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Multiple DNA Processing Reactions Underlie Tn7 Transposition

Patricia A. Gary¹, Matthew C. Biery¹, Roland J. Bainton² and Nancy L. Craig^{1,2*}

¹Howard Hughes Medical Institute, Department of Molecular Biology & Genetics, Johns Hopkins University School of Medicine, Baltimore MD 21205, USA

²Department of Biochemistry & Biophysics, Department of Microbiology & Immunology George W. Hooper Foundation, San Francisco CA 94143, USA

The bacterial transposon Tn7 uses a cut and paste mechanism to translocate between non-homologous insertion sites. In the first step of recombination, double-strand breaks at each transposon end disconnect the element from the donor backbone; in the second step, the now exposed 3' transposon ends join to the target DNA. To dissect the chemical steps in these reactions, we have used mutant transposons altered at and near their extreme termini. We find that the initiating double-strand breaks result from a collaboration of two distinct DNA strand processing activities, one mediating cleavages at the 3' ends of Tn7, which can be blocked by changes at the transposon tips, and another mediating cleavages at the 5' ends. The joining of exposed 3' transposon ends to the target DNA can be blocked by changing the transposon tips. Our results suggest that the target joining step occurs through two usually concerted, but actually separable, reactions in which individual 3' transposon ends are joined to separate strands of the target DNA. Thus Tn7 transposition involves several distinct DNA processing reactions: strand cleavage and strand transfer reactions at the 3' ends of the transposon, and separate strand cleavage reactions at the 5' ends of the transposon.

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*Corresponding author

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Introduction

Transposable elements are discrete DNA segments that can translocate between non-homologous sites. Mobile elements encode two types of functions for transposition: recombination proteins, which often work in collaboration with host proteins, and DNA sequences at the ends of the transposon, which participate directly in recombination, i.e. are the substrates upon which recombination proteins act (Berg & Howe, 1989; Craig & Kleckner, 1989; Mizuuchi, 1992b). The recombination sequences at the two ends of an element include specific binding sites for transposition proteins, and DNA sequences at the tips of the element critical for steps following recombinase

Abbreviations used: ELT, excised linear transposon; DSB, double-strand break; SEJ, single-end join; DI, double insertion; BSA, bovine serum albumin; Chaps, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propansulfonat; TBE, Tris-borate/EDTA. binding, i.e. the breakage and joining events (Derbyshire *et al.*, 1987; Huisman *et al.*, 1989; Baker & Mizuuchi, 1992; Derbyshire & Grindley, 1992; Jilk *et al.*, 1993; Haniford & Kleckner, 1994). These terminal recombination sequences are generally arranged as inverted repeats so that recombination proteins are similarly positioned with respect to the element termini where breakage and joining occur.

A variety of different DNA products can result from transposition, depending upon which strands of the transposon ends undergo breakage and joining (Craig & Kleckner, 1989; Mizuuchi, 1992b). Central to all transposition reactions examined in biochemical detail are DNA strand breakages that expose the 3' ends of elements and subsequent joining of these exposed ends to the target DNA. Whether breakage at the 5' ends also occurs has profound effects on the nature of the recombination products. If the 5' transposon ends remain unbroken and thus connected to the donor site, transposition results in a fused structure containing the transposon, the donor backbone and the target DNA called a Shapiro or strand transfer intermediate (Arthur & Sherratt, 1979; Shapiro, 1979; Craigie

Present address: R. J. Bainton, Department of

Anesthesiology, University of California, San Francisco, CA 94143, USA.



Figure 1. Tn7 transposition proceeds through two distinct steps: donor cleavage and target joining. Various substrates, which differ in whether they have undergone donor cleavage, and their recombination products are shown. The *cis*-acting recombination sequences at each end of Tn7 are designated by an open triangle (Tn7L) and a filled triangle (Tn7R); it should be noted that the required sequences in Tn7L and Tn7R include and extend beyond Tn7's terminal 30 bp inverted repeats. The target DNA is shown as a broken line. In the upper panel, reactions involving an intact donor substrate in which the transposon is embedded in donor backbone are shown. Double-strand breaks (DSBs) have undergone a doublestrand break at either Tn7L (DSB.L) or Tn7R (DSB.R); the major transposition product, a simple insertion, results from joining of the two transposon ends of an excised linear transposon (ELT) to a target DNA; a single-end join (SEJ) results from the joining of a single transposon end to the target DNA (DSB.L-SEJ and DSB.R-SEJ). In the lower panel, reactions involving an ELT substrate are shown. A simple insertion results from joining of both ends of a single ELT to a target DNA. A single-end join (ELT-SEJ) results from the joining of one end of an ELT to the target DNA. A double-insertion ELT-SEJ ((DI) ELT.R-SEJ) results from the joining of two Tn7R ends from two different ELTs into a single attTn7 site.

& Mizuuchi, 1985). This fused structure can be converted by host-mediated replication reactions into a cointegrate that contains two copies of the transposon linking the donor backbone and target DNAs. By contrast, breakage of the 5' ends in addition to 3' end cleavage results in excision of the element from the donor backbone and direct formation of a simple insertion.

The bacterial transposon Tn7 transposes *via* a cut and paste mechanism in which the element is excised from the donor backbone by breaks at both the 3' and 5' ends of Tn7 to generate an excised linear transposon (ELT) that can be inserted into the target DNA (Figure 1; Craig, 1989, 1991, 1995; Bainton et al., 1991, 1993). Insertion occurs through the covalent linkage of the 3' tips of the left end of Tn7 (Tn7L) and the right end of Tn7 (Tn7R) to staggered positions in the target DNA; thus Tn7R becomes covalently linked to one strand of the target and Tn7L to the other, and the newly inserted transposon is flanked by 5 nt gaps (Figure 2(a)). Tn7 is distinguished by its ability to insert into two different classes of target sites. One class is represented by attTn7, a specific site in the Escherichia coli chromosome, and the other class is represented by many different sites not structurally related to attTn7 or to each other (Barth et al., 1976; Rogers, et al., 1986; Waddell & Craig, 1988; Kubo & Craig, 1990; Craig, 1991, 1995). Tn7 insertion into these two different classes of target sites is mediated by distinct but overlapping sets of the Tn7-encoded transposition proteins TnsA, TnsB, TnsC, TnsD and TnsE. Insertion into attTn7 is mediated by TnsABC + D while TnsABC + E mediate insertion into other, non-attTn7 non-related sites. The key recombination proteins that act at the Tn7 ends and at the point of element insertion to execute breakage and joining are TnsB, a sequence-specific DNA binding protein that binds to multiple sites within each end (Arciszewska et al., 1991; Tang et al., 1991) and TnsA; the remaining Tns proteins are regulators that activate TnsA + TnsB and control target site selection (Craig, 1991, 1995, unpublished results).

In Tn7, the *cis*-acting DNA sequences that are the substrates of recombination are contained within the terminal 166 bp of Tn7L and 90 bp of Tn7R; the same Tn7 end sequences appear to be involved in both TnsD- and TnsE-dependent transposition (Arciszewska et al., 1989, unpublished). Tn7L and Tn7R contain distinct arrays of TnsB binding sites (Figure 2(a); Arciszewska & Craig, 1991; Arciszewska et al., 1991; Tang et al., 1991) and the different patterns of TnsB binding sites in the interior regions of Tn7L and Tn7R likely play a key role in providing the functional differences between the ends of Tn7. Examples of such functional differences are that Tn7 insertion can be orientationspecific as well as site-specific (Lichtenstein & Brenner, 1981; Hauer & Shapiro, 1984; McKown et al., 1988; Bainton et al., 1993) and that Tn7 derivatives formed from two Tn7R segments can transpose whereas elements formed from two Tn7L segments cannot (Arciszewska et al., 1989). Here, we extend this functional distinction between Tn7R and Tn7L by in vitro analyses and we show that while Tn7R segments in the absence of Tn7L segments can recombine, no recombination is observed with only Tn7L.

