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Transposon Tn5

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Key Words

transposition, transposon, Tn5, IS50, synaptic complex, target DNA, donor DNA, strand transfer

Abstract

Tn5 was one of the first transposons to be identified (10). As a result of Tn5's early discovery and its simple macromolecular requirements for transposition, the Tn5 system has been a very productive tool for studying the molecular mechanism of DNA transposition. These studies are of broad value because they offer insights into DNA transposition in general, because DNA transposition is a useful model with which to understand other types of protein-DNA interactions such as retroviral DNA integration and the DNA cleavage events involved in immunoglobulin gene formation, and because Tn5-derived tools are useful adjuncts in genetic experimentation.

Tn: transposon
IS: insertion sequence
Tnp: transposase
ES: end sequence

BACKGROUND

This chapter describes the molecular mechanism of Tn5 transposition drawing from genetic, biochemical, and structural data gained from over 40 years of work. To say that we will be discussing Tn5 transposition is misleading because the transposition process specifically involves the IS50 element that is a component of Tn5. The chapter also examines the regulatory phenomena that act to maintain Tn5 transposition as a very infrequent event in vivo. The development of Tn5-related systems as molecular genetic tools is described. Finally, the use of Tn5 transposition as a surrogate for studying HIV-1 integration is presented.

Tn5 was initially discovered as a side product of studies on R factor–encoded kanamycin resistance (10). In the course of these studies, it was discovered that the gene encoding kanamycin resistance could be transferred from the R factor to the bacteriophage λ genome by means of a process called transposition. Analysis of the transferred DNA structure revealed that it was composed of two inverted, almost identical sequences called IS50L and IS50R that bracket three genes encoding antibiotic resistances: kanamycin resistance, bleomycin resistance, and streptomycin resistance (see **Figure 1**) (6, 9, 41, 47). The study of these antibiotic resistance genes and their expression is an interesting story but is not presented in this chapter. Rather I focus on the mechanism that gave rise to the λ –Kan^r phages, DNA transposition.

Tn5 transposition occurs through a “cut and paste” mechanism in which the transposon is precisely excised from its original location in the donor DNA and then is inserted into a target sequence (9, 47). As a consequence of the insertion, 9 bp of the target is duplicated, as shown in **Figure 2** (58). The fate of the donor DNA has not been studied. All of the steps in transposition are catalyzed by a 476-residue transposase protein (Tnp) encoded by IS50R (IS50L encodes an inactive truncated version of Tnp) (26, 55). Tnp must interact in a sequence-specific fashion with inverted 19-bp DNA sequences (end sequences or ESs) at the termini

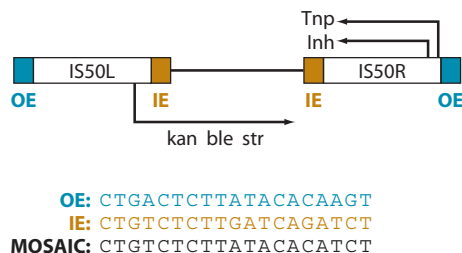


Figure 1

Transposon Tn5. Tn5 is a composite transposon in which two almost identical IS elements (IS50L and IS50R) bracket three antibiotic resistance genes [kan (kanamycin resistance), ble (bleomycin resistance) and str (streptomycin resistance)]. Each IS50 is defined by two ES sequences, OE and IE. IS50R is functional for transposition since it encodes a functional transposase (Tnp) as well as an inhibitor of transposition (Inh). IS50L encodes two inactive, C-terminal truncated versions of Tnp and Inh. The ES sequences OE and IE as well as a hyperactive version of ES (Mosaic) are presented below. This figure is derived from one presented before (42) where it is described in more detail.

of IS50 in order to catalyze transposition but the interaction with target DNA has only modest specificity (25, 34, 57, 60). No host proteins are required for Tnp's action (26).

IS50 has been classified as belonging to the IS4 family of transposable elements based upon the amino acid sequence of its transposase and the nucleotide sequence of the ES DNA (16, 51). The IS4 family has been divided into subgroups (each composed of Tnps with residue identities of between 20% and 50%) of which the one including IS50 has 19 elements found through sequence analysis of 500 bacterial and archaeal genomes (22, 50). It is presumed that all of the elements in the IS50 subgroup transpose via an identical mechanism, although only the canonical IS50 has been studied, and that all IS4 family elements also share mechanistic similarities, although only IS10 has been the object of a similar amount of studies as IS50 (30).

The following pages describe the molecular mechanism of Tn5 (and IS50) transposition separated into the following steps (see **Figure 3**).

1. Tnp first has to identify and bind to the ES sequences within a complex compacted DNA polymer consisting of over a million other sequences (4.6 million for the *Escherichia coli* genome). This must be a daunting challenge.
2. Tnp forms a binary synaptic complex composed of two ES-bound Tnps thus forming the required scaffold for the subsequent catalytic steps. Ordering step 2 following step 1 makes a conceptually easy presentation but is likely to be mechanistically misleading since it is probable that Tnp:Tnp dimeric complex formation occurs prior to Tnp ES binding, as described below.
3. Tnp catalyzes the DNA cleavage that releases the ES sequence at each end from the donor sequence. Cleavage occurs through three catalytic steps involving a water-mediated nicking of one strand (called the transferred strand) generating a 3' OH group, and a strand transfer reaction in which the 3' OH group attacks the opposite strand to generate a hairpin structure. DNA cleavage also results in displacement of the donor DNA. Tnp catalyzes cleavage of the hairpin DNA structure using water as the nucleophile.
4. The resulting cleaved dimeric Tnp-ES DNA complex then binds to target DNA to form a target capture complex.
5. Tnp catalyzes insertion of the ES DNA into the target sequence through nucleophilic attacks by both 3' OH groups on either side of a 9-bp target DNA sequence.

Following completion of the above steps, the Tnp complexes with ES and target DNA are disassembled and the 9 base gaps on either edge of the inserted Tn5 are repaired presumably by host functions (1, 47, 48, 61).

