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Factors responsible for target site selection in Tn*10* transposition: a role for the DDE motif in target DNA capture

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Tn*10*, like several other transposons, exhibits a marked preference for integration into particular target sequences. Such sequences are referred to as integration hotspots and have been used to define a consensus target site in Tn*10* transposition. We demonstrate that a Tn*10* hotspot called HisG1, which was identified originally *in vivo*, also functions as an integration hotspot *in vitro* in a reaction where the HisG1 sequence is present on a short DNA oligomer. We use this *in vitro* system to define factors which are important for the capture of the HisG1 target site. We demonstrate that although divalent metal ions are not essential for HisG1 target capture, they greatly facilitate capture of a mutated HisG1 site. Analysis of catalytic transposase mutants further demonstrates that the DDE motif plays a critical role in 'divalent metal ion-dependent' target capture. Analysis of two other classes of transposase mutants, Exc$^+$Int$^-$ (which carry out transposon excision but not integration) and ATS (altered target specificity), demonstrates that while a particular ATS transposase binds HisG1 mutants better than wild-type transposase, Exc$^+$Int$^-$ mutants are defective in HisG1 capture, further defining the properties of these classes of mutants. Possible mechanisms for the above observations are considered.

Keywords: DDE motif/DNA transposition/protein–DNA interactions/target DNA

Introduction

Transposons are mobile genetic elements which have important effects on genome structure and gene expression in a large variety of organisms. The specific consequences of transposition depend on where in the genome and by what means the transposon integrates (Arber, 1990). Many transposons and insertion sequences transpose preferentially into a specific sequence, while others exhibit regional preferences which may reflect structural features of the target induced by unusual base composition, DNA replication and/or transcription (reviewed in Sandmeyer *et al.*, 1990; Craig, 1997). The molecular basis for target site selection is not well understood and remains an important question in transposition. The bacterial transposon Tn*10* provides a good model system for studying target site selection because target capture (the non-covalent association between excised transpososome and target DNA) can be analyzed *in vitro* independently of donor cleavage and strand transfer reactions (Bolland and Kleckner, 1996), and the DNA sequence requirements for establishing a good target site are relatively simple (Bender and Kleckner, 1992a).

The basic steps in Tn*10* transposition are shown in Figure 1A (reviewed in Kleckner *et al.*, 1995). The pairing of two transposon ends (paired ends complex formation; or PEC for short), which is a critical early step in Tn*10* transposition, is not dependent on a divalent metal ion; however, all of the chemical steps require Mg^{2+} (Chalmers and Kleckner, 1994; Sakai *et al.*, 1995). Similar to many other transposases and retroviral integrases (Kulkosky *et al.*, 1992; Rezsohazy *et al.*, 1993; Polard and Chandler, 1995), IS10 transposase contains three acidic amino acid residues (DDE motif) which are critical for catalysis. Mutations at D97, D161 and E292 block all of the chemical steps in Tn*10* transposition without influencing PEC formation. The fact that each of the above amino acids is critical for steps in donor cleavage and strand transfer supports the idea that transposase contains a single active site (Bolland and Kleckner, 1996; Kennedy and Haniford, 1996). Evidence that D97, D161 and E292 function in catalysis by binding a divalent metal ion(s) comes from metal ion substitution experiments with mutant proteins; when the aspartic acid at either position 97 or 161, or the glutamic acid at position 292, is mutated to cysteine, the thiophilic divalent metal ion Mn^{2+} rescues the cleavage and strand transfer defects (J.Allingham and D.Haniford, unpublished observations). Similar experiments with the Mu and Tn*7* transposase proteins have been performed and are in agreement with the role of the 'DDE' motif in transposase proteins being one of divalent metal ion binding (T.Baker, personal communication; Sarnovsky *et al.*, 1996).

In addition to their role in catalysis, there is evidence that divalent metal ions also function in the target capture step in Tn*10* transposition. The most direct evidence to support this comes from the finding that Ca^{2+} stabilizes a target capture complex formed with a supercoiled plasmid DNA (Sakai and Kleckner, 1997). In addition, changing the divalent metal ion from Mg^{2+} to Mn^{2+} results in a relaxed target specificity (Junop and Haniford, 1996). Given the role of DDE residues in metal ion binding and the effect that divalent metal ions have on the integration reaction, it is possible that the DDE motif is involved in target capture in addition to its role in catalysis. Such a dual function has not been shown previously in any other transposase or retroviral integrase protein.

Although Tn*10* can insert into a wide variety of target sites, it exhibits a strong preference for a specific target sequence. This sequence, 5′ NGCTNAGCN 3′, defines the Tn*10* consensus target, and includes six consensus base pairs which comprise an interrupted 3 bp inverted

