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The Organization of the Outside End of Transposon Tn*5*

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The end sequences of the IS*50* insertion sequence are known as the outside end (OE) and inside end. These complex ends are related but nonidentical 19-bp sequences that serve as substrates for the activity of the Tn*5* transposase. Besides providing the binding site of the transposase, the end sequences of a transposon contain additional types of information necessary for transposition. These additional properties include but are not limited to host protein interaction sites and sites that program synapsis and cleavage events. In order to delineate the properties of the IS*50* ends, the base pairs involved in the transposase binding site have been defined. This has been approached through performing a variety of in vitro analyses: a hydroxyl radical missing-nucleoside interference experiment, a dimethyl sulfate interference experiment, and an examination of the relative binding affinities of single-site end substitutions. These approaches have led to the conclusion that the transposase binds to two nonsymmetrical regions of the OE, including positions 6 to 9 and 13 to 19. Proper binding occurs along one face of the helix, over two major and minor grooves, and appears to result in a significant bending of the DNA centered approximately 3 bp from the donor DNA-OE junction.

Since Barbara McClintock's discovery of transposable elements in maize (22), transposons of one sort or another have been discovered in nearly every organism that has been studied (4). While transposons have shown a great diversity of form, there are several underlying principles that unite the various transposable elements that have been described, from simple insertion sequences of bacteria to retrotransposons of higher eukaryotes. Because of these similarities, the study of the relatively straightforward bacterial transposons has proven to be important in advancing the understanding of all transposable elements (for a review, see reference 23). One emerging principle is that most of these elements have a single protein that is responsible for carrying out several reactions, including recognition of and binding to the transposon end sequences, the alignment of two such ends in the proper orientation to form a nucleoprotein synaptic complex, endonucleolytic cleavage to expose the 3′-OH at the ends, and association and strand exchange with the target DNA in order to insert the element (23). Obviously these proteins need to be complex in their domain structure in order to carry out this diverse set of reactions. The end sequences themselves also contain a complex domain structure in order to support these varied reactions (6, 13, 15, 40). It is the study of the domain organization of the bacterial transposon Tn*5* end sequences that is the focus of the present series of experiments.

Tn*5* is a simple composite transposon that consists of two IS*50* insertion sequences in an inverted orientation about a unique region that carries the genes encoding resistance to the antibiotics neomycin, bleomycin, and streptomycin (for a review of Tn*5*, see references 3 and 26). The Tn*5* and IS*50* mobile elements are defined by the unique 19-bp DNA sequences at their ends. While IS*50* is flanked by two similar but nonidentical ends, called the outside end (OE) and inside end (IE), Tn*5* is flanked by two OEs (Fig. 1a). The insertion sequence IS*50*R encodes the *cis*-acting transposase (Tnp) and its *trans*-acting inhibitor (Inh). Tn*5* is speculated to transpose through a conservative mechanism (for a review, see reference 3). This mechanism requires that Tnp cleave the transposon DNA in a double-stranded manner out from the donor site and insert the transposon into the target site without replication of the element (2, 30). Inh is an in-frame version of the full-length Tnp that is missing the N-terminal 55 amino acids (18). Inh alone cannot bind the specific end sequences and is thought to inhibit transposition by forming inactive dimers with Tnp (5, 37).

The OE and IE are the sites of action of Tnp. Tnp is known to be able to bind the OE in a specific manner (5). The OE and IE also have consensus sites for the action of the host proteins DnaA and deoxyadenosine methylase (Dam), respectively (Fig. 1b). The action of both of these host proteins is tied to the cell cycle of *Escherichia coli*, and it is attractive to think that these host proteins play a regulatory role in Tn*5* transposition (3). Such a role has, in fact, been clearly established for Dam (39). Single-base-pair substitutions at nearly every position in the OE or IE greatly reduce the frequency of transposition (20, 25). Furthermore, it was found that single-base-pair substitutions at just one OE could lead to the formation of deletions adjacent to another wild-type (wt) OE. These adjacent deletions fell into two classes which were completely dependent on the type and location of the base pair substitution, implying different activities for the different base pair positions (16). These in vivo effects suggested a domain structure for the ends but do not define the functions of the different domains. This led to the present set of in vitro experiments, whose aim it is to establish the location of the Tnp binding site within the ends. Various in vitro studies of other bacterial transposons have allowed the delineation of specific binding domains from other possible domains within their respective ends (1, 6, 15a, 19, 35, 40).

The removal of single nucleosides before binding by hydroxyl radical treatment allowed the determination of those nucleosides that were not necessary for binding. A dimethyl sulfate (DMS) modification-inhibition experiment was done in order to find those positions where methylation of the nucleoside inhibited Tnp binding. Finally, the relative binding affinities of Tnp for various end derivatives that contained single-base-pair substitutions were assayed in order to determine those substitutions that disrupted binding. The bending of the OE induced by Tnp upon binding was also characterized. From

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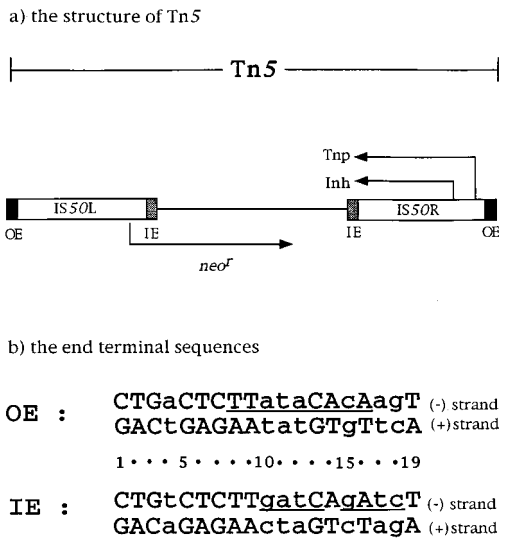


FIG. 1. Structure of Tn*5*. (a) Tn*5* consists of two IS*50* elements in inverted orientation around a unique region that contains genes encoding resistance to antibiotics. IS*50*R is the element that encodes the transposase (Tnp) and inhibitor (Inh). Inh is encoded in the same frame as Tnp but primarily by a second transcript that has a separate downstream translational start site. (b) Each IS*50* is flanked by two similar but nonidentical end sequences, the OE and IE. The sequences of each of these ends are shown. Positions of homology between the ends are shown with capital letters. Underlined bases represent sites of interaction with the host proteins DnaA (OE) and Dam methylase (IE). Both strands of each end are shown for reference to strand differences examined in the experiments. The 5′-to-3′ top strand has been arbitrarily referred to as the minus strand throughout. Also, all base substitutions referred to are relative to this minus strand. The position numbering is also shown.

our results, we propose that the primary binding site of Tnp is at positions 6 to 9 and 13 to 19, that it involves major groove contacts, and that Tnp binding is associated with a significant bend of DNA that contains an OE centered close to the junction between the OE and donor DNA.