At each end of Tn7, the most terminal of the TnsB binding sites form part of Tn7's highly related 30 bp inverted repeats (Arciszewska & Craig, 1991). The extreme Tn7 termini are -CA-3', a motif that is highly conserved among transposable elements including retroviruses (Polard & Chandler, 1995). To probe the DNA transactions that underlie Tn7 transposition and to further dissect the functions of



Figure 2. Tn7 transposition substrates and products are shown at the nucleotide sequence level. (a) An intact donor substrate is a plasmid containing a 1.6 kb miniTn7 element comprised of minimal Tn7L (166 bp) and Tn7R (199 bp) segments separated by a 1.2 kb kanamycin resistance (Km^r) gene segment. TnsB binding sites in Tn7L and Tn7R are indicated by arrows; the extreme tips of Tn7 at both Tn7L and Tn7R are-CA-3'. Tn7 is excised from the donor backbone by staggered cleavages that cleanly expose the 3'-terminal A and leave 3 nt of flanking donor DNA (indicated by x) attached to the 5' ends of the excised element (see also Figure 8). The target DNA is a 3.2 kb plasmid containing a –342 to +165 attTn7 segment. Tn7 inserts into attTn7 in site and orientation-specific fashion; the 3' end of Tn7L joins to a particular position in the bottom strand of attTn7 and the 3' end of Tn7R joins to a specific position in the top strand of attTn7. The positions of end joining are staggered by 5 bp in attTn7; host-mediated repair of the resulting gaps removes the flanking nucleotides from the 5' ends and regenerates intact DNA duplexes. Relevant SalI (S), SmaI (Sm) and BsaHI (Bs) sites are indicated. (b) The nucleotide sequences at the tips of Tn7 and flanking donor sequences of some miniTn7 elements are shown. Each segment shown is flanked by a BamHI site (GGATCC) that is joined to the vector BamHI site; MluI (M) and ScaI (Sc) sites are indicated. The elements have various combinations of wild-type (L+, R+) and mutant (L-, R⁻) ends. Xs mark changes at the tips of Tn7 and * indicate sequence variations from the pMIM flanking sequences. The released -GT-3' transposons have neither the 3 nt 5' extensions of wild-type miniTn7 ELTs nor the 4 nt 5' extensions of ELTs obtained from pMIM following MluI digestion but these 5'-terminal extensions are not essential to recombination (Bainton et al., 1991). It should be noted that the terminal 16 bp of Tn7L of such an engineered ELT are different at two interior positions from authentic Tn7, having been changed to the terminal sequences of Tn7R during element construction (Bainton et al., 1991).

the Tn7 ends, we have analyzed the recombination properties of Tn7 end derivatives in which the highly conserved -CA-3' dinucleotide at the transposon tips has been altered. Our analyses reveal that these mutations differentially effect the cleavages at the 3' and 5' ends of Tn7 that underlie the double-strand breaks that initiate recombination and suggest that Tn7 transposition involves multiple DNA processing activities that may be mediated by distinct active sites. Our results suggest also that target joining by Tn7 occurs *via* two separable DNA strand transfer steps in which 3' transposon ends join individually to single strands of the target DNA.

Results

Characterization of the target joining step: recombination of an ELT substrate

Efficient transposition of Tn7 into attTn7 is observed in an in vitro reconstituted system containing highly purified TnsA, TnsB, TnsC, TnsD, Mg²⁺ and ATP with a donor plasmid containing a miniTn7 element and an attTn7 target plasmid (Bainton et al., 1993). Recombination is observed with both supercoiled (Bainton et al., 1993) and relaxed (data not shown) donor plasmids. Tn7 transposition in vitro generates a variety of transposition intermediates and products (Figure 1). Recombination initiates with double-strand breaks at each end of Tn7, which can result in the formation of an excised linear transposon (ELT); the joining of both ends of the ELT to the target DNA results in formation of a simple insertion. Recombination intermediates in which a double-strand break (DSB) has occurred at only one transposon end can be observed (DSB.L and DSB.R). The exposed Tn7 end of a DSB can join to the target DNA, forming a DSB single-end join (DSB.SEJ) but these species are likely to be recombination by-products rather than intermediates in simple insertion formation (Bainton et al., 1993; data not shown). SEJs involving either Tn7L or Tn7R are observed (Bainton et al., 1993; see also below), indicating that there is no preferential order of end utilization during target joining. The chemistry of SEJs is considered in more detail below.

The use of an ELT substrate allows analysis of the target joining step in the absence of the initiating double-strand break step. An ELT substrate can be generated by restriction digestion of an appropriately engineered element (Bainton *et al.*, 1991). An ELT generated by restriction can be an effective substrate in the reconstituted system (Figure 3), as previously observed in the crude extract Tn7 transposition system (Bainton *et al.*, 1991). With an ELT substrate, the major product is a simple insertion in which both ends of a single transposon are joined to the target DNA. Other species are, however, present at lower levels. A species observed at low level is an ELT-SEJ. Single-end joins involving either Tn7L or Tn7R are observed, indicating that



Figure 3. To uniquely analyze the target joining step of recombination, recombination reactions were performed with an ELT donor substrate containing wild-type Tn7 ends generated from pMIM by digestion with MluI. Reaction mixtures contained different combinations of Tns proteins as indicated and products were analyzed by native agarose gel electrophoresis followed by hybridization with a mini-Tn7 specific probe. The substrates and products are labeled as in Figure 1. Tn7R is shown as filled segments and Tn7L is shown as open segments; the target is indicated by broken lines. $R \rightarrow Top$ indicates that Tn7R joined to the top strand of attTn7 and $R \rightarrow Bottom$ indicates that Tn7R ioined to the bottom strand of attTn7.

there is no preferential order of end utilization during target joining of these substrates (Bainton et al., 1993; see also below). The major alternative product observed with an ELT substrate results from the concerted joining of the two R ends from two different ELT molecules to a single attTn7. In this "double-insertion single-end join", (DI) ELT.R-SEJ, one R end is joined to the top strand of *attTn7* $(R \rightarrow Top in Figure 3)$ and the other end is joined to the bottom target strand ($R \rightarrow Bottom$); the observation that this (DI) ELT.R-SEJ product is formed with an ELT substrate with a mutant Tn7L end and with substrates that contain only Tn7R ends reveals that this product involves only Tn7R joints (see below). Since two distinct Tn7 elements join to attTn7 in this reaction, attTn7 is apparently cleaved into two segments during recombination.

Although an ELT substrate has bypassed the double-strand break step that usually initiates recombination, ELT recombination, like recombination of an intact donor substrate, requires all the Tns proteins, i.e. TnsA, TnsB, TnsC and TnsD (Figure 3). Thus, although Tn7 transposition occurs in two distinct steps, initiation by double-strand breaks followed by joining of the exposed 3' ends to the target DNA, all of these Tns proteins play essential roles in both the excision and target joining steps.