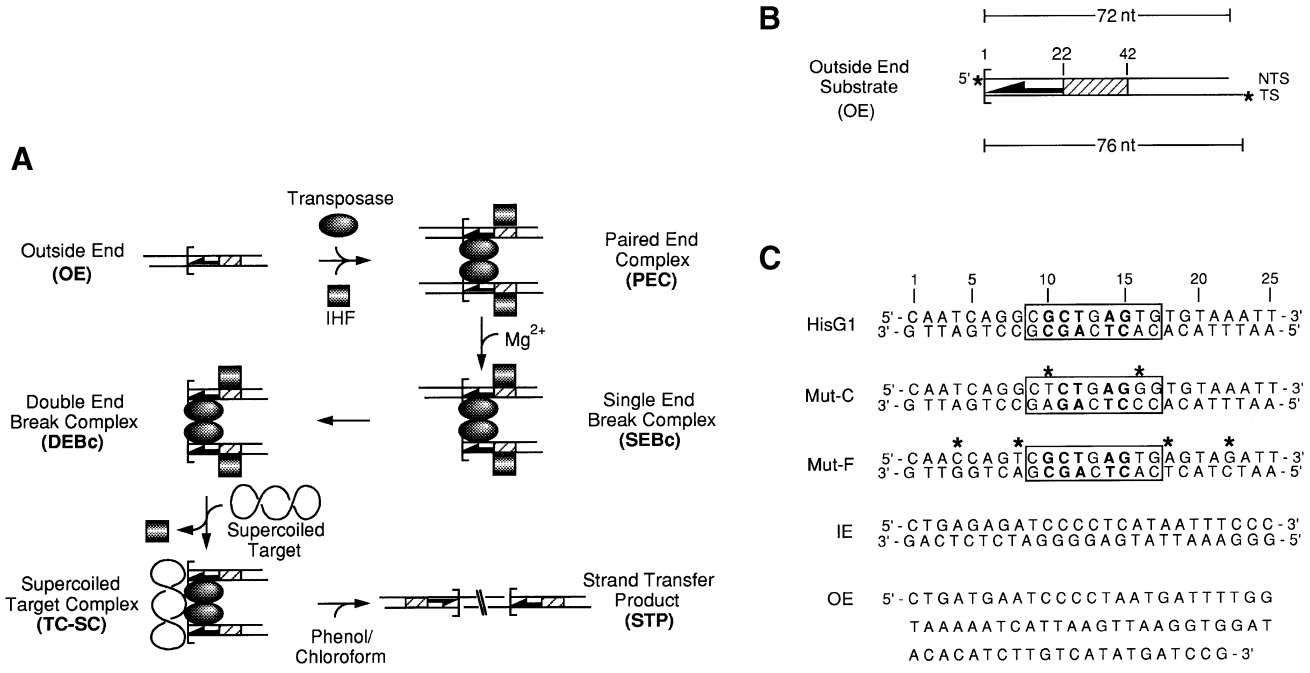

Fig. 1. Tn*10* transposition with a linear outside end substrate. (**A**) Reaction intermediates obtained *in vitro* using a linear outside end (OE) substrate and supercoiled target DNA are shown (Sakai *et al.*, 1995). Transposase and IHF are represented by shaded ovals and squares, respectively. Transposase- and IHF-binding sites in the OE substrate are represented by the half arrow and striped box, respectively. It is not yet known how many molecules of transposase are present in each complex, but for simplicity we show a dimer. (**B**) The pre-cleaved OE fragment and (**C**) the 25 bp target oligomers used as substrates in the reactions presented in this work are shown. NTS (non-transferred strand) and TS (transferred strand) are 5′-end labeled with ^{32}P (*). The HisG1 target is a known hotspot for Tn*10* integration. It contains a 9 bp core (box) which differs from the Tn*10* target consensus by only a single base pair. Matches to the perfect consensus are indicated in bold. Target oligomers Mut-C (consensus) and Mut-F (flanking) differ from HisG1 at positions marked by asterisks. The IE target, which consists of the terminal 25 bp of the inside end of IS10 Right, does not contain a sequence that resembles the Tn*10* target consensus. The sequence of the pre-cleaved OE fragment (one strand) is also shown.

repeat (Halling and Kleckner, 1982). Genetic analysis of one particular hotspot for Tn*10* integration, the HisG1 site, has demonstrated that mutations in either the consensus or flanking sequences can reduce the integration frequency into this site. Specifically, mutations in the most terminal positions of the consensus sequence and within the adjacent 10 bp of flanking DNA on either side of the consensus have strong inhibitory effects *in vivo* (Bender and Kleckner, 1992a). While it is clear that both the consensus and flanking sequences contribute to the integration frequency into a site, the specific role(s) of these sequences has not been determined with respect to target capture and/or catalysis of strand transfer.

Experiments by Sakai and Kleckner (1997) with supercoiled target plasmids suggest the existence of two distinct forms of the target capture complex. One form is observed very rapidly after target addition to double end break complex (DEBc), and is sensitive to challenge with competitor DNA. The second form takes longer to form and is resistant to challenge with competitor DNA. The differences in rates of formation and stability may reflect differences in the target DNA sequences which are engaged; the fast rate of formation and low degree of stability are consistent with a target complex in which transposase engages DNA in a sequence-independent manner, while the slow rate of formation and high degree of stability are consistent with a target complex in which transposase engages a target site resembling the Tn*10* consensus . This interpretation is supported by experiments which show that pre-incubation of DEBc with a target DNA significantly enhances the rate of strand transfer, as is expected if tracking to a productive target sequence is the rate-limiting step in strand transfer. The formation of a stable target capture complex is expected to be critical for strand transfer for kinetic and/or thermodynamic reasons.

Although efficient target capture and strand transfer can be achieved with plasmid targets, the presence of multiple target sequences in any given plasmid makes it difficult to identify the factors that are important for target site selection in a systematic manner. In the work presented here, we use short DNA oligomers (25 bp) of defined sequence as targets. We establish that target capture in this system is highly dependent on target DNA sequence and that the target capture complex formed with the HisG1 oligomer is a viable transposition intermediate. The development of this assay has permitted us to identify factors involved in target site selection and to further characterize the classes of transposase mutants which might be affected in this process.

Results

Experimental system

Tn*10* transposition can be carried out *in vitro* using a short DNA fragment which contains the terminal segment (70 bp) of the outside end of IS10 Right (Figure 1B) (Sakai *et al.*, 1995). This fragment, designated outside end substrate (OE), contains binding sites for transposase and the host protein, IHF. Flanking donor DNA has been removed from OE substrate by prior digestion with the restriction endonuclease *Pvu*II. *Pvu*II cuts precisely at the transposon–donor junction, leaving the same blunt end as

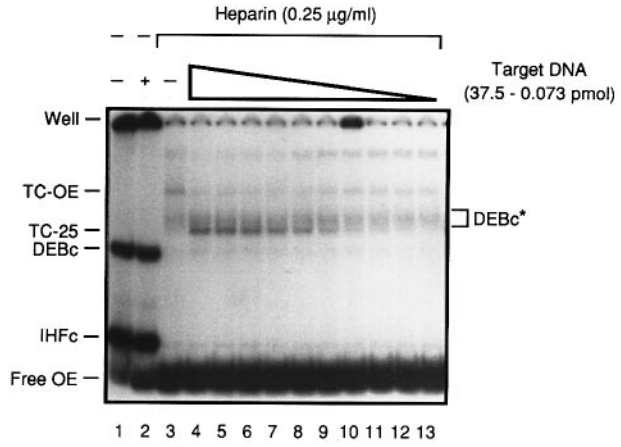

Fig. 2. Heparin treatment of DEBc facilitates capture of the HisG1 target oligomer. A mobility shift assay was used to detect the formation of a non-covalent target capture complex, TC-25, which contains DEBc and HisG1 oligomer. Briefly, the double end break complex (DEBc) was first assembled by mixing ^{32}P-labeled OE substrate with IHF and then transposase. After 2 h at 20°C the reaction mix containing DEBc was divided into equivalent aliquots for the treatments described below. DEBc was or was not treated for 20 min with heparin as indicated and then, where indicated, target oligomer (HisG1) was added and incubation was continued for another 6 h at 20°C. The amount of target added is indicated, except in lane 2 where it was 37.5 pmol. Reactions were mixed with loading dye and applied to a 5% native polyacrylamide gel. Species present include TC-25, outside end target complex (TC-OE), DEBc, DEBc without IHF (DEBc*) which usually runs as a diffuse band, integration host factor complex (IHFc), unreacted OE substrate (Free OE) and high molecular weight material which gets stuck in the wells. The species migrating between DEBc and IHFc has not been characterized. An autoradiograph is shown.

transposase (Benjamin and Kleckner, 1992). In the work described here, OE substrate is used to form DEBc, a cleavage intermediate in Tn*10* transposition. We subsequently use the DEBc to analyze the way in which transposase engages target DNA by examining target capture under a variety of conditions. The use of pre-cleaved OE substrate permits the direct study of target capture since the donor cleavage step, which is necessary for target capture (Sakai and Kleckner, 1997), has been by-passed. In addition, effects of divalent metal ions can be evaluated specifically on target capture since divalent metal ions are not required for DEBc formation when the pre-cleaved OE substrate is used (Bolland and Kleckner, 1996; Kennedy and Haniford, 1996). Performing reactions in the absence of a suitable divalent metal ion for catalysis and/or with transposase mutants that do not carry out strand transfer efficiently also allows us to distinguish target capture complexes from strand transfer complexes in which transposon and target sequences are covalently linked. Experimentally, DEBc is assembled by first mixing ^{32}P-end-labeled OE substrate with IHF and then transposase. Following the addition of a 25 bp target oligomer (Figure 1C), we assess target capture using a gel mobility shift assay in which the mobility of DEBc decreases if it makes a stable interaction with the target.