MATERIALS AND METHODS

Media and reagents. Bacterial strains were generally grown in LB medium containing 10 g of Bacto-trypyone, 5 g of Bacto-yeast, and 10 g of NaCl in 1 liter of distilled water. The antibiotics ampicillin (100 μg/ml) and tetracycline (15 μg/ml) were purchased through Sigma. The restriction enzymes and T4 polynucleotide kinase were purchased from Promega and New England Biolabs. The enzyme avian myeloblastosis virus (AMV) super-reverse transcriptase was purchased from Boehringer-Mannheim. Radioisotopes were obtained from Amersham. Ferrous ammonium sulfate and Na^+ ascorbic acid were acquired from Aldrich and Sigma, respectively. DMS was also purchased through Amersham.

Bacterial strains and plasmids. Plasmid DNA was grown in *E. coli* DH5α [*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]. Unmethylated DNA was prepared by growth in strain D47.01 [Δ(*pro-lac*) *ara dam*::Tn*9*] (39). The OE derivatives wt, 1A, 2A, 3C, 5G, 5T, 6A, 6C, 7G, 8C, 8G, 12C, 13G, and 16T as well as IE were all propagated in plasmid pRZ7067. This series of plasmids were made by cloning the *Kpn*I-*Sph*I fragment of the appropriate pRZ1495 OE derivative (20, 24) into the same sites on a pUC19 plasmid (38). The OE derivatives 2C, 10T, 11A, 12T, 15T, 18C, and 19G were all propagated in the plasmid series pRZ7725. This plasmid series was constructed by cloning purified single-stranded oligonucleotides containing the desired substitution into the *Kpn*I and *Sph*I sites of pUC19. This was possible because the restriction sites give overhangs from the same strand of DNA. The oligonucleotide was annealed to the two overhangs, filled in with T4 DNA polymerase (Promega), and ligated. The OE derivatives 9G, 14G, and 17G were propagated in the plasmid series pRZ7730. This series of plasmids were constructed by cloning the *Kpn*I-*Sph*I fragment from the appropriate pDB44 derivative (pDB4423 [9C], pDB4407 [14G], or pDB4414 [17G]) (25) into pUC19. The pDB44 plasmids were a gift from Doug Berg, Washington University, St. Louis, Mo. The larger OE DNA probes used for the interference studies were obtained from plasmid pRZ7011 (37). The 182-bp DNA-bending probes were prepared from plasmid pRZ9012, a pBend3 derivative (17). pRZ9012 was made by inserting a 67-bp fragment that contained the 19-bp OE sequence into the *Xba*I and *Sal*I sites of pBend3. pBend3 was kindly supplied to us by S. Adhya.

Transposase overproduction was done in *E. coli* BL21(DE3) [F^- *ompT hsdS* (r^- m^-) *dcm gal* (DE3)] with plasmid pRZ7074 as described by Weinreich et al. (34).

Transposase purification. Transposase purification was done essentially as described by Weinreich et al. (34). This procedure involves cell lysis followed by consecutive high-speed spins, ammonium sulfate precipitation, polyethyleneimine precipitation, and heparin-agarose column chromatography. The purity of the transposase preparation was determined to be >90% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining. The Tnp final concentration was determined to be 0.83 μg/ml by a standard Bradford assay.

Gel retardation assay. The ability of Tnp to bind OE DNA in a specific manner has been described (5). We have essentially followed the same conditions. The binding affinity assays were performed with a 60-bp OE probe isolated by *Hin*dIII-*Eco*RI digestion of the derivatives of pRZ7067, pRZ7725, and pRZ7730 described above. These oligonucleotides were radiolabeled by filling in the overhangs with [α-^{32}P]dATP and AMV super-reverse transcriptase. The binding conditions were as follows. In 25 μl of binding buffer (5), approximately 0.5 ng of labeled OE (0.5 nM in terms of binding sites) was incubated with increasing amounts of purified Tnp (0, 0.075, 0.15, 0.30, and 0.6 μg [0 to 440 nM]) at 30°C for 40 min. The bound and free fractions were separated by electrophoresis on an 8% (30:1) native acrylamide gel. The polyacrylamide gels were dried and exposed to film for autoradiography. The percentage of DNA that was bound was quantified with a Betascope 603 blot analyzer (Betagen Corporation, Waltham, Mass.) and calculated by adding the counts in the bound complex to those in the wells and dividing by the total counts in the reaction. The counts in the wells were considered to be specifically retarded because the appearance of DNA in the wells is dependent upon site-specific binding (data not shown).

Missing-nucleoside and DMS inhibition assays. These assays required us to use larger oligonucleotides than above, and we needed to differentially label the two strands of DNA. The minus strand was visualized by digesting pRZ7011 with *Eag*I-*Pvu*II (318 bp) and labeling the *Eag*I overhang with [α-^{32}P]dGTP and AMV super-reverse transcriptase. The plus strand was visualized by digesting pRZ7011 with *Eco*RI-*Hpa*I (266 bp) and likewise labeling the *Eco*RI end with [α-^{32}P]dATP. The hydroxyl radical needed for cleavage of the DNA was generated by the Fenton reaction (8). Our conditions involved incubating 100 fmol of DNA in 1 mM ferrous ammonium sulfate–2 mM EDTA–4 mM Na^+ ascorbate–0.003% H_2O_2. After 5 min, the reactions were stopped with 0.1 M thiourea and 0.2 M EDTA. The cleaved DNA was then used in the binding reaction as described above but at a larger scale (100 fmol of DNA and 20 pmol of Tnp) so that approximately half of the DNA was bound. The bound and free DNAs were separated on a 4% (30:1) polyacrylamide gel. The DNA was isolated from this gel as described by Sambrook et al. (27). The purified DNA was then denatured and run on a 10% polyacrylamide (20:1)–8 M urea gel, next to a Maxam-Gilbert G/A reaction (21). The results discussed are based upon multiple experiments. In these experiments, bound DNA refers to DNA isolated from retarded complexes and not DNA found in the wells.

The methyl modification of the DNA by DMS was done essentially as described for the G/A reaction (21). Our conditions involved incubating 100 fmol of labeled DNA with 0.5% DMS and 100 ng of calf thymus DNA for 5 min at room temperature. The DNA was purified, bound to Tnp, isolated, and analyzed as described for the missing-nucleoside experiment except that the DNA was cleaved with 1 M piperidine as described elsewhere (21) prior to loading on the 10% polyacrylamide gel. Again, the results discussed are based upon multiple similar experiments.