Alteration of the terminal -CA-3' blocks target joining

The extreme termini of Tn7 end with -CA-3', a dinucleotide that is highly conserved at the termini of many transposable elements (Polard & Chandler, 1995). The terminal A-3' plays a particularly critical role in recombination, as it becomes covalently joined to the target DNA during the strand transfer step, having been precisely exposed during the strand breakage step (Bainton *et al.*, 1991). We probed the role(s) of the terminal -CA-3' dinucle-otide by analyzing the recombination properties of

Tn7 end derivatives whose terminal sequences were changed to -GT-3' (Figure 2(b)). We generated transposons whose terminal sequences were altered to include restriction enzyme recognition sequences such that digestion with the cognate enzyme released Tn7 ends with -GT-3' termini, similar to the above strategy of releasing -CA-3' transposons (Figure 2(b)).

No recombination is observed with an ELT substrate in which both termini ends have been changed to -GT-3' (miniTn7 SIM L-R-; data not shown), revealing the importance of the -CA-3' in target joining. Chimeric ELTs have one mutant end (-GT-3') and one wild-type (-CA-3') Tn7 end, i.e. (Chi L^-R^+ and Chi L^+R^-). When a chimeric ELT is used as a substrate, the wild-type transposon end is joined to the target DNA but the mutant end is not, resulting in formation of ELT-SEJs, which are by far the major products of recombination evaluated by ethidium bromide staining as (Figure 4(a)). The considerable level of ELT-SEJ recombination product is notable and indicates that recombination involving only the strand transfer of a single transposon end to the target DNA can occur efficiently. Other recombination products formed at lower levels are detectable by hybridization probes (data not shown). With a chimeric ELT containing a wild-type Tn7R end (Chi $L^{-}R^{+}$), products resulting from the concerted joining of transposon ends from two ELTs are observed (DI-SEJs), whereas these species are not observed with ELTs that lack a wild-type R end (Chi L^+R^-). Another low-level product observed with both chimeric substrates is a simple insertion, indicating that the mutant end (-GT-3') can, albeit at low level, join to the target. It is interesting that simple insertions are not observed with substrates containing two mutant ends (-GT-3'); thus the presence of a wild-type end rather than a mutant end appears to modestly suppress the defect in a chimeric mutant (-GT-3') end. Suppression of defective



Figure 4. The polarity of target strand joining of recombination products obtained using different ELT donor substrates was examined. Transposition reactions were performed with various ELT donor substrates with wild-type (MIM L^+R^+) and mutant (Chi $L^+\hat{R}^-$ and Chi $L^{-}R^{+}$) ends. (a) The reaction mixtures were digested with NdeI and analyzed by native agarose gel electrophoresis. (b) The resulting simple insertion and ELT-SEJ recombination products were extracted from the agarose gel and analyzed by denaturing gel electrophoresis. The substrates and products are labeled as in Figure 1. Tn7R is shown as filled segments, Tn7L is shown as open segments and the target is indicated by broken lines. (a) The recombination products obtained using wild-type (lane 1) and chimeric (one wild-type -CA-3' end and one mutant -GT-3' end; lanes 2 and 3) ELT transposons as substrates are shown after NdeI digestion as detected on native agarose gels by ethidium bromide staining. The resulting recombination products are diagrammed; the mutant ends of the ELTs are indicated by X. (b) The recombination products obtained with chimeric ELT substrates obtained as in (a)

were extracted from an agarose gel, digested with *Sal*I (S), *Hin*dIII (H) and *Xba*I (Xb) and then analyzed by denaturing gel electrophoresis, followed by hybridization with Tn7 end and strand-specific oligonucleotides NLC 97 and NLC 163 (shown adjacent to their complementary strands.) The drawings depict the species obtained when the transposon is inserted in *attTn7* in its correct orientation (boxed drawing and labels), with Tn7R joined to the top strand of *attTn7* (right diagram) and when the transposon is inserted into *attTn7*, in the incorrect, opposite orientation, with Tn7R joining to the bottom strand to *attTn7* (left diagram).

transposon ends by wild-type ends in chimeric elements have been observed with other elements (Derbyshire *et al.*, 1987).

The observation of SEJ recombination products provides an important insight into the mechanism of strand transfer during Tn7 transposition. In SEJ products, one transposon end is joined to one strand of the target DNA, while the other target strand remains intact. This reveals that transposon insertion into the target DNA occurs by two independent, but usually concerted, attacks of individual transposon ends on single, individual strands of the target DNA. Such separate attacks on each strand of the target contrasts with an alternative pathway for target joining that can be imagined: the introduction of double-strand breaks at the transposon ends and at the insertion site, followed by joining of exposed transposon and target strands. No double-strand break at the target site can be detected when reactions are examined by hybridization with a target-site specific probe (data not shown). Moreover, the efficient formation of ELT-SEJs (as shown here) and DSB-SEJs (see below) suggests that Tn7 transposition proceeds through separate attacks of individual transposon ends on each target strand, i.e. without a double-strand target break.

Chimeric ELTs maintain site- and orientation-specificity during target joining

Simple insertion of wild-type Tn7 into *attTn7* is site-specific and orientation-specific, deriving from specific joining of the Tn7R 3' end to a particular position in the top strand of *attTn7* and the Tn7L 3' end to a particular position in the bottom strand of attTn7 (Figure 2(a)). To determine if this target strand specificity is preserved with chimeric transposons containing one wild-type -CA-3' end and one mutant -GT-3' end, we directly examined the transposon-target joints generated with ELT chimeric substrates using denaturing gel analysis and strand-specific hybridization probes recognizing Tn7L and Tn7R (Figure 4(b)). When a simple insertion at the standard target position in the correct orientation is formed with a wild-type substrate, new species resulting from joining of the 3' end of Tn7L to the bottom strand of attTn7



(531 nt) and joining of the 3' end of Tn7R to the top strand of attTn7 (375 nt) are observed (lane 1); opposite (incorrect) orientation insertion would result in species 564 nt and 342 nt in length. Analysis of the joints made between chimeric ELTs and the target DNA reveal that ELT-SEJ joints are predominately site and orientation-specific, i.e. the Tn7R end joins to the top strand of attTn7 at the standard position (lane 2) and Tn7L joins to the bottom strand of attTn7 at the standard position (lane 3). The small amount of opposite orientation insertion that is observed with Chi L^-R^+ (564 nt, lane 2) reflects the formation of DI-SEJs in which wild-type R ends join to both strands of *attTn7*. Thus the chimeric transposons containing one wild-type -CA-3' end and one mutant -GT-3' end maintain target site-selectivity and their ability to discriminate between the top and bottom strands of the target DNA, i.e. also maintain orientation specificity.

The special role of Tn7R in target joining

The observation that only Tn7R ends are involved in the DI-SEJ reaction of an ELT substrate indicates that Tn7R and Tn7L are not functionally equivalent in vitro, as has been observed in vivo (Arciszewska et al., 1989). To further probe the properties of Tn7L and Tn7R, we used split-end substrates in which the L and R ends of an ELT were separated by restriction digestion. Recombination can still occur with such split end substrates, but all the resulting products exclusively involve recombination of R ends (Figure 5). With split end R segments, the major product observed results from the concerted joining of two R END segments to a single attTn7, a double-insertion-single-end join ((DI) END.R-SEJ; lanes 1, 5, 7 and 9). Also present is a product that results from the joining of a single R end segment to attTn7, an END.R-SEJ that is the equivalent of the single-end join of ELTs and DSBs.