Effect of heparin on target capture

The results in Figure 2 (lanes 4–9) show that a target capture complex is made with the HisG1 oligomer when the DEBc is treated with heparin prior to target addition; the 25 bp HisG1 oligomer contains the well characterized HisG1 hotspot for Tn*10* insertion (Halling and Kleckner, 1982; Bender and Kleckner, 1992a). We call the resulting target capture complex, TC-25. The formation of TC-25 is not dependent on the presence of a divalent metal ion in the reaction buffer. In the reaction in lane 4, the HisG1 target oligomer is in 1000-fold molar excess relative to DEBc; at <30-fold excess, HisG1 becomes limiting for TC-25 formation. In the experiment presented, ~5% of DEBc is converted to TC-25 (lane 4); however, it is possible to get ~50% conversion under the same reaction conditions (see Figure 4).

Addition of heparin is critical for efficient TC-25 formation (compare lanes 2 and 4 of Figure 2). The role of heparin is to strip IHF from the DEBc. Sakai (1996) has shown previously that target complex formation requires removal of IHF from the DEBc. Furthermore, we have found that treatment of the DEBc with heparin alters its electrophoretic mobility in a way that is consistent with IHF loss; this is best seen in reactions where target oligomer is omitted (lane 3) or a sub-optimal amount of target is used (lanes 10–13). The observed reduction in mobility can be explained by an unfolding of the DNA within the DEBc so that the overall volume of the complex increases. IHF is known to be a DNA-bending protein which facilitates the compaction of many different higher order protein–DNA complexes (Nash, 1996). A similar reduction in electrophoretic mobility has been noted upon removal of IHF from the paired ends complex (Sakai, 1996). We call the IHF-stripped form of the DEBc, DEBc*. Direct evidence that DEBc* is in fact a form of DEBc, as opposed to being material freed up from the well, comes from an experiment where DEBc is eluted from a gel slice, treated with heparin and then examined on a second gel; essentially all of the DEBc (>95%) is converted to DEBc*. In addition, upon adding IHF back to gel-purified DEBc*, complete conversion of DEBc* to DEBc is observed, indicating that only IHF and not transposase is removed from the DEBc by heparin treatment (data not shown).

The amount of heparin required for optimal TC-25 formation causes a substantial amount of DEBc* to fall apart, presumably accounting for the upper limit of target capture being ~50% of the starting DEBc level. The presence of target appears to stabilize DEBc* as yields of TC-25 can be moderately increased if target is present in the reaction prior to heparin addition (data not shown). TC-25 is an extremely stable complex as it withstands incubation at 60°C for 30 min, and challenge with competitor DNA (data not shown).

Another target capture complex (TC-OE) which consists of DEBc* bound to free OE substrate is observed in Figure 2. This species is formed at highest levels in either the absence of target oligomer (Figure 2, lane 3) or when a target oligomer which does not contain a good hotspot for Tn*10* integration is used (Figure 4A, lane 3–5). The identity of this species has been confirmed by gel purifying TC-OE formed in the presence of Mg^{2+} and characterizing strand transfer events (which occur at a very low frequency) in this complex, as described in Figure 3 (data not shown). Unlike TC-25, TC-OE is sensitive to challenge with competitor DNA (data not shown).

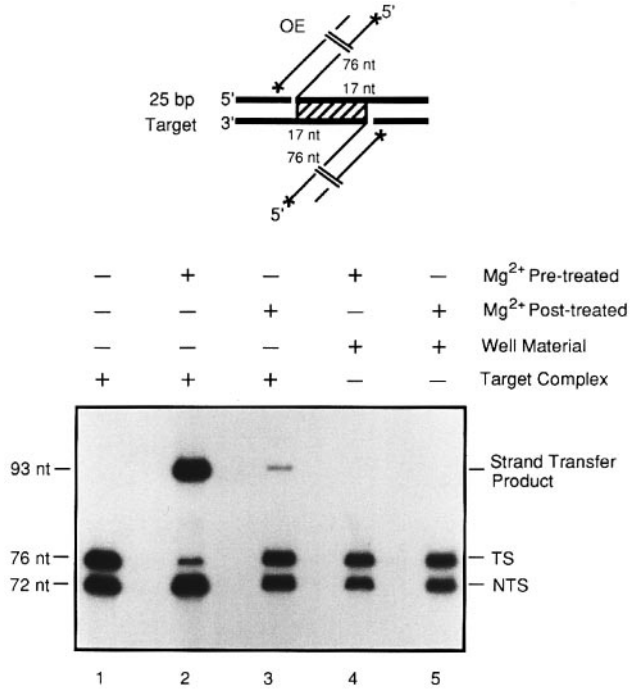

Fig. 3. Conversion of TC-25 into a single strand transfer product in the presence of Mg^{2+}. TC-25 was formed with HisG1 oligomer (37.5 pmol) as described in Figure 2. The formation of strand transfer product was then assayed under various conditions by loading samples on an 8% denaturing polyacrylamide gel. Whole reactions were either treated (lane 2) or not treated (lane 1) with Mg^{2+}. Target capture complex formed in the absence of Mg^{2+} was eluted from a gel slice and then treated with Mg^{2+} (lane 3). Well material formed in the presence and absence of Mg^{2+} (lanes 4 and 5, respectively) was eluted from gel slices and the latter was treated with Mg^{2+}. All samples were phenol/chloroform extracted before gel electrophoresis. The illustration shows the size (93 nucleotides) of the predicted strand transfer species that would be formed upon insertion into the HisG1 hotspot (thin lines, transposon DNA; thick lines, target DNA; hatched box, HisG1 hotspot; asterisk, ^{32}P label). TS and NTS are transferred (76 nucleotides) and non-transferred (72 nucleotide) strands, respectively. A Sanger sequencing reaction was used as a size standard (not shown).

