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A Minimal System for Tn*7* Transposition: The Transposon-encoded Proteins TnsA and TnsB Can Execute DNA Breakage and Joining Reactions that Generate Circularized Tn*7* Species

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In the presence of ATP and Mg^{2+}, the bacterial transposon Tn7 translocates via a cut and paste mechanism executed by the transposon-encoded proteins TnsA + TnsB + TnsC + TnsD. We report here that in the presence of Mn^{2+}, TnsA + TnsB alone can execute the DNA breakage and joining reactions of Tn7 recombination. ATP is not essential in this minimal system, revealing that this cofactor is not directly involved in the chemical steps of recombination. In both the TnsAB and TnsABC + D systems, recombination initiates with double-strand breaks at each transposon end that cut Tn7 away from flanking donor DNA. In the minimal system, breakage occurs predominantly at a single transposon end and the subsequent end-joining reactions are intramolecular, with the exposed 3′ termini of a broken transposon end joining near the other end of the Tn7 element in the same donor molecule to form circular transposon species. In contrast, in TnsABC + D recombination, breaks occur at both ends of Tn7 and the two ends join to a target site on a different DNA molecule to form an intermolecular simple insertion. This demonstration of the capacity of TnsAB to execute breakage and joining reactions supports the view that these proteins form the Tn7 transposase.

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Introduction

Transposable elements are discrete DNA segments that move between non-homologous sites (Mizuuchi, 1992b; Craig, 1996). The DNA breakage and joining reactions that underlie the movements of transposable elements are mediated by transposases, specialized recombinases that are usually encoded by the mobile element itself. The transposase is positioned at the termini of the mobile element by specific binding to cognate recognition sequences, then acts to cleave the transposon away from the donor DNA and to join the newly exposed transposon ends to the target DNA.

The bacterial transposon Tn7 is unusual in that it encodes an elaborate array of proteins that are involved in its transposition; TnsA, TnsB, TnsC, TnsD and TnsE (Barth *et al*., 1976; Craig, 1995a). This multiplicity of Tn7 transposition proteins has obscured definition of the transposase. Previous work has demonstrated that Tn7 recombination *in vitro* can occur with just TnsABC when the non-hydrolyzable analog AMP-PNP is used instead of the usual ATP cofactor, revealing that these proteins form a ''core'' transposition machine that execute breakage and joining (Bainton *et al*., 1993). In the presence of ATP, this core machine can direct transposition to different classes of target sites using the alternative target site-selectors/activators, TnsD and TnsE. In the presence of TnsABC + D, Tn7 transposes to *attTn7*, a specific site in the *Escherichia coli* chromosome; *attTn7* target activity is determined by specific binding of TnsD to a particular sequence in *attTn7* (Bainton *et al*., 1993). Alternatively, TnsABC + E direct Tn7 to many sites whose locations are related by their presence on conjugable plasmids (Wolkow *et al*., 1996). Several observations suggest that TnsAB actually forms the transposase. TnsB binds specifically to the ends of Tn7, interacting with the DNA segments that are necessary for recombination

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(Arciszewska *et al*., 1991; Tang *et al*., 1991). Furthermore, sequence homology indicates that TnsB is a member of the retroviral integrase superfamily, a large group of recombinases that can execute the DNA breakage and joining reactions underlying transposition (Craig, 1995b; Grindley & Leschziner, 1995; Polard & Chandler, 1995a). Members of the retroviral integrase family contain a signature sequence, a conserved cluster of amino acids know as the DD(35)E motif, that binds the essential Mg^{2+} cofactor. Mutation of this sequence in TnsB, as in other members of the superfamily, blocks Tn7 recombination (Sarnovsky *et al*., 1996). The demonstration that a block to recombination imposed by mutation in TnsA of a catalytically essential glutamate residue to cysteine can be rescued by the presence of Mn^{2+} suggests that TnsA likely also contains an active site that uses Mg^{2+} as a cofactor (May & Craig, 1996; Sarnovsky *et al*., 1996). Recent crystallographic analysis of TnsA (unpublished results) has revealed that TnsA resembles most a type II restriction enzyme.

