A new set of Mu DNA transposition intermediates: alternate pathways of target capture preceding strand transfer

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Mu DNA transposition occurs within the context of higher order nucleoprotein structures or transpososomes. We describe a new set of transpososomes in which Mu B-bound target DNA interacts non-covalently with previously characterized intermediates prior to the actual strand transfer. This interaction can occur at several points along the reaction pathway: with the LER, the Type 0 or the Type 1 complexes. The formation of these target capture complexes, which rapidly undergo the strand transfer chemistry, is the rate-limiting step in the overall reaction. These complexes provide alternate pathways to strand transfer, thereby maximizing transposition potential. This versatility is in contrast to other characterized transposons, which normally capture target DNA only at a single point in their respective reaction pathways.

Keywords: DNA transposition/strand transfer/target complexes/transpososomes

Introduction

Fundamental processes of DNA, including transcription, replication, recombination and transposition, are often mediated by higher order nucleoprotein structures (Echols, 1990; Grosschedl, 1995). These complexes involve multiple proteins, numerous DNA sites, a high level of co-operativity as well as DNA bending and/or wrapping. These features of assembly confer multiple layers of regulatory interactions as is evident in the study of Mu DNA transposition (see Craig, 1995; Grindley and Leschziner, 1995; Lavoie and Chaconas, 1995; Chaconas *et al.*, 1996; Craigie, 1996; Craig, 1997). The chemical steps of Mu DNA transposition are also found in other bacterial and eukaryotic transposons, including human immunodeficiency virus (HIV) and other retroviruses (Mizuuchi, 1992a,b; Polard and Chandler, 1995; Andrade and Skalka, 1996; Rice *et al.*, 1996). In Mu, this process is mediated by a series of higher order complexes, or transpososomes (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Mizuuchi *et al.*, 1992; Watson and Chaconas, 1996).

The earliest characterized complex, the LER, results from synopsis of the transpositional enhancer with the left and right Mu ends (Watson and Chaconas, 1996). Release of the enhancer and subsequent engagement of the Mu

ends by the transposase active site results in formation of the Type 0 transposome (stable synaptic complex), which is poised for the first chemical step (Mizuuchi *et al.*, 1992). Specific nicking at the 3'-terminal nucleotides results in the formation of the Type 1 transpososome (cleaved donor complex) (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987). This intermediate is subsequently processed in a single-step transesterification reaction (Mizuuchi and Adzuma, 1991) whereby the 3' hydroxyl termini of Mu become covalently linked to a target DNA. This reaction is greatly stimulated by Mu B and ATP. The product of this strand transfer is the very stable Type 2 transpososome (strand transfer complex). Before replication can occur to give the final transposition end products, the Type 2 complex must be dismantled by the action of the *Escherichia coli* ClpX chaperone along with other host factors (Levchenko *et al.*, 1995; Nakai and Kruklytis, 1995; Kruklytis *et al.*, 1996).

The Mu A protein (transposase) is responsible for the structural and functional integrity of the transpososome. Mu A binds as a monomer (Kuo *et al.*, 1991) to its six consensus sequences at the Mu ends, but its active form in the transpososome is a tetramer (Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1992). The 663 residue polypeptide can be divided into three domains by partial proteolysis (Nakayama *et al.*, 1987). Domain I (1–243) is comprised of distinct subdomains which are able to bind the enhancer and Mu end sites independently (Leung *et al.*, 1989; Mizuuchi and Mizuuchi, 1989). Domain II (243–574) comprises the core of the transposase, containing both a non-specific DNA-binding activity and the D-D-(35)-E metal ion-binding motif (Baker and Luo, 1994; Kim *et al.*, 1995). Domain III (575–663) contains a 26 residue region (575–600) which is believed to be involved in catalysis of donor DNA cleavage (Wu and Chaconas, 1995) and is also required for protein–protein interactions with Mu B (Baker *et al.*, 1991; Leung and Harshey, 1991; Wu and Chaconas, 1994).