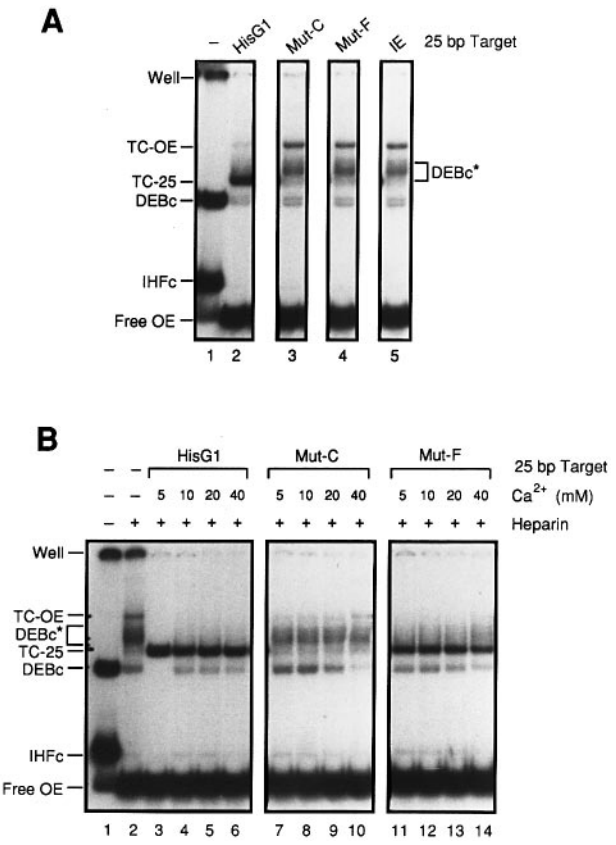

Fig. 4. TC-25 formation with HisG1 and HisG1 variants in the presence and absence of Ca^{2+}. (**A**) DEBc was assembled as in Figure 2, treated with heparin and incubated with the indicated target oligomers (37.5 pmol). No divalent metal ion was added. The appearance of a doublet at the position of DEBc may be the result of incomplete titration of IHF in this experiment. The reaction in lane 1 was not treated with heparin and did not receive target. In (**B**), Ca^{2+} was added to reactions immediately after target at the indicated concentrations. Ca^{2+} does not support strand transfer in Tn*10* transposition so only non-covalent target complexes are formed. Heparin was added to the reaction in lane 2 but both target and Ca^{2+} were omitted.

Analysis of TC-25

Evidence that TC-25 is in fact a target complex comprised of DEBc* and HisG1 target sequence comes from the analysis of strand transfer products formed in the presence of Mg^{2+} (Figure 3). When a standard reaction is performed in the presence of Mg^{2+}, >90% of DEBc* within the target complex is covalently joined to the HisG1 hotspot present in the HisG1 target oligomer. This is indicated by the disappearance of the 76 nucleotide species (transferred strand) and the appearance of a 93 nucleotide species (strand transfer product) on a denaturing gel (lane 2). Furthermore, when a gel slice containing TC-25, formed in the absence of Mg^{2+}, is incubated with Mg^{2+}, the 93 nucleotide species is observed (lane 3). The low level of strand transfer observed in the gel slice relative to that observed in solution (lane 2) may be a result of inhibitors present in the gel. No strand transfer product arises from material stuck in the wells (lanes 4 and 5), supporting the conclusions that strand transfer product, formed in the reaction in lane 2, arises from TC-25 and that well material does not contain 'trapped' reactive target capture complexes.

DNA sequence dependence of TC-25 formation

The results from Figure 2 demonstrate that target capture with the HisG1 sequence is very efficient and that this step is not dependent on a divalent metal ion. Furthermore, since only one strand transfer product was generated (Figure 3), target capture in this reaction appears to be highly dependent on target DNA sequence. However, this result does not rule out the possibility that TC-25 is comprised of a population of different target capture complexes. We have examined the DNA sequence requirements for TC-25 formation further by testing variants of the HisG1 target oligomer for target capture. Mut-C and Mut-F are 25 bp target oligomers which differ in sequence from HisG1 at consensus and flanking base pair positions, respectively (Figure 1C). *In vivo*, base pair changes present in Mut-C and Mut-F reduce the usage of the HisG1 site by at least 1000-fold (Bender and Kleckner, 1992a). A third 25 bp oligomer (IE), derived from the inside end of IS10 Right, which contains an 'end-type' transposase-binding site but does not contain a close match to the consensus target site, was also tested. The results in Figure 4A show that TC-25 is not detected in reactions with Mut-C, Mut-F or IE oligomers. Densitometric analysis

reveals that the level of TC-25 formed with HisG1 (lane 2) is at least 50 times higher than in reactions with Mut-F, Mut-C and IE oligomers (lanes 3–5). Similar results were obtained when longer incubation times were used (up to 17 h; data not shown), indicating that stability and not time is the limiting factor in TC-25 formation with these oligomers. We conclude that formation of a stable target capture complex (TC-25) with a 25 bp target is highly dependent on the target sequence. Furthermore, for the HisG1 hotspot, both consensus and flanking base pairs contribute to this stability.

TC-OE is a prominent product in reactions with Mut-C, Mut-F and IE oligomers (Figure 4A, lanes 3–5). This is somewhat surprising since the relative concentration of OE in these reactions is ~250-fold less than that of the above target oligomers. The higher reactivity of OE fragment relative to Mut-C, Mut-F and IE oligomers could either be due to increased stability of target capture complexes formed on longer DNAs, or the presence of an 'end-type' transposase-binding site on the OE fragment.

While the results presented thus far demonstrate that TC-25 formation with the HisG1 oligomer is not dependent on a divalent metal ion, we were interested in determining if conditions could be defined where TC-25 formation is divalent metal ion dependent. In Figure 4B, we have analyzed the effect of Ca^{2+} on target capture with the suboptimal target sequences, Mut-C and Mut-F. Ca^{2+} was used as opposed to either Mg^{2+} or Mn^{2+} because it does not support strand transfer in Tn*10* transposition and therefore we can examine specifically effects on target capture. In addition, we have shown previously that Ca^{2+} can inhibit strand transfer competitively in the presence of Mg^{2+}, suggesting that it does bind to the relevant Mg^{2+}-binding site(s) necessary for strand transfer (Junop and Haniford, 1996). The results demonstrate that Ca^{2+} strongly stimulates TC-25 formation with Mut-F (lanes 11–12), without significantly effecting the HisG1 (lanes 3–6) or Mut-C (lanes 7–10) reactions. It should be noted that at suboptimal concentrations of HisG1 target, Ca^{2+} does significantly increase the level of TC-25 formed (data not shown). At the Ca^{2+} concentrations tested, the level of TC-25 with Mut-F is ~70% of the level observed with HisG1 (compare TC-25 levels in lanes 11 and 3). No TC-25 was observed with Mut-F in the presence of spermidine (0.6–40 mM), verifying that non-specific charge neutralization is not sufficient to promote TC-25 formation with this suboptimal target sequence (data not shown). In addition to showing that TC-25 formation can be divalent metal ion dependent, the above experiment also suggests that consensus base pairs may be involved more intimately in divalent metal ion interactions in the target capture complex, since TC-25 formation was observed only with Mut-F.

The addition of Ca^{2+} also influences the amount of TC-OE formed. A comparison of lanes 3 and 4 from Figure 4A with lanes 7–10 in Figure 4B shows that under conditions where no TC-25 is formed (i.e. with Mut-C), Ca^{2+} causes a decrease in the level of TC-OE. Thus, Ca^{2+} can have both positive and negative effects on target capture, with the nature of the effect being related to the DNA sequence in the target that is engaged.

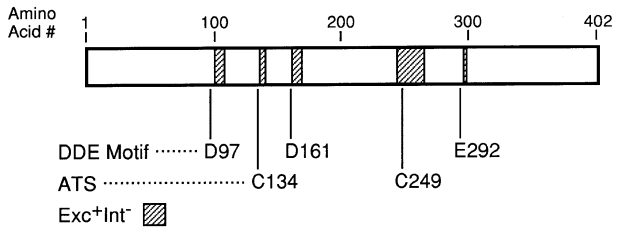

Fig. 5. Linear representation of IS10 transposase. The positions of mutations which confer catalytic defects (DDE motif), ATS and $Exc^{+}Int^{-}$ phenotypes (see text) are shown.