With Tn7 split end segments containing various combinations of wild-type -CA-3' and mutant -GT-3' termini, recombination is observed only with the Tn7R -CA-3' ends; notably, no recombination is observed with mutant Tn7R -GT-3' ends in any

Figure 5. The recombination products obtained using combinations of split Tn7 end substrates (obtained by digestion of a miniTn7 ELT with Smal) with either wildtype (-CA-3') or mutant (-GT-3') tips as detected by native agarose gel electrophoresis and hybridization with a miniTn7 specific probe are shown. Reaction mixtures contained all Tns components (+) except TnsB where indicated (-). The products are labeled as in Figure 1. Tn7R is shown as filled segments, Tn7L is shown as open segments and the target is indicated by broken lines.

combination with Tn7L wild-type -CA-3' or mutant -GT-3' ends (lanes 3 and 11). While Tn7L ends are efficient recombination partners with Tn7R end segments when these ends are covalently linked, as in an ELT or in an intact donor substrate, no recombination is observed with L end segments that are not covalently linked to Tn7R (lanes 1, 11, 13 and 15); thus the reactivity of Tn7L is greatly enhanced by covalent connection to Tn7R.

The donor cleavage step requires two Tn7 ends in an intact donor substrate

When Tn7 is embedded in flanking donor DNA, recombination requires both a DNA breakage step to release the transposon and a strand transfer step to join the transposon ends to the target DNA. We examined recombination with a supercoiled plasmid substrate containing only a single wild-type



Figure 6. Alteration of the transposon terminus can block double-strand breaks. The recombination products obtained using various miniTn7-containing plasmids as intact supercoiled substrates are shown as detected by native agarose gel electrophoresis. The products are labeled as in Figure 1; reaction mixtures contained all Tns proteins (+) except TnsB where indicated (–). The recombination substrates were plasmids containing a wild-type Tn7R end (MIM R⁺ Δ L; lanes 1 and 2) or mini-Tn7 plasmids containing two wild-type -CA-3' and/or mutant -GT-3' ends in various combination (lanes 3 to 8). Recombination substrates and products were analyzed by hybridization with a miniTn7-specific probe.

-CA-3' Tn7R end (MIM $R^+\Delta L$). No initiating double-strand break or intra- or intermolecular recombination is observed with this substrate containing a single Tn7 end (Figure 6). MiniTn7 elements containing two Tn7R ends do undergo breakage and joining in vivo, indicating that Tn7R sequences are sufficient to promote these steps (Arciszewska et al., 1989). The finding here that no donor breakage is observed with a substrate containing only a single Tn7R end reveals that the initiation of recombination by double-strand breaks requires the presence of two transposon end segments on the substrate DNA molecule. Thus the initiation of recombination requires the presence of two Tn7 ends in the substrate molecule. A reasonable hypothesis is that synapsis between the two transposon ends is a critical early step in recombination.

Alteration of the transposon terminus blocks double-strand breaks

Analysis of the transposition of supercoiled substrates containing wild-type -CA-3' and mutant -GT-3' ends reveals that alterations of the terminus can also block the double-strand breaks that initiate recombination. With supercoiled chimeric substrates containing one wild-type -CA-3' end and one mutant -GT-3' end, both breakage and joining occur at the wild-type ends but no double-strand breakage or target joining is observed at the mutant -GT-3' ends (Figure 6, lanes 7 and 8). The products of recombination with these chimeric substrates are DSB-SEJs: doublestrand breaks and single-end joins of the wildtype transposon end to the target DNA are formed but the donor backbone remains attached to the mutant transposon end. It is notable that equivalent high levels of recombination products are observed with both the wild-type and chimeric mutant substrates. No broken substrate molecule is observed with elements containing two mutant ends (lanes 5 and 6). Thus, changing the terminal -CA-3' to -GT-3' also blocks the double-strand breaks that initiate recombination in addition to blocking target joining as demonstrated above. It should be noted that in some cases these constructions involved changing the donor sequences immediately flanking Tn7 termini (Figure 2(a)); the donor substrates pMIM and pSIS, however, differ only in the miniTn7 -CA-3' sequences.

These experiments reveal that, although the initiation of recombination, i.e. a double-strand break, does require two transposon ends in the donor substrate as shown above, two fully functional ends are not required. A reasonable hypothesis is that alteration of the -CA-3' blocks the chemical steps of recombination, i.e. breakage and joining, but that the other functions of the ends such as the binding of recombination proteins remain intact.



Figure 7. Double-strand breaks at the ends of Tn7 result from two activities: 5' cleavage processing can be separated from 3' end breakage and joining. The cleavages at the 3' and 5' ends of Tn7 were examined by analysis of recombination products on a denaturing agarose gel. (a) The donor plasmid pSIM is shown, which contains mutant (-GT-3') Tn7L and Tn7R segments. The sizes of single-strand fragments obtained by 5' end processing and digestion with *Bsa*HI are diagrammed. (b) The recombination products obtained following incubation of pSIM with various combinations of Tns proteins are shown as detected by denaturing agarose gel electrophoresis following digestion with *Bsa*HI and hybridization with a miniTn7-specific probe.

Substrate mutations can separate the cleavages of the 3' and 5' transposon strands

As described above, changing the terminal -CA-3' to -GT-3' blocks the introduction of the doublestrand breaks that initiate recombination (Figure 6). However, examination by denaturing agarose gel electrophoresis of the fates of individual transposon strands in a substrate mutant at both Tn7L and Tn7R revealed a surprising result (Figure 7). Cleavage at the 5' ends still occurs with this substrate, whereas cleavage at the 3' ends does not. Thus alteration of the terminal -CA-3' sequence blocks only 3' end cleavage (and hence doublestrand breaks). This finding suggests that the cleavages of the 3' and 5' ends of Tn7 result from separable and distinct activities. It should be noted that in the mutant substrate examined in this experiment, miniTn7-SIM, the sequences at both tips of Tn7 were changed from the wild-type -CA-3' sequence to -GT-3' and several donor nucleotides immediately flanking the Tn7 tips were changed.

Like the other chemical steps in Tn7 transposition, these 5' end cleavages require the full complement of Tns proteins (Figure 7(b)). Furthermore, 5' end cleavage and 3' end cleavage both require an appropriate DNA substrate, i.e. one containing two Tn7 ends, since no cleavage is observed with a DNA substrate containing only a single wild-type Tn7R -CA-3' end (data not shown). These results suggest that the double-strand breaks that initiate recombination actually reflect a collaboration of two distinct and separable reactions: a 3' end cleavage reaction and a 5' end cleavage reaction, which can be separated by alteration of the DNA sequence at the ends of the transposon.

Identification of the position of the 5' strand cleavage reactions with wild-type -CA-3' ends

Where do the cleavages at the 5' ends of Tn7 actually occur? Previous experiments using a crude extract system for Tn7 transposition established that the 5' end cleavages occur within the flanking donor DNA (Bainton et al., 1991). However, it was not possible in those crude extract experiments to establish the primary location of 5' end cleavages. To map the location of these cleavages at high resolution, we have analyzed recombination products generated in the reconstituted system using denaturing gel electrophoresis and hybridization with strand-specific oligonucleotides, following restriction of the recombination reactions (Figure 8). An oligonucleotide complementary to the top strand of Tn7L (NLC 95) hybridizes to a species 171 nt in length; this maps the 5' cleavage to a position 3 nt outside the Tn7L end, the other terminus of the 171 nt fragment being generated by restriction with SalI (S) within the transposon end. Similarly, the oligonucleotide complementary to the 5' strand of Tn7R (NLC 100) identified a 204 nt species, corresponding to cleavage at a position 3 nt displaced from the 5' end of Tn7R. In this experiment, the uncleaved donor substrate fragments are greater than 250 nt in length and thus are not visible in this gel.