The Mu B protein is also an important component of the transposition machinery. Mu B captures target DNA (Craigie and Mizuuchi, 1987; Maxwell *et al.*, 1987; Surette *et al.*, 1987; Mizuuchi and Mizuuchi, 1993), mediates transposition immunity (Adzuma and Mizuuchi, 1988, 1989) and is an allosteric activator of Mu A (Baker *et al.*, 1991; Surette and Chaconas, 1991). Mu B also stimulates the reaction with certain mutant substrates (Baker *et al.*, 1991; Surette and Chaconas, 1991; Surette *et al.*, 1991; Wu and Chaconas, 1992) or a transposase carrying an N-terminal deletion (Mizuuchi *et al.*, 1995). Furthermore, kinetic experiments involving pre-incubation of the Type 0 complex with Mu B, ATP and target DNA (Mizuuchi *et al.*, 1992) also demonstrated the ability of Mu B to interact with both the transpososome and target DNA at early stages of the reaction. These associations were found

Fig. 1. Formation of a Type 1 target capture (TC-1) complex. Type 1 complexes were pre-formed with either wild-type Mu A or Mu A₆₁₅, the latter of which is unable to interact with Mu B. Mg²⁺ was then chelated with EDTA, following which Mu B, ATP and supercoiled target DNA were added and allowed to incubate at 30°C for 20 min. Reactions were then cross-linked with DSP as noted and, where indicated, samples treated with SDS prior to agarose gel electrophoresis. The TC-1 band is labeled on the gel with a white arrow. The positions of supercoiled donor (SD), supercoiled target (ST), relaxed donor (RD) and relaxed target (RT) as well as the Type 2 and θ structure products of the strand transfer reaction are indicated. The band sometimes visible just above the supercoiled donor is unreacted substrate which is bound by proteins. BT is supercoiled target DNA which has been complexed with Mu B protein.

to accelerate both donor cleavage and strand transfer. However, complexes in which Mu B has recruited target DNA to the transpososome in a non-covalent association have not been directly observed.

Here we report the direct observation of a new set of transpososomes (target capture complexes) in which Mu B has recruited target DNA to the transpososome at various steps along the reaction pathway. These complexes provide alternate pathways for Mu DNA transposition. This versatility is in contrast to Tn7 and Tn10; each of these elements apparently capture target DNA at a defined point, before or after donor cleavage, respectively (Bainton *et al.*, 1991, 1993; Sakai and Kleckner, 1997).

Results

Identification of a Type 1 target capture complex

The Mu B protein is known to capture target DNA and recruit it to the Type 1 transpososome for the strand transfer reaction. However, Type 1 complexes containing Mu B and target have not been observed prior to the actual strand transfer. Expecting that such complexes might be inherently unstable or may dissociate during electrophoresis, we began a search for such complexes utilizing conditions which blocked strand transfer (the absence of Mg²⁺), coupled with the use of a protein cross-linking reagent to stabilize any such complexes. Using these conditions and an electrophoretic assay, we were able to observe a target capture (TC) complex (Figure 1, lane 7). Observation of the complex involved pre-forming

Fig. 2. Disruption of gradient-purified Type 1 target capture complex. TC-1 complexes were formed as described in the legend to Figure 1 and purified by sucrose gradient centrifugation as described in Materials and methods. Purified target complexes were then loaded for agarose gel electrophoresis in the absence or presence of SDS. Markers are labeled as in Figure 1.

a Type 1 transpososome, chelating Mg²⁺ with EDTA followed by addition of Mu B, target DNA and ATP and subsequent cross-linking with dithiobis(succinimidyl propionate) (DSP). The migration of the Type 1 target capture (TC-1) complex was substantially faster than that of the Type 2 transpososome, as expected for a complex in which the target DNA was supercoiled rather than relaxed. DSP was required to see the TC-1 (compare lanes 5 and 7), but the cross-linking reagent itself did not affect gel migration of the complexes as shown with a Type 1 control (lane 3 versus lane 1). Formation of the TC complex required Mu B, target DNA and ATP; however, ATP hydrolysis was apparently not required since ATP- γ S also supported TC-1 formation (data not shown). As expected, domain III β of Mu A (which interacts with Mu B) was also required (lane 9). Other cross-linking reagents (glutaraldehyde and SANPAH) were also tested but were found to be less effective than DSP in stabilizing the TC-1 complex (data not shown). Observation of TC complexes required some supercoiling in the target DNA (data not shown). Although formation of Type 2 complexes does not require a supercoiled target (Craigie *et al.*, 1985), the reaction is more efficient than when relaxed or linear targets are used (data not shown), and target supercoiling may stabilize the TC complexes.