Effects of different transposase mutations on target capture

We have also used our target capture assay to characterize further different classes of IS10 transposase mutants. Several classes of mutants (see Figure 5) could potentially be affected in their ability to select target sites resembling the Tn*10* consensus. $Exc^{+}Int^{-}$ mutants perform donor cleavage at, or close to, wild-type levels but form little or no strand transfer products. Single amino acid substitutions at >20 amino acid residues, clustered primarily in four regions, confer this phenotype (Haniford *et al.*, 1989; Bolland and Kleckner, 1996). ATS (for altered target specificity) mutants relax the target sequence requirements for Tn*10* integration without significantly affecting the overall transposition frequency. Single amino acid substitutions at only two different residues (C134 and C249) confer this phenotype (Bender and Kleckner, 1992b). Finally, catalytic residues (DDE motif), which appear to bind divalent metal ions (J.Allingham and D.Haniford, unpublished data), may also play a role in target site selection by mediating the divalent metal ion effects noted in Figure 4B.

In the experiment shown in Figure 6A, three different catalytic mutants, including EA292, the double mutant DA97/DA161 and the triple mutant DA97/DA161/EA292, are analyzed for TC-25 formation with HisG1 in the absence of a divalent metal ion. The double mutant DA97/DA161 forms TC-25 at roughly the same level as wild-type (compare lanes 3 and 12), indicating that with this target, D97 and D161 are not important for TC-25 formation. This is consistent with the result that TC-25 formation with HisG1 and wild-type transposase is not dependent on a divalent metal ion (Figure 2). In contrast, the single mutant EA292 and the triple mutant DA97/DA161/EA292 show severe defects (<5% of wild-type) in TC-25 formation (lanes 9 and 6, respectively). Since EA292 and the triple mutant have essentially the same phenotype and we know that the D/D double mutant is not affected for TC-25 formation with HisG1, we infer that the EA292 mutation is responsible for the absence of TC-25 in Figure 6A.

We also examine the ability of DA97/DA161 to form TC-25 with Mut-F, which requires a divalent metal ion for TC-25 formation. The results in Figure 6B indicate that DA97/DA161 is severely inhibited in its ability to form TC-25 with Mut-C, Mut-F and IE oligomers even in the presence of a divalent metal ion (lanes 5, 7 and 9). Thus, when capture of the HisG1 target is dependent on a divalent metal ion, D97 and/or D161 are essential. This is consistent with the idea that the same divalent metal ion(s) used in catalysis also functions in target site

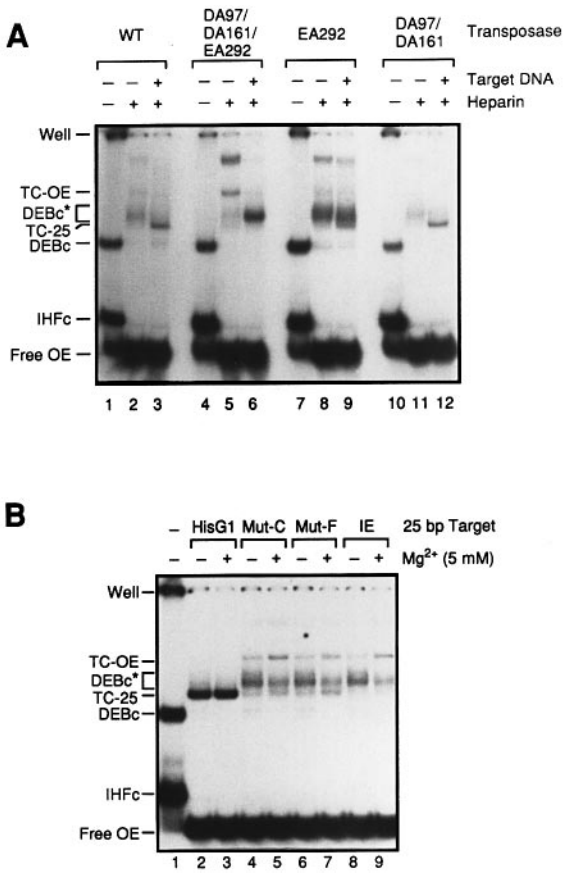

Fig. 6. TC-25 formation with transposase DDE mutants and different target oligomers. (**A**) Heparin-treated DEBc formed with each of the indicated transposase proteins was mixed with HisG1 target (37.5 pmol) and TC-25 formation was assayed as described in Figure 2. No divalent metal ion was added. In (**B**) TC-25 formation was analyzed in reactions with DA97/DA161 transposase and the indicated target oligomers (37.5 pmol) in both the presence and absence of Mg^{2+}. DA97/DA161 does not form detectable levels of strand transfer product, so only non-covalent target complexes are formed. In both (A) and (B), a portion of each of the DEBc reactions was removed before heparin treatment and analyzed separately to compare the levels of DEBc formation in each reaction. DEBc formed by each of the mutants did not differ from wild-type by more than 2.5% (data not shown). The DEBc* formed by the triple mutant DA97/DA161/EA292 is much more homogeneous in nature than that formed in reactions with any other version of transposase examined in this work.

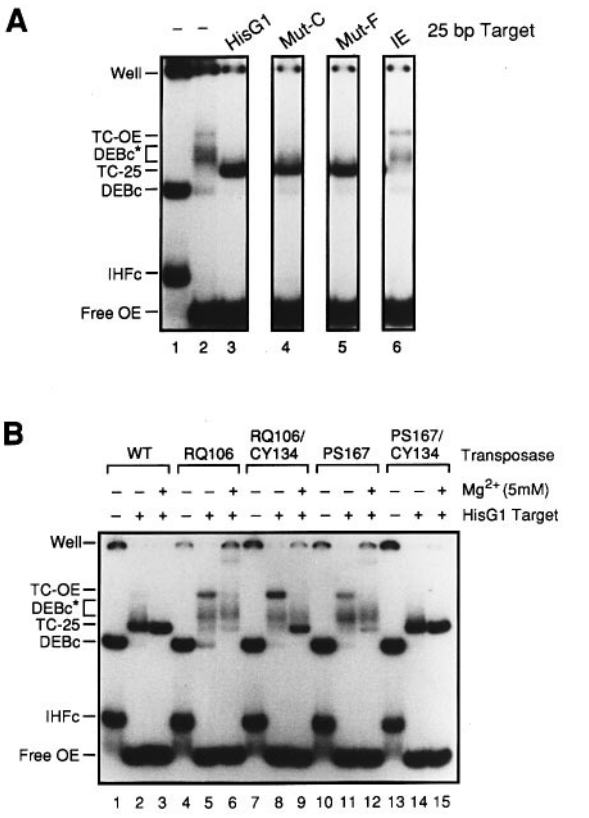

Fig. 7. TC-25 formation with Exc^+Int^-, ATS and Exc^+Int^-/ATS mutants and different target oligomers. Heparin-treated DEBc formed with each of the indicated transposase proteins was mixed with the indicated target oligomers (37.5 pmol) and TC-25 formation was assayed as described in Figure 2. In (**A**), CY134 transposase (ATS phenotype) was used. No divalent metal ion was added. In (**B**), Exc^+Int^- mutants RQ106 and PS167 and the double mutants RQ106/CY134 and PS167/CY134 were used with the HisG1 target. Mg^{2+} was added where indicated. The relative transposition frequencies of the above mutants as determined by a conjugal mating out assay are: RQ106, 0.001; PS167, 0.02; RQ106/CY134, 0.06; PS167/CY134, 0.75 (Junop *et al.*, 1994). PS167 forms only a very low level of strand transfer product in the presence of Mg^{2+}. As in Figure 6, DEBc levels were measured in each reaction and found to differ by no more than 2.5% (data not shown).

selection. Interestingly, the previously noted destabilizing effect of divalent metal ion on TC-OE is not observed with the DA97/DA161 mutant, suggesting that the binding of a divalent metal ion to one or both of these residues is responsible for TC-OE destabilization.