Figure 8. The positions of the cleavages at the 5' ends of Tn7 that occur during transposition were defined by displaying recombination products obtained using intact supercoiled pEM Δ and pMIM as substrates on a denaturing gel after digestion with *Sal*I (S) and hybridization with oligonucleotide probes as indicated; the hybridization positions of the strand-specific oligonucleotides NLC 95 and NLC 100 on their complementary strands are indicated.

Thus 5' end cleavage at wild-type Tn7 ends occurs within the flanking donor DNA, 3 nt displaced from the actual transposon terminus (Figure 2). It should be noted that these cleavages occur at a single flanking position even when the flanking sequences differ, as in different donor plasmids pMIM and pEM Δ ; thus, the precise

Figure 9. The positions of breaking and joining reactions are defined at the nucleotide level by displaying the products of recombination reactions using a variety of DNA substrates on denaturing polyacrylamide gels and hybridization to Tn7 strand-specific oligonucleotide probes (shown adjacent to their complementary strands). (a) Diagrams of the various DNA substrates used and the expected sizes of DNA fragments resulting from restriction and recombination are indicated. The positions of hybridization of the oligonucleotide probes are indicated. The transposon is depicted in the donor site with some elements (MIM L⁺R⁺-TnsB, SIS L⁻R⁻ and SIM L⁻R⁻) since no 3' end breakage or strand transfer is observed with these substrates; these DNAs are also reference substrate sizes for other elements (MIM L^+R^+ , Chi L^-R^+) and Chi L^+R^-) that do undergo recombination and are drawn as recombination products, i.e. joined to the target DNA. The sizes of end segments vary slightly because of sequence variations at and flanking the transposon tips (see Figure 2(b)). (b) The patterns of DNA products after digestion with SalI (S), XbaI (X) and HindIII (H) as displayed on a denaturing gel are shown. The left gel displays reactions at the 3' ends of the transposon, both Tn7L and Tn7R, and the right gel shows reactions at the 5' ends, as detected using strand-specific hybridization probes. The sizes of the substrates and products vary by a few nucleotides because of the slightly different sequences at the transposon tips in different substrates (Figure 2(b)) and because of single nucleotide variations in the positions of 5' end cleavage as described in the text. Note that an unreacted R end is represented by two species because of incomplete restriction at the flanking HindIII (H) site.



Figure 9. (legend on p. 308)

position of these 5' cuts appears to be dictated by information within the transposon.

Identification of the position of the 5' strand cleavage reactions with mutant -GT-3' ends

We have similarly examined the 5' and 3' cleavage reactions at high resolution in a variety of DNA substrates containing both wild-type and mutant ends (Figure 9). We used strand-specific probes to examine cleavage at 3' ends (NLC 97 for Tn7L and NLC 163 for Tn7R) and 5' ends (NLC 95 for Tn7L and NLC 94 for Tn7R). With unreacted donor substrate DNA (MIM L⁺R⁺-TnsB), specific fragments resulting only from restriction are detected: 190 nt for Tn7L and 241/256 nt for Tn7R, the two different lengths reflecting partial digestion at an intervening *Hin*dIII (H) site (lane 2). Recombination of MIM L^+R^+ (lane 1) results in 5' end cleavage (generating fragments of 171 nt from Tn7L and 204 nt from Tn7R, as shown above) and 3' end cleavage and transfer to the target DNA (a fragment of 531 nt revealing a Tn7L-target joint and of 375 nt revealing a Tn7R-target joint); 3' end cleavage without strand transfer is not observed. In chimeric DNA substrates containing wild-type -CA-3' and mutant -GT-3' ends (lanes 5 and 6), both donor cleavage and strand transfer occur at the wild-type -CA-3' end, indicated by Tn7L-target species at 531 nt and the Tn7R-target species at 375 nt; 5' strand cleavage at a wild-type (CA-3') end cleavage is revealed by the 171 nt Tn7L species and the 204 nt Tn7R species. This analysis reveals that correct 5' processing is observed at both mutant and wild-type ends but no 3' cleavage or transfer is observed at the mutant ends. Thus in these chimeric substrates, only the 3' processing reactions are specifically blocked at the mutant ends.

In the case of a substrate that contains mutant -GT-3' Tn7L and Tn7R ends, and has flanking donor changes (miniTn7-SIM, lane 4), no 3' end breakage or joining is detected but 5' end cleavage is observed at both Tn7L and Tn7R, consistent with the denaturing agarose gel analysis shown above. Cleavage at the 5' ends of Tn7 results in the generation of species about 204 and 171 nt in length, deriving from Tn7R and Tn7L, respectively. With miniTn7-SIM, most (90%) of the cleavages occur at positions 3 nt displaced from the 5' tips of Tn7, as is observed with wild-type -CA-3' ends of the transposition-competent miniTn7-MIM substrate; however, at the mutant -GT-3' ends of miniTn7-SIM, a low level of cleavage is also observed 2 nt from the 5' end. In the case of the mutant transposon miniTn7-SIS, whose terminal sequences are -GT-3' but whose flanking donor sequences are identical with miniTn7-MIM (lane 3), 3' end cleavage is also blocked at both Tn7 ends but variable 5' end cleavage is observed: 5' end cleavage occurs at 3 nt and 2 nt from the 5' tip of Tn7L but 5' end cleavage is not detectable at Tn7R. Since 5' end cleavage is observed with a -GT-3' transposon flanked by different donor sequences, i.e. miniTn7SIM, we conclude that 5' end cleavage can be influenced by both the transposon tips and the flanking donor sequence.

These analyses of mutant transposon substrates have revealed that a critical initiating step in recombination, the cleavages at the 3' ends that expose the transposon terminus for attachment to the target DNA, can be specifically and uniquely blocked by changing the conserved terminal -CA-3' dinucleotide; by contrast, processing of the 5' strands appears to be a separable event that can occur in the absence of the -CA-3' but can be influenced by flanking donor sequences.

Discussion

The chemical steps of transposition: DNA breakage and joining

We previously established that Tn7 transposition proceeds through a cut and paste mechanism in which the element is excised from the donor backbone by double-strand breaks at each end of the element and then inserted into the target site by joining of the exposed 3' transposon ends to the target DNA (Bainton et al., 1991, 1993). We have demonstrated here that these double-strand breaks result from two separable DNA cleavage activities, one that cleaves to expose the 3' ends that will subsequently be joined to the target DNA and another that cleaves at 5' ends. These different cleavage activities are separable by their different responses to changing the sequences at the extreme tips of Tn7, including the -CA-3' dinucleotide highly conserved among many retroviral-like elements (Polard & Chandler, 1995). In Tn7, changing this dinucleotide to -GT-3' and changing some flanking nucleotides blocks the cleavages at the 3' ends but cleavage at the 5' ends can still proceed. These 3' and 5' cleavage activities are similar in that both require the entire complement of Tns proteins necessary for formation of a simple insertion, likely reflecting the requirement for the proper assembly of a nucleoprotein complex containing the Tns proteins and DNA substrates prior to the initiation of recombination (Bainton et al., 1991, 1993). The utilization of two separable activities to execute a concerted double-strand break is unusual among transposable elements (Mizuuchi, 1992b) and among nucleases. We have established here that cleavage of the 3' ends is not essential for cleavage at the 5' ends; we have demonstrated also that 3' end cleavage and strand transfer can occur in the absence of 5['] end cleavage (unpublished results). As will be described elsewhere, TnsB executes the 3' end cleavages that can be blocked by changing the terminal -CA-3' sequence and TnsA executes the 5' end cleavages.