The maximum yield of TC-1 complex, corresponding to >95% of the Type 1 complex, was found to occur after a 20 min incubation at 30°C. Assay conditions for TC-1 formation were identical to standard reaction conditions except that Mu B was doubled in concentration; this doubling resulted in the formation of approximately three times as much TC-1 as when standard amounts of Mu B were used (data not shown).

Fig. 3. Electron microscopy of the Type 1 target capture complex. Electron micrographs of the TC-1 complex are shown together with schematic illustrations. Digestion with restriction endonucleases *EcoRV* or *XhoI* followed purification via sucrose gradient centrifugation. Samples were mounted for electron microscopy using formamide spreads with cytochrome c and are shown in reverse contrast. In 58 complexes digested with *EcoRV*, the arm lengths were found to be 1305 ± 80 bp (1307 bp expected) and 2590 ± 145 bp (2559 expected). In 54 *XhoI*-digested complexes, the sum of the two target arms was found to be 5260 ± 175 bp, while the expected length was 5250 bp. Molecules with decreased levels of supercoiling are shown for ease of interpretation.

Characterization of the Type 1 target capture complex

TC-1 was purified by fractionation in a sucrose gradient as described in Materials and methods and analyzed via agarose gel electrophoresis (Figure 2, lane 1). To demonstrate that TC-1 was in fact composed of one molecule of donor DNA complexed non-covalently with one molecule of target DNA, the complex was disrupted in the presence of 1% SDS (Figure 2, lane 2). As expected, disruption of the complex liberated relaxed donor DNA, indicative of the Type 1 complex, and supercoiled target.

Densitometry of the ethidium bromide-stained gel yielded a molar donor to target ratio of 1 to 0.93.

TC-1 complexes were characterized further by electron microscopy (Figure 3). TC-1 complexes were mounted directly, or digested with either *EcoRV*, which cuts once in the Mu domain, or *XhoI*, which cuts once in the target DNA. The uncut complexes (Figure 3A) showed the expected three domains: a supercoiled Mu and relaxed vector, which together comprise the Type 1 complex, linked at the junction to a supercoiled target molecule. Complexes cut with *EcoRV* (Figure 3B) all displayed two

Fig. 4. Kinetics of conversion of Type 1 versus Type 1 target capture complex into strand transfer product (Type 2). Type 1 complexes were pre-formed as described in Materials and methods. In half of the reaction, TC-1 complexes were formed as described in the legend to Figure 1. The remaining Type 1 was incubated for the same time on ice. To initiate conversion of TC-1 complexes to Type 2, $MgCl_2$ was added to a final concentration of 10 mM while conversion of Type 1 to Type 2 was initiated by addition of Mu B, ATP, target DNA and 10 mM $MgCl_2$. Reactions were incubated at 20°C with time points taken as indicated. Reactions were stopped by addition of loading dye, either in the absence or presence of SDS. Densitometry of the ethidium bromide-stained gels was as described in Materials and methods.

Mu arms of correct length attached to the target and vector domains. Finally, cleavage with *XhoI* (Figure 3C) produced Type 1 complexes with two arms whose lengths varied from molecule to molecule due to attachment at different sites in the target; the sum of the lengths of the two arms always added up to the total length of the target DNA.

Conversion of Type 1 target capture complex to strand transfer product

To assess whether TC-1 could function as a legitimate reaction intermediate, we attempted to convert the TC-1 complex into the strand transfer product (Type 2) and compare the kinetics of conversion with that of the Type 1 complex. Type 1 complex was pre-formed and the reaction was divided into two equal parts. Target capture complex was formed with one aliquot by addition of Mu B, ATP and target DNA, while the remaining aliquot was incubated on ice for an equal length of time. Conversion into Type 2 was initiated by addition of Mg^{2+} to the pre-formed TC-1 or by addition of Mu B, ATP, target DNA and Mg^{2+} to the Type 1 complex. As shown in Figure 4, there was a slow conversion of Type 1 into Type 2 product. In sharp contrast, however, was the very rapid conversion of TC-1 into the Type 2 product. Addition of Mg^{2+} to TC-1 resulted in the strand transfer step being completed in <1 min. Although the extent of reaction with TC-1 plateaued at ~45% of the input donor DNA, this corresponded to conversion of >95% of pre-formed TC-1 into Type 2 complex. Hence TC-1 can serve as an efficient reaction intermediate.