In Figure 7A, we examine TC-25 formation with the ATS mutation CY134 in the absence of a divalent metal ion. Unlike wild-type transposase, CY134 forms TC-25 at high levels with Mut-C and Mut-F (compare lanes 3, 4 and 5). The absence of TC-25 formation with the IE oligomer (lane 6) indicates that the effect of the CY134 mutation is to increase the stability of the DEBc on a suboptimal target site as opposed to increasing the stability of DEBc on DNA sequences which bear no resemblance to the Tn*10* target consensus.

In Figure 7B, we examine TC-25 formation with HisG1 and Exc^+Int^- mutants and Exc^+Int^-/CY134 double mutants. Neither of the Exc^+Int^- mutants form significant levels of TC-25 in the presence or absence of a divalent metal ion, although, specifically in the absence of a divalent metal ion, both mutants form significant levels of TC-OE (lanes 5 and 11). These observations are consistent with the idea that while these mutants can still form target capture complex, they are defective in (HisG1) target site selection. Experiments with supercoiled target plasmid confirm that these mutants can form target capture complexes and, as expected, these complexes are sensitive to challenge with competitor DNA (data not shown). Other members of the Exc^+Int^- class that we have examined, including DE100, RH243 and EK263, behave similarly to PS167 and RQ106 with respect to TC-25 formation and target capture using a supercoiled plasmid (data not shown). Bolland and Kleckner (1996) have identified an Exc^+Int^- mutant (KA299) which fails to capture a supercoiled plasmid target, indicating that not all Exc^+Int^- mutants behave in the same way with respect to interactions with target DNA.

CY134 was identified previously as an intragenic second site suppressor to both PS167 and RQ106 (Junop *et al.*,

1994). If CY134 suppresses these mutations by specifically affecting target site selection, as opposed to influencing another step, then the double mutants should show increased levels of TC-25 formation. This result is observed with PS167/CY134 (Figure 7B, compare lanes 11 and 14); however, formation of TC-25 with RQ106/CY134 requires a divalent metal ion (lane 9).

Discussion

The work described here focuses on the target capture stage of the Tn*10* transposition reaction. We show that it is possible to form a stable target capture complex (TC-25) with short DNA oligomers, that TC-25 formation is highly dependent on target DNA sequence and that this sequence dependence mimics that observed *in vivo* (Halling and Kleckner, 1982; Bender and Kleckner, 1992a). Importantly, TC-25 formed with HisG1 undergoes strand transfer specifically into the hotspot when incubated with Mg^{2+}, indicating that it is a viable transposition intermediate. The assay developed here is unique in transposition systems as we are able to study target selection directly using an intact transpososome. Using this assay we have begun to investigate the factors involved in target site selection in Tn*10* transposition.

DNA sequence and topological requirements for target selection and strand transfer

Target selection in our system is extremely sensitive to DNA sequence; changing either 2 (Mut-C) or 4 (Mut-F) bp out of 25 prevents TC-25 formation. The complete absence of TC-25 in these reactions was not expected, given that the initial target capture complex formed is expected to involve transposase–target interactions which are independent of DNA sequence (Sakai and Kleckner, 1997). Why then do we not see TC-25 in reactions with Mut-C, Mut-F and IE oligomers? The most likely explanation is that in the situation where transposase is not able to make a sufficient number of sequence-specific contacts with a target DNA, the half-life of the DEBc on a small piece of DNA is too short to detect the corresponding TC-25 species in our assay. Our ability to detect TC-OE in these reactions, even though the OE fragment is ~250-fold less abundant than the 25 bp oligomer, and does not contain a close match to the Tn*10* target consensus, could be due to either the increased size of this fragment (76 versus 25 bp) or the presence of an 'end-type' transposase-binding site. However, our observation that the IE target, which also contains an 'end-type' transposase binding site (Kleckner *et al.*, 1995), does not form TC-25 suggests that the presence of this site is not sufficient to allow the formation of a stable target capture complex. Thus, we favor the interpretation that TC-OE is a target capture complex in which transposase predominantly makes non-specific interactions with the target DNA and that its presence in our reactions reflects the increased stability afforded by the relatively large size of this fragment. This idea is also supported by the sensitivity of TC-OE to competitor DNA (data not shown).

The observation that TC-25 formation and strand transfer occur efficiently with a linear DNA target indicates that target selection and strand transfer do not require DNA supercoiling. Target DNA supercoiling is not required for strand transfer in either Mu (Savilahti *et al.*, 1995) or Tn*7* transposition (Bainton *et al.*, 1993). Furthermore, we conclude that all of the DNA sequence information required for target site selection and strand transfer in Tn*10* transposition can be accommodated within a stretch of 25 bp. By comparison, in the Tn*7* system, all of the DNA sequence requirements for target selection appear to be contained within a 28 bp segment which is adjacent to as opposed to being within the site of integration (Waddell and Craig, 1989).

Role of consensus and flanking base pairs in target site selection

Mutations in both consensus and flanking regions of the HisG1 target oligomer prevent the formation of detectable levels of TC-25. For mutations in the consensus region, this effect is likely to be a result of disrupting some of the base-specific contacts made by transposase (Lee *et al.*, 1987). The lack of conserved sequence in the DNA flanking the consensus base pairs of Tn*10* integration hotspots makes it unlikely that transposase makes any significant base-specific contacts within this region. It is possible, however, that specific contacts are made with phosphates of the DNA backbone, and that mutations in this region result in the loss of these contacts by altering the local DNA structure. Evidence that transposase does make significant contacts with flanking DNA comes from the observation that following strand transfer into a supercoiled plasmid, target supercoiling is retained even though nicks are generated on opposite strands of the target site (Sakai *et al.*, 1995). It is difficult to explain how the DNA would remain topologically constrained without protein–DNA contacts being formed on both sides of the nick. Thus, it is likely that formation of a stable target capture complex involves significant interactions between transposase and both flanking and consensus base pairs of a target site. The ability of Ca^{2+} to rescue TC-25 formation with Mut-F but not Mut-C suggests that target site selection is less sensitive to mutations in flanking relative to consensus base pairs. Additional studies with different mutations in both the consensus and flanking regions of HisG1 and other target sites will be required to determine how general our observations are with regard to the effects of consensus and flanking mutations on target site selection.