MECHANISM OF Tn5/IS50 TRANSPOSITION

The key molecular structure for all of the steps in the Tn5 transposition pathway is the dimeric

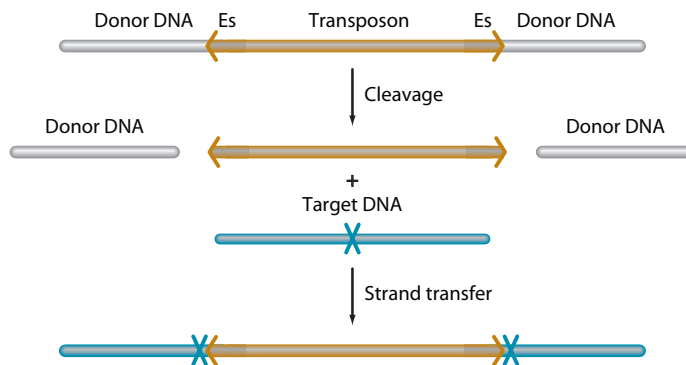


Figure 2

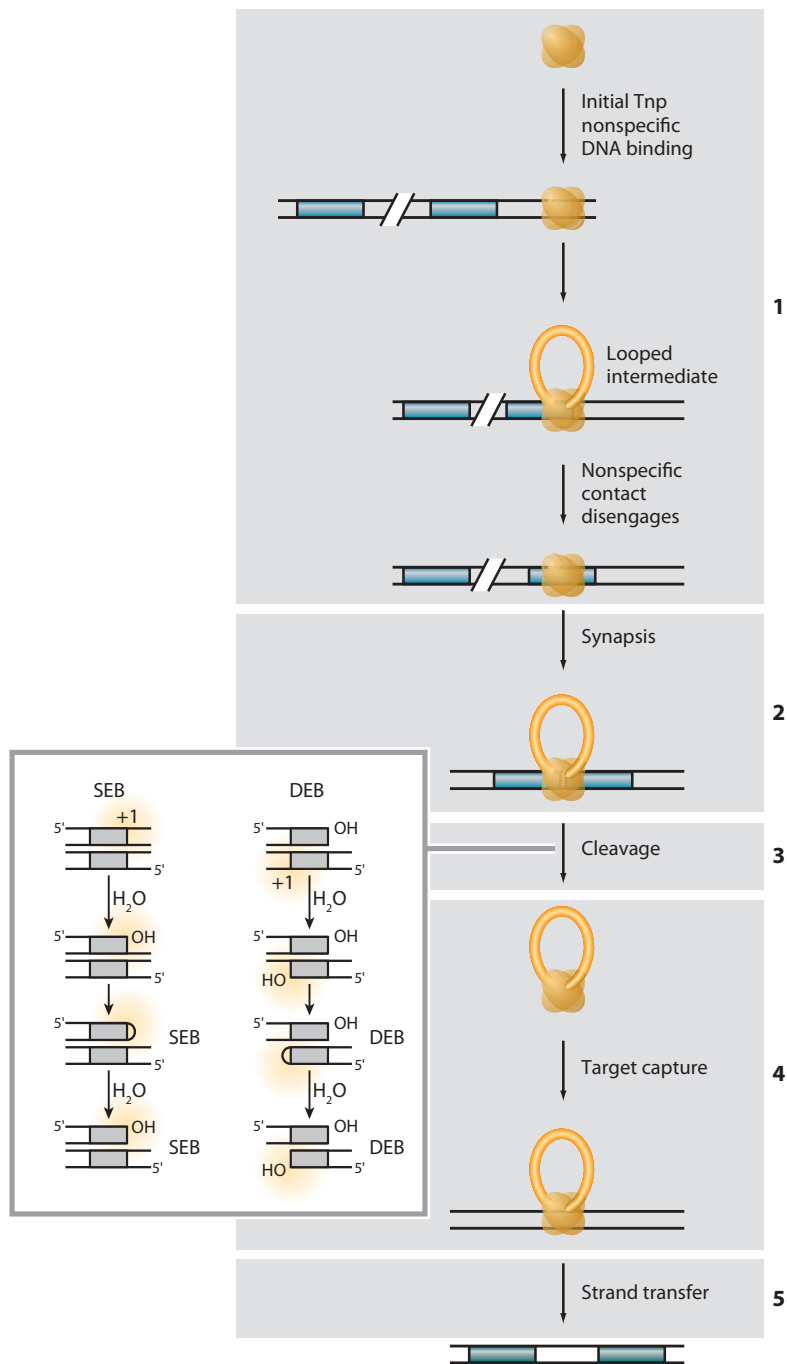
Cut and paste transposition. A simplified version of cut and paste transposition is presented in which the transposon is cleaved free of donor DNA and then inserted into target DNA with an associated micro duplication of the target DNA sequence.

synaptic complex of the transposase with ES DNA. Understanding the features of this complex and the conformational changes that it undergoes throughout the process of transposition is a necessary prerequisite for developing a molecular understanding of how the various steps occur (20, 42). We are very fortunate to have two molecular structures of the Tn5 synaptic complex as they appear sequentially after release of blunt-ended ES sequences following ES-donor DNA junction and hairpin cleavage (20, 37). The structures that exist at other steps would be very desirable but, unfortunately, they have yet to be determined. Nonetheless, genetic, biochemical, and modeling studies provide us with some insights as to how these structures appear as compared to the structures that are well known.

The two known structures for the Tn5 synaptic complex were determined through X-ray crystallography of Tnp-ES complexes formed with two versions of the ES DNA (20, 37) (see **Figure 4**). The structures clearly show the active site residues (D97, D188, and E326), and one of the two divalent metals critical for catalysis by each monomer of the Tnp. Each of the Tnps is shown bound to both ESs, bound to internal nucleotides of one ES and overlapping but more terminal ES nucleotides of the other

Figure 3

Mechanism of Tn5 transposition. Tn5 transposition involves multiple steps including: 1. Tnp finds the transposon-specific ES DNA (blue) through a direct transfer mechanism. 2. Tnp-bound ES DNA forms the dimeric synaptic complex. 3. Tnp (containing two bound Mg^{2+}) catalyzes cleavage of the transposon free of the donor DNA through 3 step processes on each side of the transposon. These cleavage processes occur first at one end (generating a single end break, SEB, product) then at the second end (generating a double end break, DEB, product). 4. The released Tnp-bound transposon then captures the target DNA sequence. 5. Tnp catalyzes strand transfer in which the transposon 3' OH groups attack the target DNA in a 9-bp displaced fashion. 6. Following strand transfer, host functions disassemble the strand transfer complex and repair the two 9-bp single-strand gaps on either side of the inserted transposon.



ES resulting in a *trans*¹ structure. The implication from the structure is that catalysis can only occur in the context of the synaptic complex because each Tnp has initially bound one ES and inserted it into the active site of the second Tnp where catalysis has occurred. All of the genetic and biochemical data support this proposal (42, 47, 48). The locations of the active sites are approximately where one might expect them to be in order to accomplish strand transfer reactions located 9 bp apart on the target.

The Tn5 Tnp has been used as a model through which other transposition systems can be analyzed.