Fig. 5. Analysis of Type 0 target capture (TC-0) and LER target capture (TC-LER) complexes. Type 0 complexes were pre-formed using the 6.5 kb substrate pBL08 as described in Materials and methods. TC-0 was formed by addition of Mu B, ATP and target DNA to the pre-formed Type 0 followed by a 20 min incubation at 30°C. TC-LER was formed with the 7.2 kb mutant substrate pMS9A1 by incubating donor DNA with Mu B, ATP and target DNA for 10 min at 30°C in the presence of 10 mM $CaCl_2$. Both reactions were cross-linked with DSP and purified via sucrose gradient centrifugation. Purified target complexes were loaded for agarose gel electrophoresis in the absence or presence of SDS. The migration position of TC-1 with pBL08 is also indicated.

Identification and analysis of LER and Type 0 target capture complexes

Since the Mu B protein can influence donor cleavage and interact with early transposition intermediates (Baker *et al.*, 1991; Surette and Chaconas, 1991; Surette *et al.*, 1991; Wu and Chaconas, 1992; Mizuuchi *et al.*, 1995), we tested whether TC complexes could be formed using Type 0 and LER complexes. Mu B, ATP and target DNA were added to pre-formed Type 0 or LER complexes and incubated at 30°C for 20 min; reactions were then cross-linked with DSP and analyzed for the appearance of new SDS-sensitive bands. TC complexes were generated efficiently for both Type 0 (TC-0) and LER (TC-LER). For studies using the LER reaction intermediate, terminal base pair mutants, which cannot proceed to the Type 0 complex, were used (Watson and Chaconas, 1996). Biochemical and electron microscopic analysis of the TC-LER complexes confirmed that they were not converted into TC-0 complexes (data not shown). The TC-LER and TC-0 complexes were subsequently purified by sucrose gradient centrifugation and analyzed by agarose gel electrophoresis as shown in Figure 5. As expected, these fully supercoiled complexes displayed an increased electrophoretic mobility compared with TC-1, which has a relaxed vector domain. Disruption of TC-0 and TC-LER with SDS liberated only supercoiled donor and supercoiled target DNA in molar ratios of 1.00–0.99 and 1.00–0.96 respectively.

To determine whether these complexes had the properties of bona fide transposition intermediates, chase experiments similar to those performed for TC-1 (Figure 4) were carried out on both TC-0 and TC-LER (Figure 6).

Fig. 6. Kinetics of conversion of TC-0 and TC-LER into Type 2. (A) Type 0 complexes were pre-formed and half of the reaction was converted to TC-0 by the addition of Mu B, ATP and target DNA with a 20 min incubation at 30°C. The remaining Type 0 was incubated on ice for 20 min. For conversion of TC-0 to Type 2, MgCl₂ was added to a final concentration of 10 mM. To initiate conversion of Type 0 to Type 2, Mu B, ATP, target DNA and 10 mM MgCl₂ were added. Reactions were incubated at 20°C with time points taken as indicated, and stopped by addition of loading dye. (B) TC-LER complexes were pre-formed in the presence of 10 mM CaCl₂ using the terminal base pair mutant substrate pMS9A1. Conversion of TC-LER to Type 2 was initiated by addition of 10 mM MgCl₂ and incubation at 20°C with time points taken as indicated. LER complexes on the mutant substrate were pre-formed in the presence of 10 mM CaCl₂. Conversion to Type 2 was induced by addition of Mu B, ATP, target DNA and 10 mM MgCl₂ at 20°C.

Conversion of TC-0 to the strand transfer product was stimulated efficiently by addition of MgCl₂. Following a 30 s incubation at room temperature, the strand transfer reaction had reached a plateau, with 65% of the total input DNA converted into Type 2 complex (Figure 6A); this corresponded to >95% of the pre-formed TC-0. However, in the Type 0 chase, a much slower conversion was observed. Efficient conversion of Type 0 to Type 2 transpososomes requires a longer incubation (30 min) at higher temperature (30°C).