Permuted DNA gel retardation assay. Gel retardation binding experiments were performed with four circularly permutated 182-bp fragments from pRZ9012 having the 19-bp consensus OE sequence for Tn*5* transposase: an *Mlu*I-*Mlu*I fragment, an *Nhe*I-*Nhe*I fragment, an *Eco*RV-*Eco*RV fragment, and an *Nru*I-*Nru*I fragment. These fragments were phosphatase treated (Boehringer-Mannheim) and end labeled with [γ-^{32}P]dATP. Reaction mixtures (15 μl) contained 0.5 ng of probe, 600 ng of transposase, 100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 0.5 mM β-mercaptoethanol, 400 mg of bovine serum albumin (BSA) per ml, 600 ng of tRNA, and 20% (vol/vol) glycerol. After 30 min at 30°C, 3.5 μl of 20% (vol/vol) glycerol–0.1% bromophenol blue–0.1% xylene cyanol was added to each sample and loaded onto a 5% (30:1) polyacrylamide gel. The gel was run at 300 V for 2 h, dried, and exposed to film overnight. The distances of migration of the individual complexes were measured by the Betascope 603 blot analyzer. The relative mobilities (R_f) reported are the relative distances of migration of the bound complexes divided by the migration distance of the respective free DNAs. The fractional distances reported are the distances between position 3 of the OE (the apparent bending center) and the left end of the fragment divided by the length of the probe. The apparent bend angle was computed by using the derivation of Zhou et al. (42) of the Thompson and Landy bending equation (32).

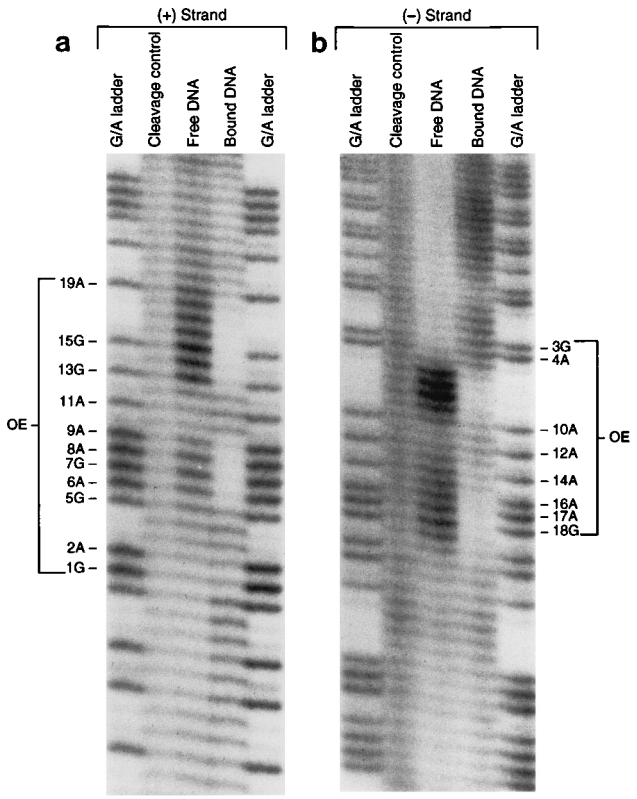


FIG. 2. Missing-nucleoside inhibition experiment. The missing-nucleoside experiment was performed in order to determine which bases are involved in binding Tnp. Experiments were performed as noted in Materials and Methods. Those polynucleotides that lacked a nucleoside necessary for binding show a decrease in the lane of bound DNAs at that position and an enhancement in the lane of free DNAs compared with the cleavage control. The results for both strands are shown. (a) Plus-strand DNA. When the nucleosides at positions 6 to 9 and 13 to 18 of this strand were removed, there was a clear inhibition of binding. Conversely, nucleosides 1 to 5 and 10 to 12 are not necessary for Tnp binding. (b) When the nucleosides at positions 6 to 9 and 13 to 19 were removed from the minus strand, there was also an inhibition of binding. It appears that an absence of nucleosides or the associated backbone cleavage at positions 1 to 5 and positions exterior to the end may enhance binding.

RESULTS

Missing-nucleoside experiment. The first step in transposition is the sequence-specific binding of Tnp to the OE sequences defining the ends of Tn*5*. The OE base pairs that are intimately involved in Tnp binding were analyzed through a missing-nucleoside–inhibition experiment. DNA containing the OE was first exposed to limited hydroxyl radical treatment (8). The hydroxyl radical attacks the ribose, leading to a cleavage in the DNA backbone and removal of the nucleoside (base plus ribose), although phosphates are conserved (14). Following the hydroxyl radical treatment, those polynucleotides that could still bind Tnp were then separated from those that could not by gel retardation (see Materials and Methods). The effects were observed by running the pooled groups on a polyacrylamide gel next to a Maxam-Gilbert sequencing ladder. A decrease in the abundance of the molecules in the pool of bound DNAs and a corresponding increase in the pool of free DNAs at any given position were observed when the removal of a nucleoside led to reduced binding (Fig. 2). The experiment was repeated for both strands of DNA.

As shown in Fig. 2a, a dramatic inhibition of binding was seen when the nucleosides of the plus strand were removed at positions 6 to 9 and positions 13 to 18. On the other hand, there appeared to be no decrease in binding when nucleosides 1 to 5 or 10 to 12 were removed. A similar trend is seen for the minus strand (Fig. 2b); the removal of a nucleoside at positions 6 to 9 is clearly inhibitory. A second critical group is also present on this strand, although it is slightly displaced relative to the plus strand, starting at position 14 and appearing strongest at positions 16 to 19.

A drawback of the missing-nucleoside approach is the fact that the DNA backbone is cleaved. This makes the effect of the removal of a nucleoside arguably due to the damage of the DNA structure rather than to removal of an interaction with the nucleoside in question. The results are more easily interpreted when the removal of a base does not impair binding. This is the situation on both strands for positions 1 to 5 and 10 to 12.

It is possible that the removal of specific nucleosides enhances Tnp binding, for instance, by enhancing the flexibility of the DNA. In fact, there does appear to be an enhancement of binding if bases 1 to 5 of the minus strand are removed. This enhancement extends to molecules that have missing nucleosides in the flanking DNA. The effect seems to be weaker for the four nucleosides immediately adjacent to the exterior of base 1 of the OE and then strengthens again for the fifth adjacent nucleoside and beyond. This effect can also be seen, although not as strongly, for the plus strand. The phase of the effect is also different; it appears weakest at positions 1 and 2 of the OE and gets slightly stronger immediately adjacent to the exterior of the OE.