Tnp Finds the ES Sequence

Sequence-specific DNA binding proteins face a daunting challenge in locating their recognition sequences within even the simplest plasmid or bacterial chromosomal sequence. In *Escherichia coli* K12, for example, a site-specific DNA binding protein might need to sample $\sim 4.6 \times 10^6$ sites in order to find its cognate sequence. In addition, the chromosome is condensed making free protein diffusion a difficult feat. The above complications in site location by sequence-specific DNA binding proteins have long been recognized. There are three generally accepted mechanisms through which proteins such as transposases can navigate complex DNA molecules to find their cognate sequences; bind and slide, hopping/jumping, and direct transfer (29). Tn5 Tnp has been discovered to find ES sequences (at least through relative short distances of ~ 1000 bp or so) through a direct transfer mechanism in which a

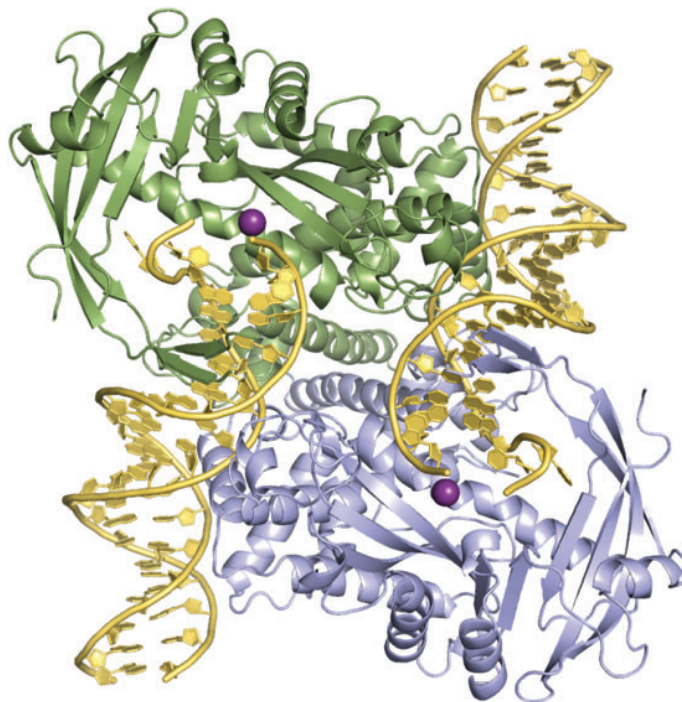


Figure 4

Molecular structure of the Tn5 synaptic complex. X-ray crystallographic analysis of Tn5 Tnp bound to precleaved ES (OE) DNA generated a molecular structure representative of the synaptic complex subsequent to ES-donor DNA cleavage. The structure is dimeric with a Tnp-ES DNA binding relationship in which each Tnp protomer (shown as *purple and green ribbon structures*) bound to both ES molecules (in *yellow*). For each Tnp protomer one ES is bound in a *cis* configuration and one ES is bound in a *trans* configuration. The overall protein-protein and protein-DNA contacts are consistent with biochemical and genetic results. The model shows one Mn^{2+} bound in each active site (*purple spheres*) but each Tnp protomer in the complex is believed to contain $2 Mg^{2+}$ under physiological conditions. This structure is a modification of that shown in Reference 20.

dimeric Tnp complex samples alternative DNA sites much like a monkey traveling through the jungle (1, 2, 61). This conclusion comes from a variety of nonspecific and ES DNA binding studies in which it was found that addition of several hundred nonspecific DNA bp to ESs facilitates the formation of synaptic complexes, that Tnp can form DNA loops between a subset of nonspecific DNA sequences, that loop formation is sensitive to the identity of Tnp residues normally involved in ES binding and Tnp dimerization, that the nonspecific complexes appear to be dimeric, and that

¹Note that the words *cis* and *trans* are used in two different ways when discussing Tn5 transposition. When discussing the structure of the synaptic complex, *cis* and *trans* have a biochemical connotation, one Tnp protomer initially binds one ES (*in cis*) and then inserts the ES into the other Tnp protomer's active site (*in trans*). However, when discussing the propensity of Tnp to act on differently located transposons *in vivo*, *cis* and *trans* has a genetic connotation. Tnp typically has a strong bias toward acting on the transposon that has encoded Tnp's synthesis (*in cis*) rather than diffusing to a transposon located on another replicon or far away on the same replicon (*in trans*).

Tnp bound in nonspecific complexes will directly transfer to ESs. However, as is discussed in greater detail below in regards to the down-regulation of Tn5 transposition, the capability of Tnp to form nonspecific complexes is likely to also play an important role in down-regulating transposition when the initial binding event occurs at longer distances from the ES and thus is likely to contribute to the strong *cis* bias of Tn5 Tnp action *in vivo*.

Before we discuss the synaptic complex, all the available evidence suggests that the nonspecific complexes formed on route to synapsis must bear a significant resemblance to true synaptic complexes. The complexes are dimeric (they require the presence of the Tnp-Tnp dimerization contacts), and they involve at least partial occupancy of the Tnp-DNA contacts found in ES-specific synaptic complexes. However, the nonspecific complexes are not as stable as the synaptic complexes (implying that not all of the Tnp-ES contacts are made to nonspecific sequences in keeping with the sequence specificity of these contacts) and the nonspecific complexes are not catalytically competent (2).

As discussed below, dimeric Tnp-DNA complexes form through a complicated series of protein-protein and protein-DNA contacts. However, prior to the formation of these contacts, Tnp must undergo a significant conformational change. Free Tnp likely exists in solution as a monomer. This monomer is believed to have a conformation different from that seen in the synaptic complex in that the N terminus and C terminus form an intramolecular contact that blocks both Tnp-Tnp dimerization (requiring a free C terminus) and DNA binding (requiring a free N terminus) (21, 28, 69, 73). In fact, biochemical experiments demonstrate that Tnp-Tnp dimerization in the absence of DNA cannot occur for full-length Tnp but can occur for an N-terminal deleted version of Tnp, and, conversely, DNA binding for the monomer version of Tnp can only occur for a C-terminal deleted version of Tnp. Molecular modeling and genetic studies suggests that the inhibitory contact involves N-terminal sequences near residue 40 and the W450 residue

(28). Thus DNA binding and dimerization must require a conformational change that moves the N terminus away from the C terminus, or else initial DNA binding must occur during translation while the partially synthesized Tnp is still tethered to its mRNA. This mechanism is discussed again in the section devoted to the regulation of transposition.

Synaptic Complex Formation

The synaptic complexes shown in **Figure 4** must have a strong resemblance to those that are formed on ES DNA in which the ES is covalently linked to donor DNA. However, there are two known caveats to this generalization: There is no donor DNA present in the **Figure 4** structure, and the synaptic complex formed on ES-donor DNA molecules is asymmetric (see below). However, genetic and biochemical experiments have demonstrated that the bulk of the important Tnp-Tnp contacts and Tnp-ES contacts found in **Figure 4** are also be present in the synaptic complex formed prior to ES-donor DNA cleavage (12, 15, 32, 33, 40, 43–45, 47, 62, 64, 66, 67, 75, 76). Thus we use the structure in **Figure 4** as a guide for understanding the structure of the synaptic complex found in transposition.

The synaptic complex shown in **Figure 4** (and present in the complex formed by an N-terminal truncated version of Tnp) demonstrates a modest Tnp-Tnp interface involving residues 458–468 and centered at residue 462 (20). This proposal is supported by the observation that mutations at residues 462 and 466 are defective in synapsis for Tnp and dimerization for the N-terminal truncated form of Tnp (64).