IS231A, a close relative of IS10, provides another example of a transposition system where transposition into a particular sequence is highly dependent on sequences flanking the consensus site. For IS231A, a hotspot for transposition is composed of the consensus sequence flanked by segments which have the potential to bend in opposite directions. This bending is thought to contribute to the formation of a stable target complex (Hallet *et al.*, 1994). Additionally, for some type II restriction endonucleases, flanking DNA can also have strong effects on cognate site usage. In the case of *Eco*RI, phosphate-specific protein–DNA contacts have been identified in flanking DNA. These contacts facilitate the formation of base-specific contacts by helping to anchor and orient protein recognition elements within the major groove and to stabilize the kinked DNA conformation within the complex. Since the DNA conformation is important for catalysis, it is apparent that in this system the flanking

DNA sequence is important for both *Eco*RI binding to its cognate site and catalysis (Lesser *et al.*, 1990). We suggest that interactions between transposase and flanking DNA perform a similar anchoring role in the formation of a stable target capture complex; however, it remains to be established if such interactions contribute to catalysis as well.

Role of divalent metal ions and DDE motif in target selection

In protein–DNA transactions which are dependent upon divalent metal ions, binding of the protein to its cognate site may or may not be divalent metal ion dependent. The restriction endonuclease *Eco*RV provides an example of a DNA cleavage reaction in which cognate site binding is dependent on a divalent metal ion (Jeltsch *et al.*, 1995; Kostrewa and Winkler, 1995), while *Eco*RI provides an example where cognate site binding is not dependent on a divalent metal ion (Vipond *et al.*, 1995). Our analysis shows that in Tn*10* transposition, divalent metal ions are not required for target site selection; however, they can facilitate usage of a suboptimal target sequence.

How might divalent metal ions influence target site selection? Considering the direct role that divalent metal ions play in catalysis in a number of phosphoryl transfer reactions (Beese and Steitz, 1991; Kostrewa and Winkler, 1995) and the expectation that divalent metal ions also participate directly in catalysis in Tn*10* transposition (Bolland and Kleckner, 1996; Junop and Haniford, 1996; Kennedy and Haniford, 1996; J.Allingham and D.Haniford, unpublished), one possibility is that divalent metal ions provide bridging interactions between transposase and a target site. In the situation where suboptimal contacts are made between transposase and a target site, additional contacts mediated by divalent metal ions could provide an important stabilizing effect. Alternatively, the divalent metal ion might be acting entirely at the protein level, helping to stabilize a conformation of transposase in which protein determinants for stable target binding are positioned optimally. We cannot distinguish between these possibilities at the present time. However, our observation that mutations in catalytic residues implicated in divalent metal ion binding block TC-25 formation indicates that the DDE motif can play an important role in target site selection.

Divalent metal ions can also contribute to cognate site binding by destabilizing non-specific interactions between protein and DNA. Evidence for this has been provided in the *Eco*RV system (Vermote and Halford, 1992; Jeltsch *et al.*, 1995). The decreased levels of TC-OE together with the increased levels of TC-25 in the reaction with wild-type transposase, Mut-F and Ca^{2+} suggest that the above phenomenon may be occurring in Tn*10* target capture. We also note that under conditions where transposase has lost its ability to respond to divalent metal ion (D/D double mutant), TC-OE levels are not reduced by the addition of a divalent metal ion. This provides additional evidence that divalent metal ion effects on target capture are mediated through the catalytic DDE motif.

How can a divalent metal ion have both positive and negative effects on target capture? The nature of the effect is likely to be dependent upon the mode of target binding. When transposase engages a target site resembling the consensus, the position of the metal ion would enhance the stability of the target capture complex. In contrast, when transposase interacts with target in a manner that is independent of DNA sequence, the position of the metal ion would decrease the stability of the target capture complex. It will be interesting to see if divalent metal ions can have similar effects on target capture in other transposition systems and, if so, whether the DDE motif also plays a prominent role in mediating these effects.

Distinct roles for DDE motif residues

Not all of the DDE mutants analyzed here have the same phenotype with respect to TC-25 formation; in the HisG1 reaction, DA97/DA161 and EA292 form TC-25 at 100% and $<5\%$ the level of wild-type, respectively. EA292 does, however, retain the ability to capture a supercoiled target plasmid (Bolland and Kleckner, 1996). A possible explanation for these results is that, in addition to its role in catalysis, E292 might also be part of the sequence-specific target DNA-binding domain. Other residues in this domain might include those defined by a subset of Exc^+Int^- mutations. Alternatively, E292 is expected to be in close proximity to the target DNA for catalysis. Thus, mutations at this position might influence target capture indirectly by inducing localized structural perturbations which affect the sequence-specific target determinants. If the former possibility is correct, then this would indicate that different amino acids in the DDE motif can be part of different functional domains. We currently are in the process of determining if other mutations in and around DDE motif residues can also inhibit TC-25 formation with the HisG1 oligomer. This analysis will help us to evaluate further the role of the segment containing E292 in target capture.

Exc^+Int^- and ATS mutations can affect target site selection

Exc^+Int^- and ATS mutations confer markedly different phenotypes. Exc^+Int^- mutants carry out excision but do not form strand transfer products at high levels (Haniford *et al.*, 1989), while ATS mutants relax target specificity without appreciably affecting the transposition frequency (Bender and Kleckner, 1992b). There are numerous ways in which mutations could block strand transfer or relax target specificity. We show that five out of five Exc^+Int^- mutants tested fail to form TC-25 in the HisG1 reaction. In contrast, CY134 (an ATS mutant) forms TC-25 (in the absence of a divalent metal ion) equally well with HisG1, Mut-F and Mut-C. Based on these results, we suggest that the respective phenotypes of these mutants observed *in vivo* can be accounted for by their effects on target selection; that is, CY134 binds suboptimal target sites more tightly than does wild-type, and Exc^+Int^- mutants do not bind with any appreciable affinity to Tn*10* target sites. Our previous observation that *in vivo* CY134 is an intragenic suppressor to a number of Exc^+Int^- mutants (Junop *et al.*, 1994) can now be explained by the CY134 effect on target selection being dominant to that of the Exc^+Int^- effect.

It is unlikely that the relaxed target sequence requirements exhibited by CY134 are a result of new contacts forming between Y134 and target DNA, since non-conservative substitutions at this position also confer an

ATS phenotype (Bender and Kleckner, 1992b). Instead, we suggest that position 134 ATS alleles promote changes in transposase structure which allow target DNA to contact residues in transposase which otherwise would not interact with DNA. This type of mechanism for achieving relaxed specificity has been proposed for the restriction enzyme *Eco*RI (Heitman, 1992).