DMS inhibition of Tnp binding to the OE. The results of the missing-nucleoside experiment were complemented by probing for information necessary for binding at a finer level. To accomplish this, DMS inhibition experiment was conducted, analyzing both strands of OE DNA. DMS methylates DNA in two positions: the N-7 position of guanine (major groove) and the N-3 position of adenine (minor groove) (29). If either of these positions is involved in a contact with bound Tnp, then the methylation of that site should inhibit binding. Tnp was allowed to bind to modified DNA, and polynucleotides with methyl groups that interfered with binding were separated from those that allowed binding by gel retardation (see Materials and Methods). The two pools were then isolated and run on a high-resolution polyacrylamide gel next to a Maxam-Gilbert sequencing ladder and a cleavage control. When the methyl modification inhibited binding, a decrease at that position in the pool of bound DNAs was observed along with a corresponding enhancement in the pool of free DNAs (Fig. 3).

There are several positions of interest on the plus strand (Fig. 3a). The strongest effect seems to be on a major groove methylation at position 7. This is consistent with the dramatic inhibition of Tnp binding by the 7C→G OE mutation described below. Other sites of major groove methylation interference are positions 13 and 15. Unfortunately, the DMS modification much preferred the G reaction to the A reaction (see cleavage control lane in Fig. 3a), making reliable statements concerning minor groove contacts impossible. There does, however, appear to be an increase in the free pool of DNA molecules with methylation at positions 6 and 8. This effect is reproducible, but our poor control cleavage does not allow us to substantiate its significance by observing the expected decrease in the bound pool. Methylation of other positions of the plus strand, whether interior or exterior to the OE, appear not to affect the binding affinity.

There are only two potential sites of major groove methylation on the minus strand, positions 3 and 18 (Fig. 3b). There appears to be a small inhibition of binding by the methylation

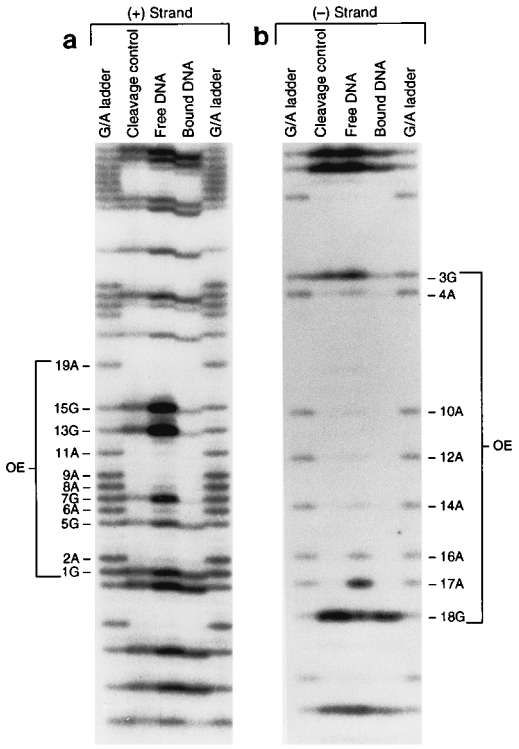


FIG. 3. DMS inhibition experiment. DMS modifies DNA by adding methyl groups to two positions, the N-7 of guanine and the N-3 of adenine. These two positions represent points of potential Tnp binding contacts in the major and minor groove, respectively. Experiments were performed as noted in Materials and Methods. Those polynucleotides with methylated bases that interfere with binding show a decrease in the lane of bound DNAs at that position and an equivalent enhancement in the lane of free DNAs. The results for both strands are shown. (a) Plus-strand DNA. When the guanines at positions 7, 13, and 15 were methylated in the major groove, there appeared to be an inhibition of binding. As is evident from the cleavage control lane, the methylation of adenines was an inefficient reaction, and we were unable to gather unambiguous information about the minor groove. (b) Minus-strand DNA. There are not many potential sights of major groove methylation on this strand, although there is perhaps a slight inhibition at position 3.

of 3G; however, densitometer scans of several experiments have shown this inhibition to be variable and not extensive (data not shown). Again, it is not possible to make reliable statements concerning the methylation of adenines. There is, however, a strong band appearing in the free lane upon methylation of the adenine at position 17.

Relative affinities of mutant end derivatives. Previously it was observed that the introduction of single base substitutions into a single OE of Tn*5* led to the formation of deletions adjacent to the wt OE. It was proposed that one class of these deletions resulted from a failure of the transposase to form a proper nucleoprotein synaptic complex (16) and that a majority of these failures to synapse were the result of impaired Tnp binding to the mutant OE. In order to establish the ability of Tnp to bind to various OE derivatives, this activity was assayed in vitro through the use of gel retardation binding experiments.

The ability to observe Tnp binding to the OE by gel retardation has been described previously (5, 36). The bound complex has been shown to consist of a full-length Tnp dimerized to an N-terminally truncated Tnp (5). The truncated Tnp lacks the DNA-binding domain, and therefore our complexes contain only one DNA polynucleotide (37). Binding was assayed by protein titration at limiting concentrations of the DNA polynucleotide (Fig. 4) (see Materials and Methods). The binding activity for any given point was determined from a plot of the percent free DNA as a function of protein concentration. These conditions gave an approximately inverse linear dependence in the range of 10 to 90% (data not shown). These plots allowed the estimation of the protein concentration at which 50% of the DNA was bound, the $K_{observed}$ (K_{obs}) of the binding reaction. The relative affinities were then determined by relating the K_{obs} of the mutant ends to that of a wt OE (Fig. 5).

Upon examining the relative binding affinities, it is surprising to find that most of the mutations do not greatly affect binding. Only one mutation, 7C→G, reduces binding by more than 10-fold. This is in sharp contrast to the effects of the mutations on transposition in vivo, which often show close to 100-fold reductions (20, 25). The reason for this diminished in vitro effect is not known (see Discussion). Despite this, however, we may draw some conclusions concerning the effects of base substitutions on binding affinities. All mutations at positions 1 to 3, 11, and 12 examined have little or no effect on Tnp binding. In contrast, mutations at positions 6, 7, and 8 reduce Tnp binding by twofold or more. These results are consistent with those of the missing-nucleoside experiment, which indicated that the same positions are either important (nucleosides 6 to 8) or not directly involved (nucleosides 1 to 3, 11, and 12) in Tnp binding.