The Tnp-ES DNA contacts are far more complex than the protein-protein dimer contacts. They involve two general categories of interactions each of which can be subdivided (see **Figure 5**) (20). One category of Tnp-ES DNA contacts is called *cis* contacts; they are believed to be the first ones formed on ES DNA. The major group of *cis* contacts involves a protein sequence extending from residue 26 to residue 66.

These residues contact ES DNA nucleotide pairs from 6 through 17 (see **Figure 5**). A second group of *cis* contacts use residues 342 and 348 and nucleotide pairs 4 through 9. The second category consists of contacts formed after the ES containing DNA is inserted into the active site of the opposite Tnp protomer. These *trans* contacts to nucleotide pairs 1 through 9 fall into several different groups. First, there are multiple contacts between residues in and around the active site. Second, there are contacts that manipulate the conformation of the DNA structure near to where cleavage is going to occur. Finally there is a region from residue 243 to 253 that is unstructured in the absence of DNA but forms a β -loop structure that appears to clamp the ES DNA into the active site. These β -loop contacts appear to play an important role in precisely orienting the ES DNA for catalysis because β -loop mutants are perturbed for all transposition steps (62).

The crystal structure of the synaptic complex as it exists after cleavage does not contain any direct information regarding where the donor DNA sits prior to cleavage. The donor DNA is believed to be closely associated with Tnp in the synaptic complex based on DNase protection studies and analyses of donor DNA preferences in synapsis, and because the presence of covalently linked donor DNA impairs synapsis (5, 27, 66).

The location of donor DNA sequences has been modeled on the structure as following a positively charged groove that would fit a

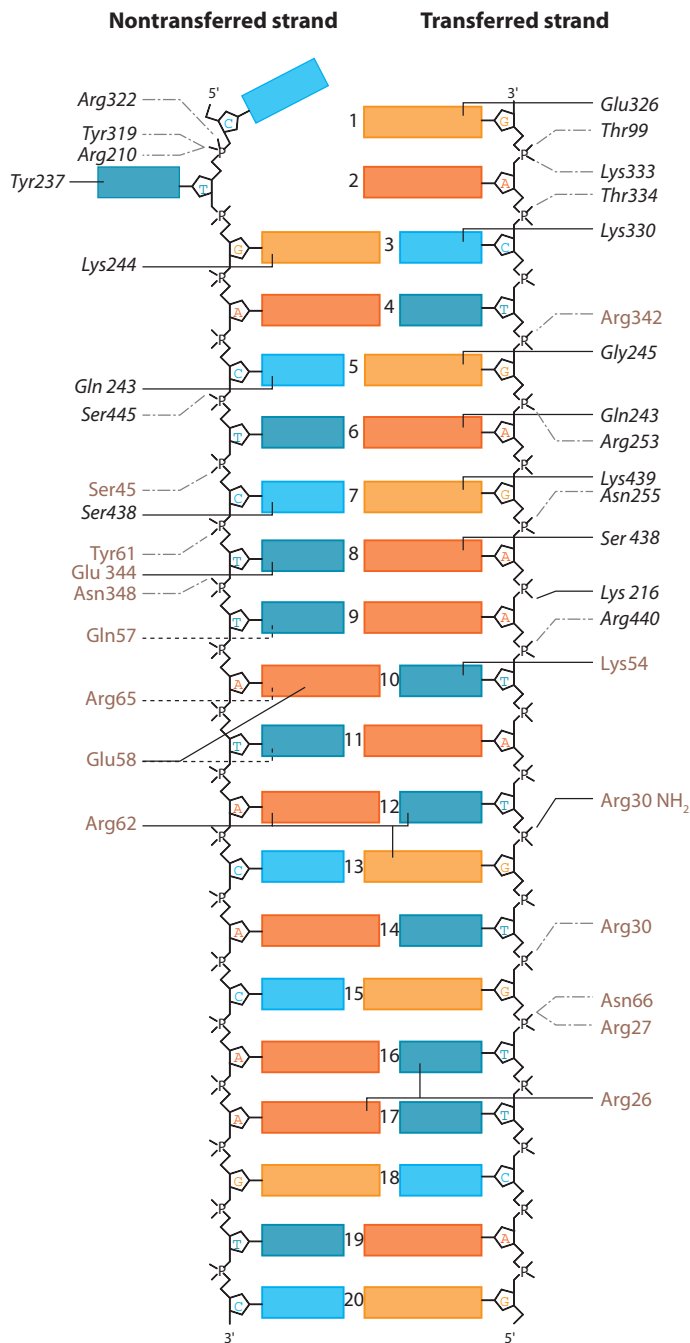


Figure 5

Tnp-ES (OE) DNA contacts found in the Tn5 synaptic complex. The Tnp-ES contacts are deduced from the X-ray crystallographic structure shown in **Figure 4** and from genetic and biochemical studies. The Tnp residues forming *cis*-ES contacts are indicated in blue roman type while the italicized, black residues indicate *trans*-ES contacts. Solid connecting lines represent base-specific contacts while dotted lines represent water-mediated interactions with bases. Phosphate interactions are also shown. This figure is an updated and simplified version of the figure first shown in Reference 46.

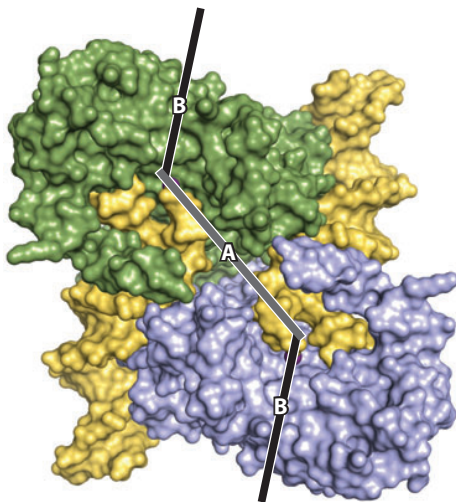


Figure 6

Proposed path of DNA in the target capture complex. Genetic and modeling studies have led to the proposal that target DNA sits in grooves B and A across the synaptic complex (27). It has been proposed that donor DNA is also situated in groove B prior to cleavage of the donor-ES DNA boundary (27). In this figure the synaptic complex is in the same orientation and has the same color codes as that shown in **Figure 4** but in this case Tnps and DNAs are in space-filled presentations. This figure is a modification of one first presented in Reference 27.

simple extension of the DNA shown in the synaptic complex structure (see **Figure 6**). This proposal is consistent with the recent finding that two mutations near that groove impair synaptic complex formation (27). This positively charged groove also plays an important role in binding another DNA molecule in a subsequent transposition step (see below).