Working model for target site selection

We have shown that, *in vitro*, divalent metal ions can contribute significantly to target site selection in Tn*10* transposition. We suggest that this contribution has two components. First, a divalent metal ion(s) bound to the DDE motif can destabilize the non-specific transposase–target DNA interactions which are responsible for the formation of the initial target capture complex. This would prevent the DEBc from being trapped on non-productive target sequences and at the same time promote linear diffusion along target DNA in a similar fashion to that reported for *Eco*RV (Jeltsch *et al.*, 1996). Second, upon engaging a target site resembling the Tn*10* consensus, the divalent metal ion(s) bound to the DDE motif can contribute to the overall stability of the target capture complex. The ability of the DDE-bound divalent metal ion(s) to have both positive and negative effects on target capture could be explained by differences in divalent metal ion position when transposase is in sequence-specific versus non-specific target-binding modes. This is supported by the observation that mutations in consensus base pairs inhibit the divalent metal ion from having a stabilizing effect on the target capture complex. We suggest that by using the DDE motif for both target DNA sequence selection and catalysis of strand transfer, the probability of initiating the reaction chemistry on a usable target site is enhanced. This would help Tn*10* avoid sites where, for instance, only one-half of the target sequence resembles the Tn*10* consensus. The consequences of initiating the reaction chemistry on such a site would probably be a single end strand transfer which is a dead end product in Tn*10* transposition. In fact, single end strand transfers are only observed at relatively low levels in reactions with wild-type transposase (Sakai *et al.*, 1995; Chalmers and Kleckner, 1996). Having a mechanism for avoiding 'unproductive' target sequences could be important for the survival of a transposable element like Tn*10*, whose transposition is subject to many levels of negative regulation (Kleckner *et al.*, 1995).

Materials and methods

Preparation of DNA substrates

Outside end substrate was generated by *Pvu*II–*Bam*HI digestion of pNK3287 (described in Sakai *et al.*, 1995). The resulting 76 bp fragment was purified from a 5% polyacrylamide gel, run in 1× TAE buffer, by the crush and soak method described in Sambrook *et al.* (1989). DNA was resuspended in 1× DNA buffer and stored at –20°C. Approximately 10 pmol of OE substrate was treated with shrimp alkaline phosphatase (USB) following the manufacturer's instructions and then phenol/chloroform extracted and precipitated with ethanol. Phosphatased DNA was resuspended in polynucleotide kinase buffer [70 mM glycine-NaOH (pH 9.0), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT)] and labeled at the 5′ termini by addition of polynucleotide kinase (NEBL) and γ-ATP (7000 Ci/mmol, ICN). After 1 h at 37°C, reactions were terminated by phenol extraction and fragments purified by gel filtration through a biospin 6 spin column (Bio-Rad) equilibrated in 1× DNA buffer. Labeled DNA was stored at 4°C and was used over a course of ~6 weeks.

Target oligonucleotides were synthesized on a 0.1 μM scale using the Applied Biosystems Model 392 DNA Synthesizer. Crude oligonucleotides were purified on a reverse phase cartridge and the dimethoxytrityl (DMT) group at the 5′ termini subsequently removed to release pure full-length oligonucleotide. After drying, the DNA oligomers were resuspended in 1× DNA buffer, and the concentrations determined spectroscopically. Complementary oligonucleotides (1 nmol) were mixed at a 1:1 ratio, heated to 70°C for 30 min in 1× DNA buffer and then allowed to anneal by slowly cooling to 30°C over a period of 3 h. Annealed oligonucleotides were stored at –20°C. Then 150 pmol of each of the annealed oligonucleotides was 5′-end-labeled as above and run on a native 15% polyacrylamide gel to assess the percentage of duplex DNA formed. We estimated that >90% of the DNA in each of the mixtures is in duplex form.

Construction of transposase mutants

DDE motif mutants EA292 (pDH166) and DA97/DA161 (pDH167) were made by the single strand method of Kunkel (1985), using pDH165, a derivative of pDH10 (Haniford *et al.*, 1989) with a *Nde*I site spanning the start codon of the IS10 transposase gene. EA292 and DA97/DA161 were subcloned on *Nhe*I–*Stu*I fragments into an appropriately prepared pET3a-IS10 transposase backbone to give pDH170 and pDH171, respectively. The triple mutant, DA97/DA161/EA292 (pDH172), was made by subcloning a *Nhe*I–*Nco*I fragment from pDH167 into an appropriately prepared backbone of pDH170. All Exc^+Int^- mutants are in the pET3a background and details of their construction are given in Junop *et al.* (1994); mutations CY134, PS167 and RQ106 are present in pDH157, pDH160 and pDH156, respectively.

Protein purification

Wild-type and mutant IS10 transposase proteins were purified from BL21(DE3)/pLysS cells containing either wild-type or mutant pET-3a-IS10 transposase plasmids, as described by Chalmers and Kleckner (1994) with the following modification: during the final step of the purification, soluble transposase was applied to a Superose 12 gel filtration column equilibrated with modified buffer B containing 2 M NaCl, 20 mM Triton X-100 instead of 2 M NaCl, 50 mM Triton X-100. Protein concentrations were determined by the BCA assay (Chalmers and Kleckner, 1994). Typical yields were in the range of 0.25–0.5 mg/ml with a purity of >90%. IHF was kindly supplied by Dr G.Chaconas.

In vitro transposition reaction

Standard conditions for *in vitro* reactions were 22 mM Tris–HCl (pH 7.5), 1 mM TES (pH 7.5), 11 mM DTT, 20% glycerol, 23 mM NaCl, 100 mM KCl, 0.05 mg/ml bovine serum albumin, 0.05 mM EDTA, 50 fmol of OE DNA fragment, 200 fmol of IHF, 25 fmol of transposase and 37.5 pmol of 25 bp target DNA. A 1× reaction was carried out in a volume of 20 μl. Transposase was first diluted in buffer containing 50% glycerol, 0.5 M NaCl, 10 mM DTT, 20 mM Tris–HCl (pH 7.5), 1 mg/ml bovine serum albumin and 2 mM EDTA, and then added to a mixture of DNA and IHF. DEBc was allowed to form for 2 h at 20°C and, where indicated, heparin sulfate (ICN) was added to a final concentration of 0.25 ng/μl for 20 min at 20°C. Following heparin treatment, target DNA and then (where indicated) $MgCl_2$ or $CaCl_2$ was added to a final concentration of 5 mM. After incubation for 6 h at 20°C, reactions were either mixed at a 5:1 ratio with loading dye [30% glycerol, 20 mM Tris–HCl (pH 7.5), 10 mM DTT, 100 mM KCl, 0.02% bromophenol blue] and applied to a 5% native polyacrylamide gel for determination of target capture complex levels, or phenol/chloroform extracted, mixed at a 2:1 ratio with loading dye [0.3% each bromophenol blue and xylene cyanol FF; 10 mM EDTA (pH 7.5) and 97.5% deionized formamide], heated to 80°C for 2 min and loaded on an 8% denaturing polyacrylamide gel for determination of strand transfer levels. Native gels (16 cm× 14 cm×0.16 cm) were run in 1× TAE at 13 V/cm for 1 h. Denaturing gels (44 cm× 35 cm×0.4 mm) were run in 1× TBE at 39.7 V/cm for 2.5 h. Gels were dried for 1 h at 80°C and autoradiography was performed with a lighting plus intensifying screen (Dupont) at –80°C. Typical exposures presented here were of 0.5–1.0 h.

Quantification

Product levels were quantified from autoradiograms by laser densitometry using a Gel Imager and Gel Print Tool Box software, Version 3.0 from Biophotonics. In order to ensure linearity, standards were loaded on each gel and multiple exposures were taken. Values were obtained from a standard curve (data not shown).

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