The effects of the mutations at other positions within the OE are more complex. However, 5C→T, 10A→T, 13C→G, and 17A→G do show a reduction in Tnp binding. In addition, it has been shown that a methylated fragment carrying the IE has a reduced Tnp binding affinity compared with either wt OE binding or unmethylated IE.

Multiple substitutions of the same base pair were examined at positions 2, 5, 6, 8, and 12. Only one position examined showed a real difference depending on the type of substitution. Whereas the 5C→G mutation binds at nearly wt levels, the 5C→T substitution shows a significant decrease in binding affinity.

Retardation of Tnp-OE DNA complexes depends upon the location of the OE sequence. The binding of a protein to DNA is often associated with a bend in the DNA. Retardation experiments were performed to examine this possibility for the

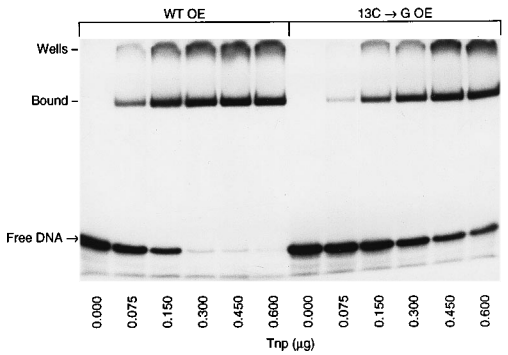


FIG. 4. Gel retardation assay of Tnp binding. A constant, limiting amount of a DNA oligonucleotide containing an OE derivative was bound to increasing amounts of purified Tnp in vitro. These reactions were then run on nondenaturing polyacrylamide gels to separate bound complexes from free DNA. This allowed us to visualize the amount of DNA involved in forming a bound complex. Some Tnp apparently forms aggregates, resulting in some bound DNA being retained in the wells. Shown are the results of the titration of two OE derivatives, wt OE and 13C→G OE. From these gels, it can be seen that more Tnp is required to bind an equivalent amount of 13C→G as of wt. Approximately 0.5 ng of labeled DNA (binding sites) was used in each reaction at the protein amounts shown.

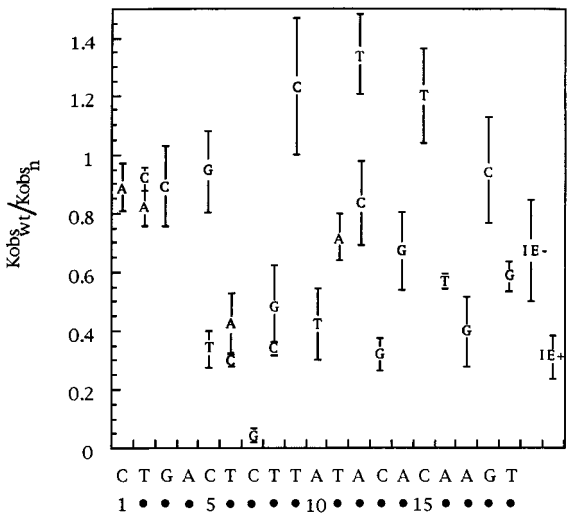


FIG. 5. Relative binding affinities of various end derivatives. The gel retardation assay was performed on 26 different end variants. Each assay was done at least in triplicate. The amount of Tnp needed to bind 50% (K_{obs}) of the DNA in each assay was determined as discussed in Materials and Methods. The relative affinities were determined by dividing the K_{obs} of wt by the K_{obs} of the end variant. The only end that showed a greater than 10-fold decrease in binding affinity was the 7C→G OE substitution. While most of the relative affinities did not vary greatly from that of the wt, some trends are evident. Substitutions at positions 6 to 8 are more likely to affect Tnp binding than substitutions at positions 1 to 3. It was also observed that Tnp binds unmethylated IE DNA with about 70% of the affinity that it has for the OE. This affinity is further reduced for methylated IE DNA.

Tn*5* Tnp-OE interaction. We digested pRZ9012 separately with various enzymes (Fig. 6a). This gave us fragments of 182 bp each, with the OE at various positions but in the same context in terms of neighboring DNA. A gel retardation analysis of these polynucleotides bound to Tnp was then done (Fig. 6b) in order to determine the relative mobilities of these complexes (Materials and Methods). That the OE-containing DNA does not have a substantial intrinsic bend can be seen by the similar migrations of the different free DNAs in Fig. 6b. Figure 6c is a plot of the relative mobilities of each fragment as a function of the position of the center of each of the bending probes (Fig. 6a). This curve allowed us to map the center of the apparent Tnp-induced bend, located at the lowest point of the curve. This position, 135, maps to the third nucleotide in the 19-bp OE sequence. A similar set of experiments with a different set of six permuted OE-containing fragments indicated that the center of the proposed Tnp-induced bend was located near the first nucleotide in the 19-bp OE sequence (data not shown).

A derivation of the Thompson and Landy bending equation formulated by Zhou et al. (42) can now be used to determine the angle of the bend. This equation is

$$\frac{\mu_i}{\mu_j} = \frac{[1 - 2(\chi_i/L)(1 - \cos\alpha) + 2(\chi_i/L)^2(1 - \cos\alpha)]^{0.5}}{[1 - 2(\chi_j/L)(1 - \cos\alpha) + 2(\chi_j/L)^2(1 - \cos\alpha)]^{0.5}}$$

where μ_i and μ_j are the relative mobilities of the complexes with Tnp bound to a centrally positioned OE and a peripherally positioned OE, respectively, χ_i/L and χ_j/L are the fractional distances from the center of bending (+3) to the left end of the bending probes of the same OEs, and α is the angle of the bend. Having determined the relative mobilities of the centermost (*Eco*RV; fractional distance, 0.47) and endmost (*Mlu*I; fractional distance, 0.74) digest to be 0.358 and 0.462, respectively (see Materials and Methods), the angle of the apparent bend was then calculated to be 119°. The same angle is found by using the fractional distances and relative mobilities of the *Nhe*I (μ = 0.68, χ/L = 0.41) and *Nru*I (μ = 0.34, χ/L = 0.40) digests. This angle is in fact outside the range (up to 100°) tested by Thompson and Landy (32). In Fig. 6b, a second, faster-migrating bound complex (complex II) can be seen. Complex II has been characterized before and found to be a contaminating monomeric C-terminally truncated Tnp fragment bound to the OE (37). It is interesting that this monomer causes the same location-dependent perturbation in complex mobility as the dimer.