To perform a similar experiment with the LER complexes, it was necessary to use a substrate which accumulates LER but has a reversible block for Type 0 complex formation. We have previously observed that substrates with a terminal base pair mutation at one end fulfil these criteria (Watson and Chaconas, 1996). Moreover, the Mu B protein in the presence of Mg²⁺ can overcome the block in the reaction so that the substrate undergoes donor cleavage and strand transfer, albeit at reduced levels. Using a plasmid with a terminal base pair change at the

left end, we generated LER in Ca²⁺ and further incubated LER with Mu B, ATP and target DNA to form the TC-LER complex. Conversion of TC-LER to Type 2 was stimulated by addition of MgCl₂, in marked contrast to the LER (Figure 6B). The LER was completely unreactive using these suboptimal conditions, i.e. the terminal base pair mutant incubated at room temperature for only 15 min. Under standard reaction conditions (30°C for 30 min), substantial conversion of the LER with a terminal base pair mutant was observed (data not shown). The experiments presented in Figure 6 indicate that TC-0 and TC-LER display the properties expected of reaction intermediates.

Discussion

Multiple target capture complexes in Mu transposition

We have demonstrated the presence of a previously unobserved set of Mu transposition intermediates in which the transpososome has recruited Mu B and target DNA prior to undergoing the chemical step of strand transfer. Observation of TC complexes required Mu B, ATP and some supercoiling in the target DNA. Transpososomes assembled using a truncated transposase lacking domain III β were unable to form TC complex, indicating that interactions between Mu A and Mu B were required for complex formation. TC complexes could only be detected in our electrophoretic assay by stabilization with a protein cross-linking reagent. Subsequent studies on the stability of the target capture complexes revealed half-lives ranging from 1 to 11 min, depending upon the experimental approach used (data not shown).

The *in vitro* reaction can be divided into two distinct chemical steps: donor cleavage and strand transfer. The chemical steps occur almost instantaneously in both donor cleavage (Mizuuchi *et al.*, 1992; Wang *et al.*, 1996) and strand transfer (see Figures 4 and 6) once the appropriate transpososomes, which mediate the reaction, are formed. Assembly of the transpososomes, however, is a much slower process: 3–5 min are required to generate complexes for donor cleavage and 20–30 min are necessary for the formation of complexes for strand transfer (TC complexes). The overall rate-limiting step of the reaction is therefore the assembly of TC complexes.

Alternate pathways

TC complexes could be formed with the three-site synaptic complex (TC-LER), the Type 0 complex (TC-0) or the Type 1 complex (TC-1), as shown in Figure 7. Formation of TC-0 was somewhat more efficient than that of TC-1 (65% of input donor versus 45%; see Figures 4 and 6A). Once formed, however, the kinetics and extent (>95%) of conversion into strand transfer product was virtually identical for both TC-0 and TC-1. These results suggest the possibility that target capture may occur preferentially prior to donor cleavage. Comparison of our TC-0 and TC-1 data with that from the TC-LER was not possible, since a partially disabled donor substrate was required to accumulate the TC-LER.

The presence of multiple Mu TC complexes is in marked contrast to other characterized transposition systems in which target capture occurs at a specific stage in the reaction pathway. In Tn7, recognition and capture of target

Fig. 7. Alternate pathways for the Mu DNA strand transfer reaction. The three target capture complexes are shown in the overall pathway for the strand transfer reaction. In each of these complexes, the target DNA is non-covalently associated with the transpososome core.

DNA precedes the donor cleavage reaction (Bainton *et al.*, 1991, 1993). Formation of a complex containing donor DNA, target DNA and all the required transposition proteins (TnsA, TnsB, TnsC and TnsD or TnsE) is the primary regulatory mechanism, decreasing the likelihood of aberrant or inappropriate recombination events. In Mu transposition, other types of regulatory mechanisms are in place which circumvent the need for such an early target complex. These include the requirement for an enhancer (Mizuuchi *et al.*, 1995; Yang *et al.*, 1995a; Watson and Chaconas, 1996), the 'interwoven architecture' of the transposase tetramer (Yang *et al.*, 1995b, 1996; Aldaz *et al.*, 1996) and cleavage *in trans* (Aldaz *et al.*, 1996; Savilahti and Mizuuchi, 1996).