We have established here that altering the terminal -CA-3' dinucleotide can block both the cleavages that expose the 3' ends and the joining of exposed 3' transposon ends to the target DNA. This finding suggests that there is a profound and

fundamental relationship between these two key reactions in Tn7 transposition. Thus Tn7 resembles other transposition systems such as retroviral integration (Engelman & Craigie, 1992; Kulkosky *et al.*, 1992; van Gent *et al.*, 1992; Leavitt *et al.*, 1993), the transposition of bacteriophage Mu (Baker & Luo, 1994; Kim *et al.*, 1995), and Tn10 (Haniford *et al.*, 1989; Bender & Kleckner, 1992; Kleckner *et al.*, 1995), where the critical 3' breakage and joining reactions that actually underlie transposition are tightly coupled and are executed by the same recombination protein, likely by the same or at least closely related active sites. In Tn7 transposition, TnsB executes the 3' end cleavage and strand transfer reactions (unpublished results).

We have established here that the joining of the transposon ends to the target site can occur through two separable but usually concerted reactions in which an individual transposon end joins to one strand of the target DNA. In analyzing target joining by chimeric transposons in which one end is proficient in target joining (has a -CA-3' terminus) and one end is incapable of joining (has a -GT-3' terminus), we found that in the resulting recombination products, the wild-type -CA-3' end is joined to one target strand while the other target strand remains intact. This observation precludes models for target joining that involve a double-strand break in the target DNA, the ends of which are subsequently joined to the transposon ends. Thus the target insertion step of Tn7 transposition coordinates two separate strand transfer reactions, each one involving one 3' transposon end and one target strand. An attractive hypothesis is that target joining in Tn7 occurs in a fashion similar to that in bacteriophage Mu and retroviruses (Engelman et al., 1991; Mizuuchi & Adzuma, 1991; Mizuuchi, 1992a), i.e. through a direct one-step transesterification reaction in which the exposed -OH of the cleaved 3' transposon end directly attacks a phosphodiester bond in the target DNA, generating a new joint between the transposon terminus and the target DNA, and a break in the target strand.

Recombination involves communication between two Tn7 ends

A hallmark of Tn7 transposition is its high degree of regulation. For example, the double-strand breaks that initiate recombination do not occur in the absence of a suitable target DNA and require all of the Tns proteins that are necessary for the completion of recombination (Bainton et al., 1991, 1993). We have demonstrated here that Tn7 recombination initiates with separable cleavage at the 3' and 5' ends of Tn7 and that the presence of two Tn7 ends in a donor DNA is a critical prerequisite to all the breakage reactions that underlie recombination; no 3' or 5' cleavage reaction is detectable in DNA substrates containing only one Tn7 end. This finding suggests that communication between the two Tn7 ends in an appropriate DNA substrate is a critical early step in recombination, i.e.

that this important "editing" or "checkpoint" step occurs prior to DNA breakage. An attractive hypothesis is that the Tn7 ends directly communicate by physical synapsis (Craigie & Mizuuchi, 1987; Surette *et al.*, 1987; Haniford *et al.*, 1991; Mizuuchi, 1992b). We speculate that such synapsis is a key feature of the nucleoprotein complex containing the substrate and target DNAs, and the Tns proteins that we have proposed must be correctly assembled to initiate Tn7 recombination (Bainton *et al.*, 1993).

It should be noted that, although the Tn7 system can execute recombination with intermolecular ends that have already been exposed by donor cleavage, for example the use of the two Tn7Rs on different ELTs, such intermolecular reactions are not observed with substrates that must undergo cleavage during the reaction itself, i.e. the recombination machinery cannot use two ends on two different intact plasmid substrates, as has been argued for some other mobile elements (Roberts *et al.*, 1991; Kleckner *et al.*, 1995). Thus Tn7 is highly regulated to prefer two transposon ends on the same donor substrate DNA.

It is likely that synapsis of the Tn7 ends is important for the target joining steps, not just for the breakage steps that initiate recombination. With a substrate that has already undergone end cleavage, for example an ELT, the predominant products, by far, involve joining of two transposon ends to a target DNA, using "intramolecular" ends, i.e. the two ends of an ELT, or "intermolecular ends", i.e. using two ends from two different ELTs. Thus it is likely that complexes in which the ends of Tn7 are physically synapsed with each other are the actual substrates of both the donor breakage and target joining steps in transposition. It is interesting that although two Tn7 ends are required for breakage and joining, it is not necessary that both of these ends actually be capable of undergoing the chemical steps of these reactions: in chimeric transposons, the mutant -GT-3' ends that are defective in breakage and joining can promote breakage and joining at the wild-type -CA-3' ends. This observation reveals explicitly that the ends of Tn7 have (at least) two functions, likely a binding interaction with proteins that can lead to synapsis and another functional interaction at the transposon tips likely involved more directly in breakage and joining. The cisacting recombination sequences of other elements have been revealed to be functionally bipartite (Derbyshire et al., 1987; Huisman et al., 1989; Baker & Mizuuchi, 1992; Derbyshire & Grindley, 1992; Jilk et al., 1993).

That the alteration of the terminal nucleotides in Tn7 so profoundly blocks both 3' breakage and joining emphasizes the importance of "editing" or "inspection" of these reactions to the Tn7 recombination machinery. It has been reported in the Tn10 system that although mutations at the tips of this element profoundly block cleavage *in vitro*, some cleavage is observed *in vivo* (Haniford & Kleckner, 1994). It will be interesting to determine how Tn7 end mutations are interpreted *in vivo*. We have been

unable to detect *in vivo* translocation of a Tn7 element in which both -CA 3' termini are changed to -GT-3' (unpublished results).

In view of the highly regulated nature of Tn7 transposition (Craig, 1989, 1991, 1995; Bainton et al., 1991, 1993), it may seem paradoxical that Tn7 recombination can efficiently proceed with a chimeric donor substrate in which only one 3' end can be broken and joined to the target DNA; we have found also that efficient 5' end cleavages occur with substrates in which the processing of both 3' ends is blocked. Certainly such mutant substrates do fulfill the perhaps most fundamental requirements of the donor DNA for Tn7 transposition, i.e. that two ends be present on the substrate DNA. One reasonable explanation for this apparent conundrum may be that the conditions of our reactions in vitro likely highly favor the formation of a target complex that we hypothesize interacts with the transposon ends to achieve formation of the complete nucleoprotein complex in which recombination actually occurs (Bainton et al., 1993). It may be that formation of the target complex effectively "pulls" the reaction in the direction of formation of the complete complex, thus allowing the bypass of certain regulatory inputs that may usually be provided by the donor DNA. Such a situation has been observed with bacteriophage Mu (Surette et al., 1991; Wu & Chaconas, 1992); in a minimal recombination system, a terminal mutation at one end of an element can block breakage and joining at a wild-type end, but cleavage at the wild-type end does proceed in a more complete system. It will be interesting to examine the effects of Tn7 terminal mutations under other minimal reaction conditions to see if such coupling between events at mutants and wild-type ends can be observed. Uncoupled singleend recombination events are not uncommon in a variety of in vitro recombination systems (Craigie et al., 1990; Katz et al., 1990; Bushman & Craigie, 1991).