The observation that the presence of covalently linked donor DNA impairs synapsis is particularly interesting. Synaptic complex formation occurs at a faster rate and has a greater yield when no donor DNA is attached to the ES, or when there is a nick in one strand between the donor DNA and the ES (27, 66). The positive effect of nicking the donor DNA-ES linkage can be understood as follows. The donor DNA-ES boundary is in an unfavorable conformation whose negative effects can be relieved by introducing a nick at the boundary and/or

by providing some partially compensatory favorable contacts between Tnp and donor DNA nucleotides. The nicked DNA is an intermediate in the DNA cleavage process as described below.

ES DNA-Donor DNA Cleavage

The cleavage process through which transposase catalyzes the release of transposon DNA from donor DNA is obviously a key event in DNA transposition. In addition, the elucidation of this event led to the discovery of two very interesting and surprising mechanisms. A key feature of the cleavage process is that it involves two versions of phosphoryl transfer chemistry: phosphodiester bond cleavage in which H_2O provides the nucleophile and strand transfer forming a new phosphodiester bond in which a DNA 3' OH provides the nucleophile (49). One of the more exciting discoveries in the field of cut and paste transposition was the observation that Tn10 DNA cleavage proceeded through a three-step hairpin mechanism in which first the donor DNA strand was nicked creating a 3' OH group, then the 3' OH group served as a nucleophile in attacking the nontransferred strand, thus creating the hairpin, and finally the hairpin was nicked (36). Almost simultaneously the same discovery was made for Tn5 (**Figure 3**) (11). This was an important mechanistic discovery because it offered an explanation for how a single active site could cleave two DNA strands with opposite polarities. It also provided a mechanistic background for understanding DNA cleavage catalyzed by RAG-1 in the immunoglobulin gene formation system, the DNA cleavage process for some eukaryotic DNA transposons such as hAT elements and the processing events for *Borrelia burgdorferi* DNA telomeres (7, 23, 74). An important observation made in the analysis of hairpin formation is that a flipped base in the ES functions to facilitate or stabilize the DNA rearrangements required for hairpin formation (4, 14).

A second surprising property of transposon DNA-donor DNA cleavage is that this event is asymmetric. That is, within the context of the

synaptic complex, the release of the transposon from donor DNA occurs with a break on one end followed by a break at the second end; these appear to be obligatory, sequential steps (18, 62). This asymmetry was discovered because a low-molecular-weight inhibitor of transposase or performing the reaction at 25° selectively blocks the second cleavage event. In addition, a mutation in one of the β -loop ES contact residues selectively impairs the second cleavage process, suggesting that the β -loop ES contact plays a roll in rearranging the synaptic complex so that the second DNA can be cleaved. It is possible that the asymmetry-dependent step is either DNA nicking or hairpin formation. It is not known which of these two steps is critically dependent on this conformational asymmetry.

The fate of the donor DNA is not known for Tn5 transposition. There are three possibilities. First, the donor DNA may be lost. Second, the donor DNA may be sealed by some sort of blunt end repair process resulting in the generation of a repaired sequence that, if done precisely, would contain a 9-bp duplication at the site from which the transposon had been excised. Finally, the donor DNA may be repaired by a homologous recombination repair reaction with another copy of the transposon-containing sequence. This last possibility would regenerate a transposon-containing sequence identical to the original donor arrangement. Note, as described below, Tn5 transposition occurs preferentially from newly duplicated DNA in *E. coli* when there are two copies of the transposon-containing sequence.

Target Capture

For the final catalytic step in transposition to occur, the transposase in the synaptic complex must bind (or capture) the target. Recent studies have demonstrated that a conformational change in the ends of the newly released ES DNA must occur to facilitate binding to target DNA. That is, the 5' end of the initial cleaved ES DNA must be moved in order to permit target DNA capture. We are fortunate to have

solved a series of synaptic complex structures. One of these structures resulted from an examination of synaptic complexes in which cleavage has occurred within a crystal lattice (S. Lovell, I.Y. Goryshin, W.S. Reznikoff & I. Rayment, unpublished; 37). The synaptic complexes were formed in the absence of Mn^{2+} with a hairpin DNA. Crystals were formed and then Mn^{2+} was added by soaking the crystals in an appropriate solution. The resulting structure contained a DNA in which the hairpin had been cleaved within the crystal lattice. The resulting structure has the 5' terminal base at the cleavage site stacked on top of the 3' base in an arrangement that would impair a target from being properly positioned for strand transfer. An important feature of this structure is that the penultimate phosphate on the 5' strand is contacted by three important residues in the active site: R210, Y319, and R322. This structure is essentially identical to the synaptic complex formed with an ES DNA lacking a 5' terminal phosphate. If one generates a synaptic complex with precleaved DNA containing, instead, a 5' terminal phosphate (the biologically correct DNA), the 5' base loses its stacking arrangement with the 3' terminal base and swings away from the transferred strand with the 5' terminal phosphate now coordinated to the R210, Y319, and R322 residues (36). This structure is now competent to bind target DNA in a fashion that allows efficient strand transfer. What is posited to have occurred is that following cleavage in solution, the 5' strand moves out of the way of the 3' end by breaking and forming phosphate contacts to the R210, Y319, R322 residues first to the penultimate phosphate then to the terminal phosphate. When cleavage occurs in the crystal, the crystal packing prevents the appropriate rearrangement.

The next questions in studying target capture are: What is recognized in the DNA as a target? And how does the synaptic complex recognize the target? In approaching these questions, note that 9 bp of the target sequence is duplicated following strand transfer. This 9-bp duplication implies that a 9-bp staggered strand transfer has occurred, which suggests, in turn,

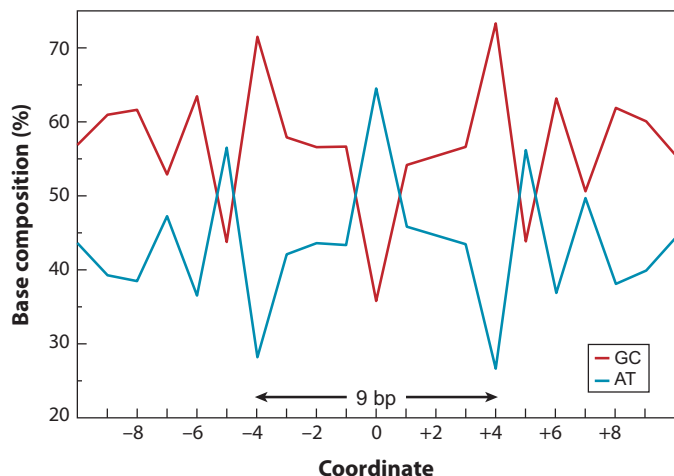


Figure 7

Tn5 transposition target preferences. The use of a Tn5-derived transposon as a mobile primer-binding site has resulted in thousands of target sites being sequenced (for an example, see Reference 59). This figure reproduces in modified form a figure presented before (59) in which the base composition preferences are plotted around the core 9-bp target.

that the fundamental target sequence for target capture is 9-bp long.