DISCUSSION

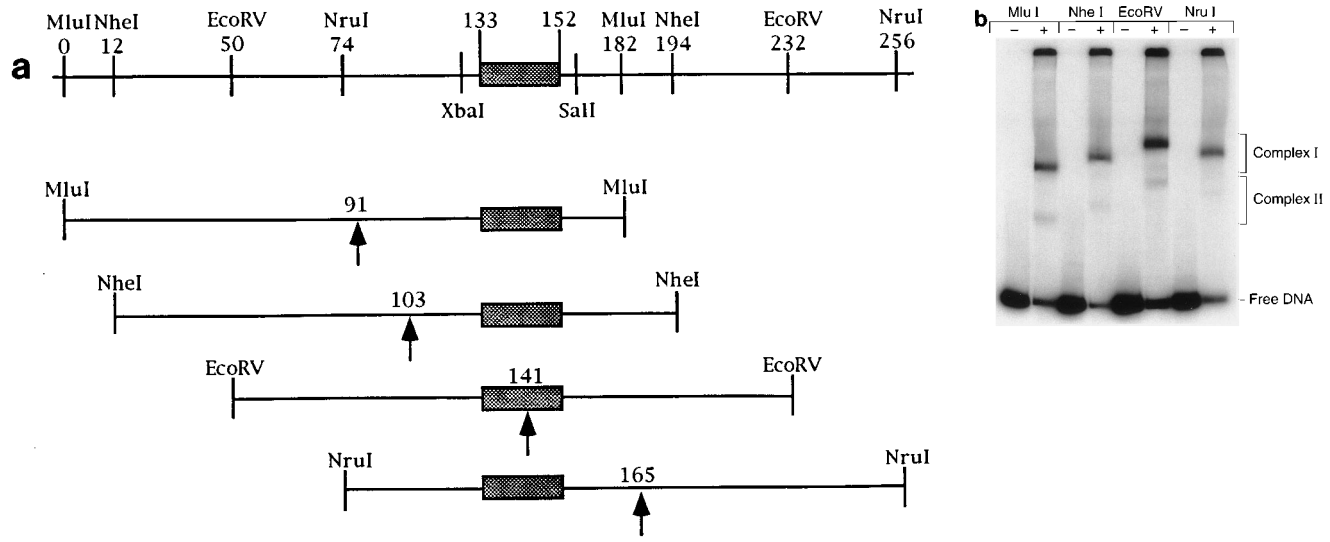
The end sequences of a transposable element offer a convenient system for studying short but complex multidomain DNA sequences. The OE and IE of Tn*5* consist of 19 bp. Nearly all base substitutions in these sequences show great decreases in transposition (10- to 100-fold) (9, 20, 25). The high sensitivity of these sequences is likely due to the fact that they contain information for Tnp binding, host protein binding, synapse formation, and DNA cleavage, all of which are vital to transposition. In order to initiate a determination of which base pairs are involved with which roles, the initial Tnp binding domain was determined. This information, together with previous data, also allows the prediction of roles for the base pairs that lie outside of the Tnp binding domain.

Tnp binds a subdomain of the OE. The OE sequences were altered in several ways, and it was determined which alterations interfered with Tnp binding. While each of these methods alone carries certain caveats, together they combine to present a strong indication that the Tnp makes initial specific binding contacts with bases between positions 6 to 9 and 13 to 19 of the OE. Bases 1 to 5 and 10 to 12, on the other hand, appear to be involved in secondary reactions.

The missing-nucleoside experiment gives perhaps the clearest evidence of the essential contacts necessary for initial Tnp binding. This experiment was performed as a way to remove critical Tnp contacts without the addition of inhibitory contacts. The drawback to the experiment is that the associated cleavage in the backbone may make an otherwise noncontributing nucleoside appear to be necessary for binding. For this reason, interpretation of the missing-nucleoside experiment is better from the approach of determining which nucleosides are not involved in binding. This evidence indicates that chemical interactions with base pairs at positions 1 to 5 and 10 to 12 are not required for Tnp binding.

The DMS inhibition experiment provides evidence supporting the conclusion that the initial Tnp binding contacts are located within positions 6 to 9 and 13 to 19. The results show that methylations of the N-7 site of the guanines at positions 7, 13, and 15 inhibit binding. Each of these positions lies within the proposed binding domain. There is also a guanine at position 18 of the minus strand whose methylation does not inhibit binding, indicating that a major groove contact at this position is probably not necessary for Tnp binding. Methylation of the guanines outside of the proposed binding regions (positions 1, 3, and 5) does not greatly affect binding. There may also be some inhibition due to the methylation of adenines at positions 6, 8, and 17.

An examination of the relative binding affinities of Tnp for various mutated OE constructs was also performed. Although this analysis was informative, it does have some limitations. One limitation lies in the ambiguity of the effects of a given



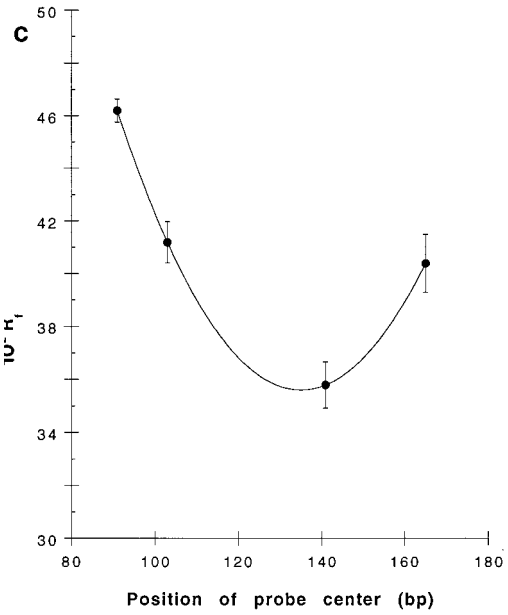


FIG. 6. Bending of OE DNA by Tnp. (a) DNA probes used in OE bending experiments. The top line shows the 256-bp *Mlu*I-*Nru*I region of the pBend 3 derivative pRZ9012. The nucleotide positions of the 19-bp OE sequence (hatched box) are indicated. Four circularly permuted probes of equal size (182 bp), each containing the OE sequence, were generated by digestion with *Mlu*I, *Nhe*I, *Eco*RV, and *Nru*I. The arrowheads mark the center of each probe. The nucleotide position of each center is also shown. (b) Gel retardation assay of Tnp binding to each of the four bending probes *Mlu*I-*Mlu*I, *Nhe*I-*Nhe*I, *Eco*RV-*Eco*RV, and *Nru*I-*Nru*I. Each probe was incubated without (−) or with (+) 600 ng of Tnp. Free and complexed DNAs were separated on a 5% polyacrylamide gel. The positions of complex I and complex II (as described in reference 37) are shown. (c) Mapping of the bending center of the OE fragment. The relative mobility, R_f (distance of migration of DNA-Tnp complex/distance of mobility of free DNA), of each bending probe was plotted as a function of the position of the center in each probe, as shown in panel a. A third-order polynomial curved fit ($y = 137.51 - 15355x + 0.0059793x^2 - 0.0000014569x^3$) of the data points allowed us to map the center of Tnp-induced bending to position 135 (position 3 in the OE sequence).