The left and right ends of Tn7 are not equivalent

Previous analysis has revealed that the ends of Tn7 are functionally and structurally distinct. The functional asymmetry was initially revealed by the fact that the ends of Tn7 join to the *attTn7* target DNA in site and orientation-specific fashion (Lichtenstein & Brenner, 1981; Hauer & Shapiro, 1984; McKown et al., 1988; Bainton et al., 1991, 1993), Tn7R being covalently linked at a specific point in the top strand of attTn7 and Tn7L being linked to a specific point in the bottom strand. Another indication of the asymmetry in Tn7 end function observed in vivo is that while miniTn7 elements containing two Tn7R segments can transpose, elements containing two Tn7L segments cannot (Arciszewska et al., 1989). Analysis of the sequences at each end required for transposition also revealed that the ends are structurally distinct. The terminal

30 bp of Tn7 are highly related inverted repeats but considerably more information at each end is required for transposition, a fully functional Tn7L segment is about 165 bp and a Tn7R segment about 90 bp (Arciszewska *et al.*, 1989, unpublished). The L and R ends of Tn7 each contain multiple TnsB sites arranged in distinct fashion and likely the interior regions beyond the terminal inserted repeats impose functional asymmetry on the ends.

We have now observed functional differences between Tn7L and Tn7R in vitro, beyond the observation of orientation-specific insertion: Tn7R is active in in vitro recombination under several conditions where Tn7L is not. When ELTs are used as recombination substrates, although the major products are simple insertions in which the two ends of a single ELT are inserted into a target DNA, we do observe recombination products resulting from the coordinated joining of two Tn7R ends of two different ELTs into the same target DNA; however, we do not observe such intermolecular reactions involving Tn7L. Another indication of the differential activity of Tn7R and Tn7L is revealed in assays in which split Tn7R or Tn7L ends are used as substrates: in these reactions, only Tn7R is observed to undergo recombination. Thus Tn7L participates in recombination only when it is covalently linked to Tn7R, i.e. as part of an ELT. In other words, the physical connection of Tn7L and Tn7R in an ELT allows Tn7R to enhance the utilization of Tn7L. These observations suggest that Tn7R plays a central role in organizing the Tns proteins necessary for recombination. Further analysis will be required to define the mechanisms underlying these observed differences in Tn7L and Tn7R function.

The effects of the donor site

The experiments reported here suggest that the DNA sequences that flank the ends of Tn7 in the donor site may also contribute to recombination; we have observed different efficiencies of 5' end cleavage in DNA substrates containing the same Tn7 end sequences but different flanking sequences. It would not be surprising that donor sequences can affect all stages of recombination: the cleavages at the 3' ends separate the Tn7 tips from flanking donor DNA, 5' end cleavages actually occur within flanking sequences and, moreover, the flanking sequences in the donor site actually derive from target sites that are acted upon by the strand transfer reactions. Although the donor effects we report here are evident under "mutant conditions", i.e. with altered Tn7 tips, they have begun to provide insight into the multitude of roles of the substrate DNA. The sequence of flanking DNA in the donor site has been shown to influence other transposition reactions (Surette et al., 1991; Wu & Chaconas, 1992). It will be useful to extend this analysis of the effects of flanking donor DNA on Tn7 recombination both in vivo and in vitro.

Concluding remarks

The work reported here has extended the view of the Tn7 transposition machinery as an elaborate assembly of multiple, specifically positioned and highly coordinated activities for DNA breakage and joining. Dissecting the macromolecular interactions that underlie these processes will provide additional insights into how transposition is executed and controlled.

Materials and Methods

DNA substrates

The standard miniTn7 element (L+R+) is 1.6 kb in length and is comprised of Tn7 end segments containing the cis-acting transposition sequences (a 166 bp Tn7L+ segment and a 199 bp Tn7R⁺ segment) flanking a kanamycin resistance gene (Arciszewska et al., 1989). In all substrates reported here except $pEM\Delta$, the terminal 16 bp of Tn7L have been changed to those of Tn7R, a 2 bp change (Bainton *et al.*, 1991). The donor plasmid pEM Δ (5926 bp) contains the L⁺R⁺ miniTn7 element inserted via TnsE-dependent transposition into a 134 bp E. coli chromosomal segment that has been cloned into the SmaI site of pTRC99 (Bainton et al., 1993). The donor plasmid pMIM L^+R^+ (4602 bp) and its derivatives (pChi-L⁺R⁻, pChi-L⁻ \mathbb{R}^+ , pSIS-L⁻ \mathbb{R}^- , pSIM-L⁻ \mathbb{R}^-) contains a miniTn7 element generated by PCR using primers complementary to the Tn7 termini and flanked by BamHI and MluI restriction sites (Figure 2(b)), inserted into the BamHI site of Bluescript pKS+, with Tn7R adjacent to the EcoRI site of the plasmid polylinker (Bainton et al., 1991); this plasmid contains a single BsaHI site located 1905 bp from the right end of Tn7. pMIM-R⁺ ΔL was constructed from pMIM-R⁺L⁺ by deletion of the HindIII fragment containing L⁺. ELT substrates were generated by digestion of the parental plasmid with appropriate mixtures of the end-specific enzymes MluI and ScaI (Figure 2(b)), and isolation of the ELT fragment by agarose gel electrophoresis followed by extraction using Qiaex (Qiagen). Split end substrates were generated by digesting isolated ELTs with SmaI which cuts within the miniTn7 element and isolation of the end segments (Tn7L about 600 bp; Tn7R about 1000 bp) by agarose gel electrophoresis followed by Qiaex extraction. The target plasmid pRM2 contains a attTn7 555 bp segment (-342 to +165) in the AccI site of pUC18 (McKown et al., 1988).

TnsB-His

TnsB-His contains amino acid residues 1 to 694 of TnsB, fused to a 15 amino acid residue linker, followed by six C-terminal histidine residues. Its properties are indistinguishable from those of authentic TnsB (data not shown). A tnsB segment extending from the ATG (replaced by an NcoI site; Orle & Craig, 1991) to the C-terminal BamI site was inserted into NcoI-BamHI digested pET21-d (Novagen) and the resulting plasmid introduced into BL21/DE3. Cells were grown at 30°C in LB medium supplemented with 100 $\mu g/ml$ carbenicillin to an A_{600} of 0.6, isopropyl- β ,D-thiogalactopyranoside added to 1 mM, growth continued for one hour, cells harvested by centrifugation and resuspended at 4°C in 60 mM imidazole, 20 mM Tris (pH 7.9), 500 mM NaCl at 0.2 g cells/ml. All subsequent steps were carried out at 4°C. The cells were lysed by sonication, centrifuged at 30,000 g for 30 minutes and the resulting supernatant passed through a 0.45 μ m filter. The filtrate was applied to a His.Bind Ni²⁺ column (Novagen); TnsB-His eluted with 200 mM imidazole, 20 mM Tris (pH 7.9), 500 mM NaCl. Peak fractions were pooled, dialyzed against 60 mM imidazole, 20 mM Tris (pH 7.9), 500 mM NaCl, reapplied to a His.Bind Ni²⁺ column and eluted with 200 mM imidazole, 20 mM Tris (pH 7.9), 500 mM NaCl. Peak fractions were pooled, dialyzed against 20 mM Tris (pH 8.0), 500 mM NaCCl, 1 mM DTT, 1 mM EDTA, 25% (v/v) glycerol, and stored at $- 80^{\circ}$ C; the resulting fraction was greater than 99% intact TnsB-His.