The Tn5 transposition system has become a convenient biotechnology tool and has been used by a number of investigators as either a knock out mutagen or as a mobile primer-binding site in large scale sequencing efforts (see below). Thus thousands of inserts have been sequenced and from these data one can study the sequence biases involved in Tn5 insertion, meaning the sequence biases involved in target capture (60). From this analysis it has been determined that the preferred target sequence extends approximately 19 bp with a 9-bp core sequence (as predicted from the observed 9-bp duplication generated during integration) surrounded by ~5 bp on either side in which some preferences are demonstrated (see **Figure 7**). Thus the target for Tn5 is not completely random, although almost any sequence can be chosen at some frequency, and the randomness is sufficient for most biotechnological applications.

How does the synaptic complex bind to the target (or, put in another way, what determines the target bias of Tn5 transposition)?

Early analysis of the synaptic complex crystal structure posited that the 9-bp core target sequence likely bound to a cleft that spans the region between the two active sites containing the two 3' transposon ends (**Figure 6**) (20, 52). This cleft has several positively charged residues suitable for binding DNA phosphates and the cleft has the right dimensions for binding 9 bp. But where do the adjoining 5-bp target sequences bind in the synaptic complex? Initial clues came from the isolation of two mutations that altered target specificity. These were located outside of the 9-bp target region to residues slightly beyond the active sites. The second clue came from an examination of possible positively charged binding grooves extending away from the active sites. These were examined by site directed mutagenesis/target-specificity assays, leading to the conclusion that groove B in **Figure 6** likely described the path occupied by the adjoining target sequences (27).

The channel B posited to be part of the target capture sequences overlaps with the sequences thought to bind donor DNA described above (see section on Synaptic Complex Formation). This suggests that target capture cannot occur until after DNA cleavage has occurred in agreement with the results of molecular studies of Tn10 transposition (56).

It has previously been discussed that nicking the boundary between donor backbone DNA and ES DNA enhances synaptic complex formation, suggesting that a DNA bend at the juncture enhances complex formation. A similar finding has been found for target binding. Introducing a nick at the predicted scissile phosphate at the end of the preferred 9-bp target sequence enhances the specificity and yield of strand transfer products, presumably due to an enhanced formation of target capture complexes (70). Thus DNA bending likely occurs at the ends of the 9-bp target during target capture.

Strand Transfer

Following formation of the target capture complex, the transposase catalyzes the strand

transfer event in which the 3' OH group of each ES sequence serves as the nucleophile. The two strand transfer events at either end of the transposon appear to occur independently. That is, in vitro one strand from the complex is transferred into the target DNA followed by the second transfer event (18, 44). No experimental evidence exists that would indicate whether these two events are actually obligatory sequential events (suggesting an asymmetry of action in the strand transfer complex) or just independent events.

Tnp Removal and Gap Repair

The strand transfer complex described above is quite stable in vitro (26). Presumably, some host function must “remove” the two molecules of transposase from the complex much as the ClpX chaperone removes the Mu transposase from its strand transfer complex (39). Following removal of the transposase the two 9-base gaps are likely filled in and sealed by host functions.

DOWN-REGULATION OF Tn5/IS50 TRANSPOSITION

One of the features of Tn5 transposition that I found most striking during my earliest studies was the very low frequency at which it appeared to occur. Various observations have determined that the frequency of Tn5 transposition is less than 10^{-5} per cell per generation. This low frequency is not a reflection of either low Tnp abundance [~ 100 molecules exist per cell (35)], the abundance of the substrate (at least one exists per cell), or a low abundance of transposition targets (at least 4.6×10^6 per *E. coli* cell). In addition, despite numerous attempts, no transposition was detected in vitro when wild-type protein and DNA components were utilized. Thus it is clear that the Tn5 transposition system is inherently very inactive. Tn5 transposition has an additional striking biological property; transposition has a strong in vivo bias for occurring in *cis* (9, 46, 47). That is, Tnp is much more effective (> 50 -fold) at catalyzing transposition of the ES-bounded sequence that

encoded the transposase molecules than it is at catalyzing transposition of a second, distantly located ES-bounded sequence.

One can rationalize the low-frequency, *cis*-biased properties of Tn5 transposition as being the product of a selected balance between sufficient transposition to allow for maintenance and spread of the transposon vs transposition's potential to kill the host cell either through donor DNA damage as a result of transposon excision or due to the formation of deleterious mutations. The general property of low transposition frequency decreases the probability of genetic damage to the host while the *cis* bias, when coupled with host regulation of transposition, leads to transposition occurring immediately following passage of the replicating fork, thus resulting in donor DNA loss not being a lethal event.

Although the general theme of low-frequency, *cis*-biased transposition makes perfectly good sense from a biological perspective, the multiplicity of molecular mechanisms leading to those properties was a total surprise to me. As described below, several of these mechanisms were discovered and studied through the isolation and analysis of Tn5 mutants that were hyperactive for transposition. The isolation of these mutants also made possible the development of the in vitro transposition system as well as the generation of synaptic complexes in vitro for the successful X-ray crystallography work.

Tnp Is Not Expressed off Read-Through Transcripts

The Tnp gene is oriented in Tn5 facing inwards toward the bulk of the transposon. Therefore read-through transcripts from the genomic region in which the transposon was located would read into the Tnp encoding gene and thus could lead to high levels of Tnp synthesis. Although read-through transcription does occur, Tnp translation off the transcript does not occur efficiently due to the formation of a read-through mRNA secondary structure that would block Tnp translation initiation. Efficient translation initiation does occur from bona fide Tnp

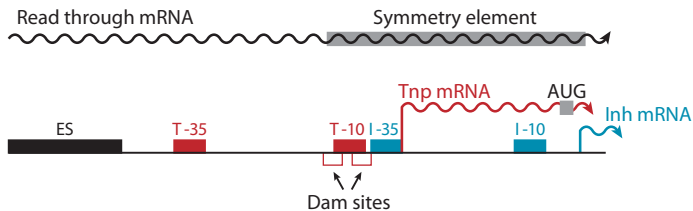


Figure 8

Regulatory elements controlling Tnp and Inh synthesis. Tnp and Inh expression is dictated by a complex series of elements that are close to the ES DNA element (38). The two promoters demarcated by -35 and -10 sites (T-35 and T-10 for the Tnp promoter and I-35 and I-10 for the Inh promoter) overlap and are likely competitive for RNA polymerase binding. The Tnp promoter is negatively regulated by Dam DNA methylation (72). Read-through transcripts fail to encode Tnp due to an RNA secondary structure (59).

transcripts starting within the transposon. This difference in translation initiation for the two transcripts is due to the positioning of a large symmetrical DNA sequence near the termini of Tnp5 (Figure 8) (38, 59).