substitution; whereas some substitutions at positions involved with binding may conserve the necessary chemical interaction and therefore still bind, other substitutions will introduce negative contacts at nearby but nonbinding positions and thereby reduce binding. Another possible limitation is that Tnp binding of the OE may impart a large bend upon the DNA. This would mean that binding is intimately involved with bending and vice versa. In order for such a large bend to occur, the DNA itself must have a certain flexibility, which is presumably sequence dependent. Therefore, base substitutions that change the DNA flexibility can inhibit binding without directly affecting chemical contacts.

The binding studies, for the most part, are consistent with the proposed binding domain. Twenty-three different single base substitutions were examined, including at least one substitution at each of the 19 bp which make up the OE except position 4 (changes at position 4 have little influence on the in vivo transposition frequency [20, 25]). Of the base substitutions examined, 10 changes at eight positions reduced binding twofold or more (Fig. 5). These changes likely either remove a contact necessary for binding or introduce an unfavorable contact. Six of the eight inhibitory substitutions are at positions that lie within the proposed binding domain. In particular, all changes at positions 6 to 8 examined significantly reduce Tnp binding. The other two are at immediately adjacent positions (5 and 10). Thirteen changes at 11 positions maintained binding greater than 50% relative to the wt sequence, including changes at positions 1 to 3, 11, and 12 that have little or no effect on Tnp binding. These substitutions probably represented positions either not directly involved in Tnp recognition or with changes that conserved necessary interactions.

The IE was the one construct examined that contained multiple substitutions relative to the OE. When not methylated, the IE, despite differing from the OE at seven positions, binds Tnp with about 60% of the affinity of OE. Unmethylated IEs in vivo may actually serve as a better substrate for transposition than the OEs, depending on conditions (9). Dam-methylated IE DNA binds with less than a third of the affinity of OE. Dam methylase adds a methyl group to the N-6 position of the adenines in the GATC consensus sites. This allows four methylated adenines between the two strands of our 19-bp IE (Fig. 1). It has been proposed that the methylation of at least one of these adenines is inhibiting an important site of interaction with the protein. The A at position 16 of the IE is the only position of the four that has the N-6 group conserved between the IE and OE.

OE mutants have modest effects on Tnp binding. The observed changes in OE binding for the various mutant sequences were more modest than the previously reported changes in transposition frequencies. Only one substitution (7G) showed an inhibition of in vitro binding greater than fivefold. This is in sharp contrast to the 100-fold effects that some of these mutations have on transposition (20). Binding studies of IS*903* showed a similar incongruity for at least one position (6). There are various possible explanations for the limited effects. One possibility is that the OE substitutions examined alter two of the functions provided by the OE and the in vivo effect is a combination of the two. For example, while a certain substitution may allow some reduced levels of binding, the substitution may also be reducing the intrinsic flexibility of the DNA for bending (see below). It has been observed that the L2 binding site of the Mu transposon, which is the weakest of six MuA binding sites, also shows the lowest amount of bending induced by MuA (19). A decrease in bending may then affect the formation of a proper synaptic complex that is required for transposition (12). Other steps that occur after Tnp binding may also be affected, such as excision or DnaA binding.

However, it may be that this low level of specificity accurately reflects the in vivo situation. Tnp binding appears to involve 9 bp of the OE. This indicates that a large number of various contacts may be made between the protein and DNA. It may be that the disruption of any individual contact is not by itself enough to abolish a Tnp-OE interaction. This could explain the divergence of sequences of the OE and IE. This is in agreement with previous studies, which suggested that a pseudo-OE-like sequence with homology only at positions 6 to 10, 13, 15, and 16 was adequate for Tnp binding in vivo and subsequent adjacent deletion formation (16).

Experiments have shown that Tnp preferentially acts in *cis* because of a rapid functional instability (33, 36). It may be that even small changes in the binding kinetics show large differences of activity in vivo due to this instability.

Tnp binds the OE from one side of the helix and bends the DNA. The DMS and hydroxyl radical data can be used to study whether Tnp binds to a single face of the helix. Figure 7 displays a planar projection of the OE sequence with the critical sites for methylation inhibition displayed. The spacing of the two sequences required for Tnp binding (6 to 9 and 13 to 19) and the locations of the sites of methylation inhibition are consistent with Tnp binding to the OE from one side of the helix over two major and minor grooves. This configuration would leave the other face of the helix available for simultaneous recognition by DnaA and/or synapse formation with a second Tnp monomer bound to the other Tn*5* OE. Similar analyses performed with other transposons have shown binding to a single face of the helix to be a common pattern (1, 6, 19, 35). Something that appears to be unique to Tn*5*, however, is the presence of two nonsymmetric and nonhomologous binding domains that are separated by an intervening spacer region.

Several lines of evidence have suggested the importance of major groove interactions for Tnp binding. Some of these are the inhibition of binding by guanine methylation, the inhibition of binding by Dam at the IE, and the strong effect of the 7C→G substitution. The involvement of minor groove contact points cannot, however, be ruled out. In fact, there are a couple of lines of evidence that suggest a role for the minor groove. Wiegand and Reznikoff (36) have shown that Tnp binding protects the minor groove of the OE from attack by copper phenanthroline. Also, six of the seven differences between the OE and IE are either C/G→G/C or A/T→T/A transversions, substitutions that preserve minor groove contacts (28). The

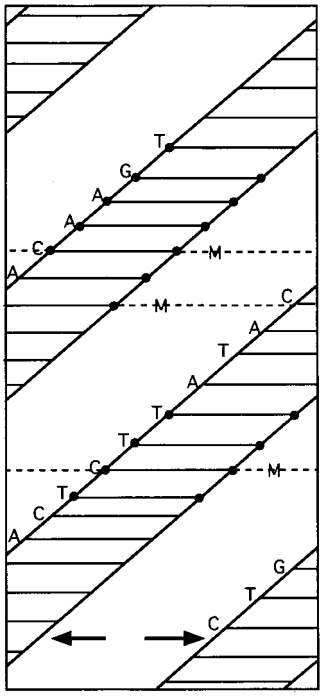


FIG. 7. Summary of the in vitro data shown in a planar projection. The double-stranded helix of the OE is shown in a planar projection to demonstrate the alignment of the important binding sites. The helix is shown with a repeat of 10 bp per twist, starting in a 5′-to-3′ direction from the lower left (position 1). The minor groove is shown through the center with solid lines, whereas the major groove is shown with dotted lines. Positions that the hydroxyl radical experiment have shown to be important are represented with black dots. Positions that showed major groove involvement in the DMS experiment are shown with capitol M's placed into the major groove. The proposed cleavage site flush to position 1 of the OE is also shown with directional arrows on both strands. The center of the Tnp-induced bend is at position 3.