Tns proteins

TnsA was generated from a GST-TnsA fusion protein, using a modification of the procedure described by Bainton et al. (1993). GST-TnsA was bound to glutathioneagarose beads, and then freed through a limited proteolytic treatment with thrombin; the fusion protein was treated with thrombin while still bound to the glutathione matrix; 5 units of thrombin (Sigma) was added per 1 ml of suspension and incubated at room temperature for 40 minutes. The glutathione beads, bound to cleaved GST and uncleaved GST-TnsA, were removed by a low-speed spin and Ca²⁺ was removed from the TnsA-containing supernatant by dialysis against one liter of TnsA storage buffer (25 mM Hepes (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) and stored at -80°C. The reactions illustrated by Figures 5 and 7 were performed with TnsB fraction IV (Arciszewska et al., 1991) and all other reactions were performed with TnsB-His; both TnsBs were stored at –80°C in 25 mM Hepes (pH 8.0), 500 mM KCl, 2 mM DTT, 1 mg/ml BSA, 25% glycerol. TnsC was fraction III (Gamas & Craig, 1992) and was stored at -80°C in 25 mM Hepes (pH 8.0), 1000 mM NaCl, 2.5 mM DTT, 1 mM ATP, 10 mM MgCl₂, 0.1 mM EDTA, 10 mM Chaps, 10% glycerol. TnsD was fraction V (Bainton et al., 1993) and was stored at - 80°C in 50 mM Tris (pH 7.5), 2 mM DTT, 500 mM KCl, 1 mM EDTA, 25% glycerol.

Transposition reactions in vitro

Reactions were performed essentially as described (Bainton, et al., 1993). Reaction mixtures (100 µl final volume) contained (final concentration) 0.25 nM donor (donor plasmid, ELT or Split End) DNA, 2.5 nM pRM2 attTn7 target plasmid, 26 mM Hepes (pH 8.0), 2.1 mM DTT, 4.2 mM Tris (pH 7.5), 100 µg/ml tRNA, 50 µg/ml BSA, 2.0 mM ATP, 0.05 mM EDTA, 0.1 mM MgCl₂, 0.1 mM Chaps, 14 mM NaCl, 21 mM KCl, 1.25% glycerol, 50 ng TnsA (20 nM), 50 ng TnsB (6 nM) or 25 ng TnsB-His (3 nM), 50 ng TnsC (10 nM), 40 ng TnsD (10 nM), 15 mM magnesium acetate. Pre-incubation reaction mixtures (92 µl) were assembled on ice by mixing target DNA, TnsC (1.2 µl) and TnsD (3.3 µl) and buffer components (Hepes, Tris, DTT, ATP, BSA, tRNA); the concentrations of the components in this mixture varied by less than 15% from the stated final concentration conditions. The preincubation mixtures were then incubated for 20 minutes at 30°C; donor DNA (1.5 µl), TnsA (1.5 µl), TnsB (1.0 µl) and magnesium acetate (4.0 μ l) were then added individually and sequentially, and the incubation was continued for an additional 20 minutes at 30°C. Reactions were stopped by extraction with phenol/chloroform (1:1, v/v), the DNA was precipitated in ethanol, digested with restriction enzymes and analyzed by gel electrophoresis.

All reaction mixtures (except those in Figures 4, 8 and 9) were digested with *Bsa*HI. In Figure 4(a), the reaction products were digested with *Nde*I, electrophoresed on a 0.8% agarose-TBE gel and visualized by staining with ethidium bromide; for Figure 4(b), the simple insertion and ELT-SEJ reaction products were extracted from the gel by electroeleution, recovered by precipitation in ethanol and digested with *Hind*III, *XbaI* and *SaII*. In Figure 8(b), the reaction products were digested with *SaII*. In Figure 9(b), the reaction products were digested with *Hind*III, *XbaI* and *SaII*.

Hybridization probes and DNA labeling

The miniTn7-specific probe was the kanamycin gene segment between Tn7L and Tn7R, and was obtained by digestion of plasmid DNA with appropriate restriction enzymes, and isolation by electrophoresis on a 0.8% agarose-TBE gel, followed by extraction with Qiaex; DNA fragments were labeled by the random priming reaction using $[\alpha^{-32}P]dCTP$ and the Klenow fragment of DNA polymerase I. Strand-specific and Tn7 end-specific oligonucleotides that hybdridized to either the 3' or 5' strand of Tn7L and Tn7R were:

- NLC 94 (5') AAAGTCCAGTATGCTTTTTCACAG-CATAAC;
- NLC 100 (5') CCATAACAAAAGTCCAGTATGCTT-TTTCAC;
- NLC 95 (5') ATAATCCTTAAAAACTCCATTTCC-ACCCCT;
- NLC 163 (5') GTGAAAAAGCATACTGGACTTTT-GTTATGG;
- NLC 97 (5') AGGGGTGGAAATGGAGTTTTTAA-GGATTAT.

Oligonucleotide probes were 5' end-labeled with $[\gamma^{-32}P]$ ATP and bacteriophage T4 polynucleotide kinase. Labeled DNA was separated from unincorporated nucleotides by chromatography elution through a Nick Spin Column (Pharmacia).

Gel electrophoresis and product analysis

The restricted products of the reactions in Figures 3, 5 and 6 were displayed by electrophoresis on 0.8% agarose-TBE gels, transferred to Gene Screen Plus (Du Pont) and analyzed by hybridization with the miniTn7specific kanamycin probe. The restricted products of the reactions in Figures 4(b) and 9(b) were displayed by electrophoresis on denaturing 5% polyacrylamide gels and those in Figure 8(b) were displayed by electrophoresis on denaturing 8% polyacrylamide gels; the gels were electrotransferred to Gene Screen Plus and hybridized with a mixture of miniTn7 end-specific oligonucleotides. The restricted products of the reactions in Figure 7(b) were displayed by electrophoresis on a denaturing 0.8% agarose gel (Sambrook et al., 1989), then transferred to Gene Screen Plus and hybridized with the miniTn7specific kanamycin probe. In some cases, non-specific cross hybridization of the Km probe to the target DNA occurred and is designated by (target). Size markers were included on all gels and are marked as M in appropriate lanes. All gels except those in Figures 4(b), 7(b), 8(b) and 9(b) included a 1 kb ladder (Gibco/BRL) that was end labeled with $[\gamma^{-32}P]ATP$ and T4 polynucleotide kinase; Figure 4(b) included ladder V (BMB) that was end labeled with $[\gamma^{-32}P]$ ATP and T4 polynucleotide kinase; Figure 7(b) included a 1 kb ladder ³⁵S-labeled (Amersham), Figure 9(b) included a 100 bp ladder end labeled with $[\gamma^{-32}P]$ ATP and T4 polynucleotide kinase. In Figure 8(b), the ladder was generated by five different digestions of a miniTn7 DNA fragment; all digestions included *Sal*I and another enzyme to generate Tn7 end fragments of the indicated sizes. Blots were analyzed by autoradiography using Kodak XAR film.

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