely less stable than the synapsis-like non-ES complexes we have recently characterized here and in other work.[20,27] Thus, of the two types of non-ES interactions, the ES-independent DNA loops are likely to contribute more to the overall down regulation of Tn*5* transposition.

Robust non-ES DNA binding activity may provide a mechanism for the widespread preference of many bacterial Tnps to bind ESs near their site of synthesis (*cis* bias[29]). Non-canonical DNA binding would tend to localize Tnp to DNA near the site of synthesis thereby contributing to Tnp's preference to transpose DNA in *cis*.[27]

The Tnp variant used in this (and many other) Tn*5* mechanistic studies may have some affect on the ES-independent looping reaction. This is because the

E54K[30] mutation alters DNA specificity and likely plays a role in the ES-dependent and ES-independent DNA binding reactions and may change the stability of the complex. That role, however, is likely to favor one set of non-ES sequences over another, not to change the overall effect of ES-independent DNA binding. The other two mutations (M56A[31] and L372P[29]) are not known to affect DNA binding.

Synapsis on single transposon-containing molecules has also been recently reported in tethered particle motion experiments using the amino-terminal DNA binding domain of the IS*911* Tnp.[32] Decreases in tether extension upon protein binding to the IS*911* sequences were studied, and were of the size expected given the known transposon size, i.e. ES-independent DNA loops were not observed. This difference between the IS*911* and Tn5 Tnps may be an intrinsic difference between those two proteins. Alternately, the lack of non-ES looping by IS*911* might be a result of use of a truncation of the IS*911* protein; a carboxy-terminal sequence that contains the catalytic domain was removed from the IS*911* Tnp in those experiments. We note that our previous work showed that mutations of specific residues in Tn*5* Tnp's DNA binding domain eliminated ES-independent looping[20] and that full length Tnp is required for ES-independent interactions with DNA.[27] It would be very interesting to determine whether ES-independent DNA looping occurs with the full-length IS*911* Tnp.

In conclusion, we have used a single-molecule approach to study Tn*5* Tnp synapsis and catalysis. We have quantitatively measured the stability of the synaptic complex and ES-independent DNA loops. The surprising stability of the ES-independent loops could play a role *in vivo* in the down-regulation of Tn*5* transposition, and in generating the well-known *cis*-bias for Tn*5* Tnp. It remains to be seen whether non-ES loops are as prevalent for other Tnps. Our approach and our measurements of the energy barrier for disassembly of the synaptic complex establish a baseline for future studies investigating other highly ordered nucleoprotein structures (e.g. synaptic complexes formed by other Tnps, as well as the mechanistically related proteins HIV-1 integrase and RAG1/2).

Materials and Methods

Proteins, molecular constructs and tethering of DNA to beads

Tnp contained E54K,[29] M56A[30] and L372P[31] changes and was purified as described.[33] This Tnp variant is required for *in vitro* experiments. The E54K change alters DNA specificity, the L372P change enhances *trans* transposition, and the M56A change prevents the expression of a Tnp inhibitor protein. Plasmids were constructed using standard cloning methods,[34] resulting in plasmids containing transposons with 0.7, 1.3 or 2.0 kb (pCDAT0.7,1.3 or 2.0) of DNA between inverted ESs, and control DNAs with one and zero ESs (pCDAdelMEl, GB1). These plasmids were linearized by digestion with SacI and NcoI (Promega). T2.0 is 11.3 kb, T1.3 is 10.6 kb, T0.7 is 10 kb, delMEl is 9.2 kb and 0 ES is 9.4 kb. For the ES containing substrates, the dbb DNA length is 4.9 and 4.4 kb for the T2.0, 1.3 and 0.7 substrates and 4.6 kb for delMEl. The linearized plasmids were assayed for cleavage as described[10] by incubating Tnp with the DNA (8:1 Tnp:ES ratio) in DNA reaction buffer (20 mM Hepes (pH7.5), 100 mM K Glu, 10 mM Mg^{2+}) for 3 h at 37 °C. Reaction products were electrophoresed on a 1% (w/v) agarose gel; DNA was visualized with SYBR green (Molecular Probes) using a Typhoon 9410 (Amersham). For tethered single DNAs the linear fragments were then modified on either end with 1000 bp fragments containing digoxigenin-11- dUTP or biotin-16-dUTP (Roche) using a ligation reaction. 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.

Sample cell preparation

Sample cells were constructed as described.[18,19,35] The 40 μl sample chamber was incubated with 4 mg ml^{-1} BSA (NEB) in PBS for 10 min. Modified DNA plus magnetic beads was then flowed into the chamber in PBS and the digoxigenin end of the molecules was allowed to react 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.[19] Extension of the single molecule tethers was measured by calibrating a library of out-of-focus images of the bead in solution as described,[18] resulting in measurements with ~20 nm accuracy in extension and data acquisition rates of 15 Hz. Forces (±5%) were determined by measuring the transverse fluctuations of the magnetic bead.[18]

Experimental protocol

Single molecule tethers were located in the sample chamber and a series of force *versus* extension measurements were performed to ensure only one DNA is tethered between the glass side and the magnetic bead and to provide force calibration data. Then, ~50 nM Tnp was flowed into the sample chamber in DNA binding buffer (20 mM Hepes (pH 7.5), 100 mM K Glu). Tnp was bound to DNA that was relaxed (i.e. no applied force) for 20 min to allow the DNA to condense. Loop formation was assayed by increasing applied force (1–15 pN) and monitoring the resulting extension versus time data over 15(±5) min. Synapsis experiments were performed at 25(±3) °C, single molecule cleavage experiments were performed at 37(±2) °C, using an objective heater to heat the flow cell.

Data analysis

Extension *versus* time data was analyzed 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).

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