DMS experiments presented here also suggest a possibility of inhibition of Tnp binding by minor groove methylation at positions 6, 8, and 17. The transposase of IS*903*, which has some similarities to Tn*5*, makes major and minor groove contacts (6), although Tn*7* and Mu appear to make only major groove contacts (1, 41).

Another common pattern evident in the interactions of transposases with their respective binding sites is the involvement of DNA bending. Bending angles of between 50° and 90° for the ends of transposons Tn*7*, Mu, and IS*903* have been reported (1, 6, 19). The results from the gel retardation analysis of permuted OE DNA fragments suggest that the binding of Tn*5* Tnp to the OE induces a bend of more than 100°. Circular permutation assays by themselves cannot unambiguously demonstrate that a Tnp-induced bend has occurred (11a); however, the large magnitude of the position-dependent effect and the enhanced Tnp binding to molecules missing nucleosides adjacent to the Tnp binding domain support the argument that Tnp binding causes a severe bend. The recurring importance of bending at the ends could be a reflection of the importance of forming a structured nucleoprotein complex.

The overall center of the bend maps to about position 3 of the OE. Such a bend center suggests the possibility of nonspecific contacts of the Tnp with the neighboring vector DNA. Nonspecific contacts between the γδ transposase and end-neighboring DNA have also been suggested based on hydroxyl radical protection footprinting (35). The proposed center of

bending also correlates with an observation that arose out of the missing-nucleoside experiments, an enhancement of binding by the absence of nucleosides outside of position 6. This may be explained by the fact that the hydroxyl radical removes the nucleoside and cleaves the DNA backbone. These two structural modifications could serve to reduce the energy involved in bending the DNA and therefore stabilize binding. In fact, in light of the large degree of bending of the OE DNA associated with Tnp binding, it is important to keep in mind that any structural change(s) in the OE, including base substitutions, could affect the intrinsic flexibility of the DNA. If bending is important to function, as appears likely, any substitutions that affect bending will also affect transposition activity.

Functional organization of the OE. If positions 6 to 9 and 13 to 19 are involved in the initial binding of Tnp, what role in transposition is played by the bases at positions 1 to 5 and 10 to 12? While the experiments presented here do not directly address this question, some inferences can be made by combining what is known from earlier genetic studies of Tn*5* with information from the work done on other transposons.

All the approaches taken here suggest that positions 1 through 5 of the OE are not involved in the initial Tnp binding domain of the OE. Earlier work, however, has clearly shown that mutations in positions 1 to 3 cause dramatic decreases in transposition (20, 25). The defect introduced by these substitutions could be an inhibition of an event that follows binding, such as bending, synapse formation, cleavage, or strand exchange. Similar roles have been suggested for the outer positions on the ends of other transposons, and this appears to be another emerging pattern general to many mobile elements (6, 13).

Earlier in vivo work suggested that one of the substitutions in the first three positions (the 2T→A substitution) did not form an active nucleoprotein complex (16). This indicates a role for the bases at position 2 in synapse formation. The manner in which the 2T→A substitution prevents synapse formation without interfering with binding is unclear, although a couple of possibilities present themselves. Perhaps the most intriguing model involves the Tnp bound specifically to one OE also making a specific contact with the partner OE, thereby bringing the ends together. Under this model, the 2T→A substitution disrupts this synapse-forming contact. Another possible model is that while the substitution allows binding, the nature of the complex is somehow different and results in an inactive conformation of either the protein or the DNA. It is interesting to compare our results with those from the mutagenesis studies of the ends of Tn*10* and Mu. Substitutions at any of the first three base pairs of the Tn*10* OE allow nucleoprotein formation and excision in vivo, but the mutants are unable to undergo strand exchange with the target DNA (13). In contrast, a mutation at position 1 of one Mu end prevents MuA cleavage at either end, perhaps as a result of an inability to form a stable synaptic complex (31).

A defined role for positions 10 to 12 is more difficult to formulate at this time. It seems that they are not involved in initial binding, as none of these three positions are conserved between the two ends and the missing-nucleoside experiments showed no important interactions. It is interesting that the base substitutions at positions 11 (T→A) and 12 (A→T) were previously shown to be unable to transpose but led to a type of adjacent deletion proposed to result from defective nucleoprotein synapse formation (16). There are a couple, not mutually exclusive, roles that could be played by the bases at these positions. These bases could play a structural role in aligning the two regions of the binding domain to the same face of the helix (see Fig. 7). Consistent with this is the observation that the addition of an A/T base pair between bases 10 and 11 completely abolished binding (data not shown). On the other hand, the bases at 10 to 12, at both the OE and IE, overlap the consensus sites for their respective host protein interactions (Fig. 1b). Interestingly, the 11T→A and 12A→T substitutions appear to bind Tnp normally, but they alter the DnaA recognition site (11). This is consistent with the observation that a 12A→C OE, which maintains the DnaA consensus site, has much higher levels of transposition (16, 25). Although the role, if any, of DnaA at the OE is unknown, these observations suggest that these positions may also play a role in synapse formation.

Conclusions. The ends of Tn*5* have a feature (displacement of the binding site away from position 1) common to many diverse types of transposons. Similar end organizations have been proposed for the relatively simple transposons IS*1* (40), IS*903* (6), and Tn*10* (15, 13) as well as the more complex bacteriophage Mu (41), Tn*7* (1), and γδ (35). This is remarkable in light of the fact that there is little or no sequence homology among the ends of these transposons. This organizational homology must represent a significant structural role for this architecture. Another feature common to many transposons is the requirement that a proper nucleoprotein synaptic complex form before excision can occur (12, 13, 31). It is likely that this requirement exists as a means of preventing excision events that involve only one transposon end and may therefore be potentially deleterious to the host genome and/or the transposable element. The need to form a synaptic complex may, in fact, partially explain some of the similarities that have been seen between the ends of various transposons, including binding to one side of the helix, bending of the DNA, and displacement of the excision site from the binding site.

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