Conversely, in *Tn10*, target capture seems to occur at a later stage of the reaction (following double end donor DNA cleavage) (Sakai and Kleckner, 1997). This limiting feature may result from the presence of a DNA-binding pocket in the transposase, which is capable of interacting with either flanking or target DNA, but not with both simultaneously. In contrast, the Mu transposase is not subject to this constraint. Because the flanking sequences are never completely severed from the Mu DNA, the transposase must utilize separate sites for interaction with the vector and target sequences. Interactions with target DNA are therefore not dependent upon donor DNA cleavage.

Alternate pathways for target capture in Mu DNA transposition can exist because of the absence of the transposition restraints imposed upon *Tn7* and *Tn10*. This, in turn, may confer a more dynamic phenotype on Mu as a transposon, allowing for greater versatility in effective completion of *in vitro* DNA transposition, as noted below.

Mechanistic implications

TC complexes are ternary structures in which target DNA has been positioned to facilitate rapid strand transfer in the active site of the transpososome. The non-covalent association between the transpososome and target DNA

is probably stabilized by interactions between Mu B and target DNA, between Mu A and Mu B, and also between Mu A and target DNA (Castilho and Casadaban, 1991; Mizuuchi and Mizuuchi, 1993). The relative strengths of these different interactions and their contributions to stabilizing the TC complexes is not currently known.

As noted earlier, the formation of TC complexes is the rate-limiting step in the overall reaction. We attempted to localize the bottleneck further by asking whether pre-incubation of Mu B with target DNA would accelerate the formation of TC complexes. We found that pre-binding of Mu B to the target actually reduced the rate of reaction, suggesting that TC complex formation uses a concerted rather than a stepwise mechanism (data not shown).

These findings have important implications for the process of target immunity whereby self-integration of Mu DNA does not occur (Adzuma and Mizuuchi, 1988, 1991). Target immunity results from a preferential association of Mu B with DNA that does not carry Mu sequences. Targets carrying Mu DNA give rise to Mu A–Mu B interactions which stimulate ATP hydrolysis and release of the Mu target by Mu B. Our results suggest that establishment of target immunity may occur in a concerted fashion concomitant with transpososome assembly, rather than as an independent step prior to synapsis. Based on this hypothesis, we expect that immunity normally occurs prior to donor cleavage at the Type 0 or LER stage. Furthermore, the early establishment of target immunity through the concerted formation of TC complexes allows for replacement of unsuitable targets at any of several stages in the reaction (LER, Type 0 or Type 1). This flexibility would be an essential feature for efficient completion of transposition, when one or more immune targets are encountered. Inclusion of the Mu B protein into the transpososome at an early stage is also suggested by the data of Levchenko *et al.* (1997), which suggests that Mu B may be required to block the ClpX-mediated disassembly of early transposition intermediates.

Finally, the association of donor and target DNA prior to the first chemical step sets up a highly concerted reaction where sequential completion of both donor cleavage and strand transfer can occur in rapid succession. Such a concerted mechanism would minimize the accumulation of partial reaction products and bias the reaction towards completion. We believe this type of concerted mechanism to mirror closely the *in vivo* properties of Mu under most circumstances.

The TC complexes described here offer new insights into association of Mu B and target DNA with early reaction intermediates, and should prove fertile ground for further experimentation into the intricacies of the DNA transposition process.

Materials and methods

DNA substrates, proteins and reagents

The mini-Mu plasmid pBL08 is a derivative of pBL03 (Lavoie *et al.*, 1991) with the addition of the pUC19 polylinker outside the Mu right end, an additional *Syl* site inside the Mu right end and the *SspI* site in Mu replaced by an *XhoI* site. The target DNA used was the 5.2 kb plasmid pSD7 (Surette and Chaconas, 1991).

Proteins were purified as described previously: Mu A (Baker *et al.*, 1993), Mu B (Chaconas *et al.*, 1985), HU (Lavoie and Chaconas, 1993) and IHF (Surette *et al.*, 1989). DSP was obtained from Pierce.