Read-through transcripts do program the synthesis of an N-terminal truncated form of Tnp called the inhibitor (Inh) (38). As discussed below, Inh is a *trans* dominant negative inhibitor of transposase activity. Thus, not only do read-through transcripts not synthesize Tnp, they also do synthesize Inh and thus these transcripts likely impair transposition.

Auto-Inhibition of Tnp ES DNA Binding and Tnp Dimerization

The monomeric form of Tnp can neither bind ES DNA nor dimerize efficiently. Removal of the N-terminal 55 residues (containing the bulk of the primary DNA determinant) allows efficient dimerization (15). Removal of the C-terminal 107 residues (containing the Tnp dimerization determinants) allows efficient ES DNA binding (73). Model building studies of monomeric Tnp from the X-ray crystallographic structures suggest that this is a consequence of the C terminus (somewhere near residue W450) nestling close to the N terminus and thus blocking both activities (28, 47). W450C and W450S hyperactive mutations (but not a W450F mutation) partially re-

lieve this impairment of both activities (28). W450 is a conserved residue among IS50 Tnp-related transposases (50). This configurational auto inhibition is also partially relieved by the hyperactive L372P mutation that likely causes a break in an α -helix that normally positions W450. L372 also partially relaxes the strong *cis* bias of Tnp5 transposition (W250C has not been tested for this property) (68). One possible explanation is that part of the *cis* bias is related to the intramolecular inhibition. The above model suggests that successful Tnp utilization can occur through a cotranslational model. The N-terminal primary DNA binding domain binds to the DNA while the transposase is still being translated, thus leading to transposition that occurs primarily *in cis*.

Note that L372P is historically important because it was incorporated into the “standard” hyperactive Tnp that has been used for all biochemical and structural studies.

Getting Lost While Searching for ES Sequences

It has been proposed that Tnp finds ES DNA through a direct transfer mechanism. However, the experiments that led to this proposal also demonstrated that nonspecific DNA-Tnp complexes are surprisingly stable and as the distance between the initial nonspecific DNA contact and the ES sequence grows longer than a few hundred bp, the enhancement of synapsis provided by the direct transfer mechanism declines. Presumably, if the transposase binds more than a few thousand bp away from the ES, ES localization will be inhibited. Thus nonspecific DNA can serve as a trap preventing active Tnp from being able to participate in transposition; in other words, depressing the frequency of transposition. This model for down-regulating transposition should also contribute to the observed *cis* bias; Tnp is most likely to find the ES if it binds close to the ES in the first place, and such neighborhood binding is most likely to occur for Tnp molecules transcribed and translated by a Tnp gene in *cis* (1, 2, 61).

Inh: inhibitor protein

Tnp Recognition of ES Sequences is Suboptimal

A whole series of quite distinct Tnp mutations have been isolated that enhance Tnp-ES DNA binding. The first group (Y41H, T47P, E54K, and E54V) is located in the N-terminal sequence of Tnp that plays a role in initial or *cis* binding to ES DNA (76). E54K is historically important because it was incorporated into the “standard” hyperactive Tnp that has been used for all biochemical and structural studies. Another residue that impairs Tnp-ES DNA *cis* binding is E344. E344 apparently has a steric clash with ES DNA (67). This can be relieved by the hyperactive E344A mutation. Finally, there is a residue (P242) at the base of the β -loop clamp that impairs formation of critical *trans* Tnp-ES DNA contacts (62). Mutant Tnps containing P242A or P242G are hyperactive possibly because these mutations increase the flexibility of the β -loop clamp.

Finally, the natural ES sequences [called OE for Tn5; IS50 has one OE and one IE (**Figure 1**)] are somewhat defective for Tnp binding. The optimal ES sequence is a merged version of the OE and IE sequence called ME (75) (**Figure 1**). Apparently, the critical base pair generating this impairment for OE is the A/T located 4 bp away from the cleavage site (63). The ME sequence has been used for many biochemical applications.

Residues E110 and E345 Impair Tnp Activity

Hyperactive mutations that were isolated early in our studies included E110K and E345K (71). E110 and E345 face each other in the structure and E345 is immediately adjacent to residues that contact the ER DNA. We previously proposed that E110 and E345 repelled each other unless either a bridging positive ion was present or one or the other residue was changed to a lysine to enable a direct ionic interaction (20). This model has yet to be tested so the mechanistic basis for the impairment caused by E110 and E345 is still a matter of conjecture.

The Roles of the Inhibitor Protein Inh, Dam DNA Methylation, DNA Replication, and DNA Transfer in Transposition Regulation

The IS50 element that is fully functional for transposition is IS50R. IS50R encodes two proteins; Tnp and an N-terminal truncated version of Tnp called the inhibitor (Inh). Inh, which lacks the first 55 residues of Tnp and thus lacks critical DNA binding residues, is a *trans* active inhibitor of transposition that under steady-state situations is found at a ~4:1 ratio relative to Tnp (13, 31, 35, 38). Inh acts by forming heterodimeric complexes with Tnp that thus removes Tnp protomers that would otherwise form functional homodimeric synaptic complexes (21).

The molecular regulators of Tnp and Inh expression form a very interesting arrangement. Although they are expressed from the same reading frame and one might expect that they reflect two different translation events from the same mRNA species, Tnp and Inh are, in fact, expressed off two different transcripts programmed by two different overlapping and competitive promoters as pictured in **Figure 8** (38). The competitive transcription arrangement in turn results in a mechanism for modulating the Tnp:Inh ratio and thus the transposition frequency. For instance, the transposition frequency is proposed to be enhanced immediately following passage of the replicating fork through the end of the transposon and following introduction of foreign DNA carrying the transposon into wild-type *E. coli* cells (9, 47). The key to these phenomena is the presence of two Dam DNA methylation sites (GATC sequences) overlapping the -10 region of the Tnp promoter (**Figure 8**) (72). Full methylation of the sites is posited to impair RNA polymerase binding to the Tnp transcript promoter that in turn allows access of RNA polymerase to the Inh promoter. This arrangement results in a 4:1 steady-state ratio of Inh:Tnp. Passage of the replicating fork generates transient hemimethylation of the two product DNAs that apparently allows RNA polymerase binding to

the Tnp promoter (and consequently reduced RNA polymerase binding to the *Inh* promoter) and thus shifts the protein balance in favor of Tnp. A similar phenomenon (only involving Tnp synthesis) has been studied in more detail in the *Tn10* system (53). Lack of full *Dam* site methylation also enhances the expression of Tnp (and conversely decreases *Inh* expression) encoded by newly introduced DNAs because DNAs introduced by conjugation should be hemimethylated and phages DNAs are typically under methylated (54).