Target complex formation

Concentrations of proteins and DNA in all reactions were 2.5× relative to standard reaction conditions, with the exception of Mu B which was at 5×. A standard (1×) *in vitro* Type 1 reaction contains CsCl-purified supercoiled mini-Mu plasmid (15 µg/ml), Mu A protein (3 µg/ml), HU (3.75 µg/ml), IHF (0.2 µg/ml), 10 mM MgCl₂, 25 mM HEPES-NaOH, pH 7.6 and 100 mM NaCl. Reactions were incubated at 30°C for 5 min. For Type 1 target capture complexes (TC-1), EDTA was added (10 mM), followed by ATP (2 mM), pSD7 (20 µg/ml) and Mu B (5 µg/ml), and allowed to incubate at 30°C for 20 min. DSP (freshly prepared) was then added to the *in vitro* reactions at a final concentration of 225 µg/ml, allowed to incubate at room temperature for 15 min and quenched with a mixture of 50 mM Tris-HCl pH 7.8 and 10 mM lysine for 15 min at room temperature. Following quenching, reactions were split into two, with and without 1% SDS.

For formation of TC-0, Type 0 complexes were pre-formed similarly to Type 1, with the exceptions that 10 mM CaCl₂ was used in place of 10 mM MgCl₂, and the incubation at 30°C was allowed to proceed for 30 min. Mu B, ATP and pSD7 were then added and incubated at 30°C for 20 min. TC-0 complexes were then cross-linked, quenched and split into two aliquots as described above.

For the TC-LER, LER complexes were initially formed as described previously (Watson and Chaconas, 1996) with the exceptions that pMS9A1 (Surette *et al.*, 1991) was used as donor substrate in place of pBL08, and 10 mM CaCl₂ was substituted for 10 mM MgCl₂. Mu B, ATP and target DNA were added from the onset of the reaction and allowed to incubate at 30°C for 10 min, following which reactions were cross-linked with DSP and quenched as described above.

Electrophoresis and DNA quantification

Electrophoresis in TAE buffer was in 1.0% agarose horizontal slab gels (200 mm in length) run at 4 V/cm for 6 h at room temperature. Loading dye consisted of 10 mM Tris pH 7.8, 1 mM EDTA, 5% sucrose and 0.08% bromophenol blue, in the presence or absence of 1% SDS. DNA was visualized by staining gels with ethidium bromide (0.5 µg/ml) for 30 min, followed by destaining in H₂O for 30 min. Gel documentation, densitometry and quantitation used a CCD camera setup and Gel Print Tools (version 3) software from BioPhotonics Corp.

Electron microscopy

Sucrose gradient centrifugation was utilized to partially purify TC complexes from other species. Seventy five µl reactions (10× relative to standard) were cross-linked, quenched and fractionated in a 10–30% sucrose gradient which was spun at 45 000 r.p.m. for 3 h. Fractions partially enriched for TCs, as determined by agarose gel electrophoresis, were mounted for electron microscopy by the basic protein film technique using cytochrome c and formamide as described previously (Lavoie and Chaconas, 1990), with the exception that 30% formamide was used in the hyperphase rather than 40%. DNA length was measured from micrographs using a Summagraphics digitizing tablet (MM960 Series) and Geocomp Easydj v5.6 software. The base pair length of DNA arms in digested complexes was estimated using uncomplexed full-length linear DNA molecules as standards, since it was generally not possible to measure the entire length of the DNA in complexes retaining supercoiling.

Conversion experiments

Seventy five µl Type 1 or Type 0 reactions were performed at 10× relative to standard reaction conditions. Reactions were then split in two, with one half containing Type 1 or Type 0 complexes, while EDTA, Mu B, ATP and target DNA were added to form either TC-1 or TC-0 in the other half, as described above. Reactions were diluted to 5× and conversion to Type 2 product was stimulated by addition of 10 mM MgCl₂ to the pre-formed target capture complexes, or by the addition of Mu B, ATP, pSD7 and 10 mM MgCl₂ to the Type 1 or Type 0 complexes. Reactions were incubated at 20°C for the times indicated and stopped by immediate addition of loading dye either in the absence or presence of SDS.

TC-LER complexes were pre-formed by incubating 75 µl reactions (5× relative to standard reaction conditions) containing Mu B, ATP and pSD7 in the presence of 10 mM CaCl₂ on the mutant substrate pMS9A1 at 30°C for 10 min. LER complexes were also pre-formed on pMS9A1 with a 10 min incubation at 30°C in 10 mM CaCl₂. Conversion to the Type 2 product was stimulated by addition 10 mM MgCl₂ to TC-LER, or by addition of Mu B, ATP, pSD7 and 10 mM MgCl₂ to LER. Reactions were incubated at 20°C for the times indicated, and stopped by immediate addition of loading dye.

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