Tn5: A MOLECULAR GENETIC TOOL

Larry Taylor's discovery of the phage/transposable element Mu introduced a powerful new experimental tool for molecular genetics (65). All one needed to do was to infect cells with Mu and isolate the Mu lysogens. The Mu lysogens represented Mu integrations at apparently random locations throughout the genome. Like other transposition systems, Tn5 transposition has also become a powerful experimental tool. There are three key experimental features to the Tn5 system. First, a variety of mutation types can be generated with Tn5 including insertions, deletions, inversions, and gene fusions. Second, Tn5 mutagenesis strategies are technically quite simple. Third, the technology is quite flexible especially in regards to the DNA found within the body of the transposon (between the two ES sequences), which, within certain general length constraints, can be any desired sequence including reporter genes, origins of conjugal transfer, site-specific recombination sequences, promoters, primer binding sites, or molecular bar codes. [For further details of the applications, the reader is referred to (8, 47, 49).]

It is useful to consider the general classes of mutations that can be generated via transposition. These include the original and most commonly used: insertion knockouts. One can also insert much smaller sequences sometimes only a few codons in length through a process that involves insertion first followed by excision of the

bulk of the transposon either using a restriction-ligation approach or site-specific recombinase-mediated excision. The generation of insertion mutations also allows the generation of gene fusions between the target gene and the gene carried within the body of the transposon. One can also generate defined deletions and inversions of adjoining DNA. Finally, one can generate plasmids carrying DNA that adjoins a transposon insert.

Another important consideration is the general strategy used to perform the transposition mutagenesis. For Tn5 there are three basic strategies. The original and still widely used approach makes use of strictly *in vivo* strategies (8). The Tn5-like transposon defined by inverted ES DNAs is imported into a target cell on a phage DNA or a plasmid DNA. Typically, the transposon carries a selectable marker (an antibiotic resistance gene), the Tnp encoding gene is located outside the body of the transposon, and the phage or the plasmid is a suicide vehicle unable to establish a permanent existence in the host cell. Introduction of the transposon containing DNA and selection for the antibiotic resistance results in selection for progeny cells that have a stable insert of the transposon in their genomes.

A second approach is to use *in vitro* transposition. For Tn5 this is a simple strategy because the only required macromolecules are the transposon DNA (versions of which are commercially available), hyperactive Tnp (also commercially available), and target DNA (26). The three macromolecules are mixed in a suitable buffer containing Mg^{2+} and incubated to yield the transposition events. The products are then typically introduced into appropriate cells.

A final strategy is a mixed protocol involving an *in vitro* step (forming intermediate synaptic complexes in which the transposase is stably bound to the transposon), electroporation of the complex into the target cells and *in vivo* selection of the inserts (24). This protocol, which is only available for Tn5 and Mu transposition systems, bypasses the need for and avoids possible barriers to the microbial genetic procedures used in standard *in vivo* approaches.

Tn5 Tnp AS A SURROGATE FOR STUDYING HIV-1 INTEGRASE

The structural studies of Tn5 Tnp and retroviral integrases indicated that these proteins were all members of a superfamily of proteins that shared similar active site architectures (**Figure 9**), the same active site DDE residues, and similar catalytic mechanisms (19, 52). HIV-1 integrase is obviously an important subject for detailed investigation but it is not an easy topic. For instance, although a huge effort has been mounted to study this protein through X-ray crystallography, we still have no structure for the full-length integrase and also no structure for the integrase bound to its cognate DNA sequences. So this circumstance opens up the possibility of using Tn5 Tnp as a surrogate for studying HIV-1 integrase especially in cases in which structure/function studies need to be pursued, a risky endeavor because the conclusions from Tn5 Tnp research can only be applied to HIV-1 integrase research with great caution. Nonetheless, in one promising study, small molecule inhibitors of Tn5 Tnp have been identified (3). Of 20 studied so far, 6 cross-inhibited HIV-1 integrase. A few hundred HIV-1 integrase inhibitors were studied against Tn5 Tnp; 15% demonstrated cross inhibition (18). Thus the cross sensitivities were not complete yet the high frequency of cross inhibition was very encouraging.

CONCLUSION

The Tn5 transposition system, with its genetic, biochemical, and structural information, obviously provides a good foundation for understanding many, but not all, transposition systems. This limitation on generalizing the findings of Tn5 research is due to an exciting finding; there are several different mechanisms that have evolved for effecting transposition. A recent description of the various known mechanisms can be found in an excellent review by Curcio & Derbyshire (17).

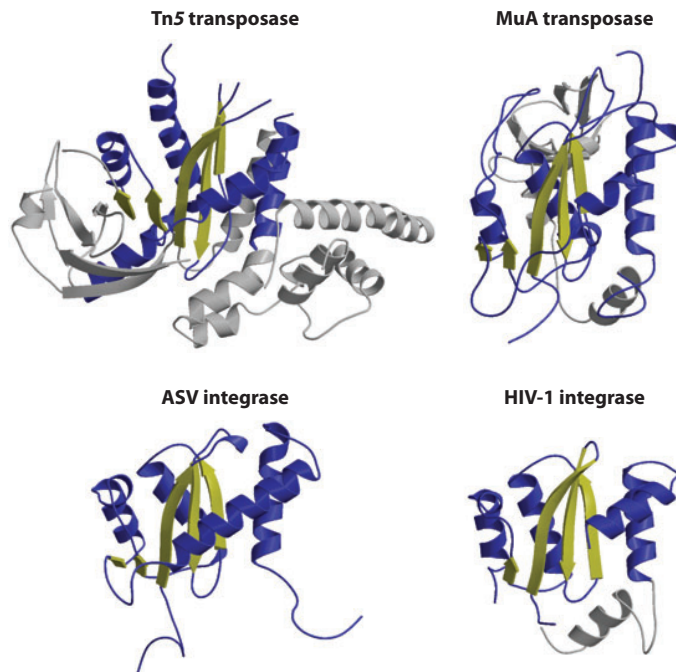


Figure 9

Tn5 Tnp belongs to the retroviral integrase superfamily. Ribbon diagrams demonstrate that the Tn5 Tnp (or an N-terminal deleted version of Tnp shown in this figure) has the same secondary structure architecture around the active site as HIV-1 and ASV integrase and MuA transposase. Closer examination demonstrates that the proteins have similar DDE active sites. This figure is copied from one presented before (19).

The understanding that we have of Tn5 transposition is, however, a static one, with a detailed picture of many of the individual events but little information about the dynamic processes that connect the events. For instance, donor-ES DNA cleavage is an asymmetric series of events; first one side of the transposon is released, then the second one (18, 62). We do not know the nature of the asymmetry nor do we know how the synaptic complex proceeds from one structure to the next. What we do know is that the Tnp β -loop, even though it is some distance from the active site residues, plays a key role in the manipulation of the DNA at this and many other steps (62).

Another example of structural dynamics involves the preparation of the cleaved tip of ES DNA to make room for target capture

(37). Again, we know critical Tnp residues for this process but we can only guess how they function. Certainly, dynamic changes at other steps are waiting to be discovered.

DISCLOSURE

The author receives licensing fees for the sale of biotechnology kits using Tn5 transposition-related systems from Epicentre Technologies, Inc.

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