Although the above observations suggest that TnsAB form the transposase, no recombination activity is observed with only these proteins (Bainton *et al*., 1993; Stellwagen & Craig, 1997a,b). Based on the fact that in Mu transposition, the activity of the MuA transposase is modulated by the ATP-utilizing protein MuB (Craigie *et al*., 1985; Baker *et al*., 1991), we have suggested that the ATP-ultilizing protein TnsC interacts with the target DNA and the transposase TnsAB to control transposition (Stellwagen & Craig, 1997a, 1998). Demonstration that TnsAB are indeed the transposase requires that breakage and joining by these proteins alone be observed.

We demonstrate here that when Mn^{2+} is used as an alternative cofactor in *in vitro* Tn7 recombination, DNA breakage and joining can occur in the presence of only TnsAB, revealing directly that these proteins execute the critical chemical steps in transposition. Thus the experiments presented here support the view that TnsAB forms the Tn7 transposase.

The breakage and joining steps promoted by TnsAB in the minimal Mn^{2+} system result in intramolecular rearrangements that generate circularized forms of Tn7, in contrast to the formation of intermolecular simple insertions by TnsABC + D. However, the same fundamental chemical events, breakage to expose 3′ transposon ends and joining of these 3′ ends to target DNA, and breakage at 5′ ends are common to both intramolecular TnsAB and intermolecular TnsABC + D transposition. A circular species generated in the minimal TnsAB system can form without the complete excision of the transposon from the donor backbone. A related intramolecular rearrangement has been observed with IS*911* (Polard *et al*., 1992; Polard & Chandler, 1995b).

Results

A minimal system for Tn*7* recombination in the presence of Mn^{2+}

The standard Tn7 transposition reaction is an intermolecular event in which Tn7 forms an intermolecular simple insertion in *attTn7*. This reaction occurs by excision of the transposon from the donor backbone by double-strand breaks at each Tn7 end, followed by the joining of both Tn7 ends to the target DNA. Tn7 transposition is efficiently promoted *in vitro* by a mixture of four Tn7-encoded proteins, TnsA + TnsB + TnsC + TnsD, in the presence of ATP and Mg^{2+} (Bainton *et al*., 1993; Gary *et al*., 1996). We have found that under alternative reaction conditions, most notably in the presence of Mn^{2+} rather than Mg^{2+} as a divalent metal cofactor, TnsAB alone can promote recombination (Figure 1(a)). In this experiment, the miniTn7 substrate DNA was incubated under various reaction conditions, digested with restriction enzymes that cut asymmetrically within the donor backbone, displayed by native gel electrophoresis and detected by hybridization with a miniTn7-specific probe. Some of the products of TnsAB transposition are donor molecules in which a double-strand break (DSB) has occurred at either Tn7L (DSB.L) or Tn7R (DSB.R). Other recombinant species result from the intramolecular joining of an exposed Tn7 end to other positions in the donor molecule, usually at the other transposon end (lanes 1, 5 and 10); detailed evidence for these structures is provided below. These recombinant molecules are (intra) DSB.R-SEJ, i.e. the intramolecular join of the Tn7R end exposed by a DSB to Tn7L as a single-end join, and (intra) DSB.L-SEJ (Figure 1(b)). The (intra) DSB.SEJ molecules migrate more slowly in the gel than the corresponding DSB molecule, consistent with their constrained topology (Bell & Byers, 1983). Also observable are excised linear transposons (ELT) in which the transposon has been completely disconnected from the donor backbone and other species that likely are transposon circles in which the ends are disconnected from the donor backbone and joined to each other. The events underlying these minimal reactions, breakage and joining at the 3′ ends of Tn7 and breakage at the 5′ transposon ends (see below), are the same chemical events that underlie standard Tn7 transposition (Bainton *et al*., 1991).

Recombination in the minimal TnsAB system is dependent on the presence of both TnsA and TnsB; no breakage or joining can be detected with either protein alone (Figure 1(a), lanes 3 and 4). Although in this experiment only double-strand breakage events are evaluated, no single-strand break is detectable when the individual DNA strands are examined (see below). It is also notable that TnsAB recombination can occur in the absence of ATP (lanes 1, 5 and 10); thus this nucleotide cofactor is not essential to DNA strand breakage and joining.

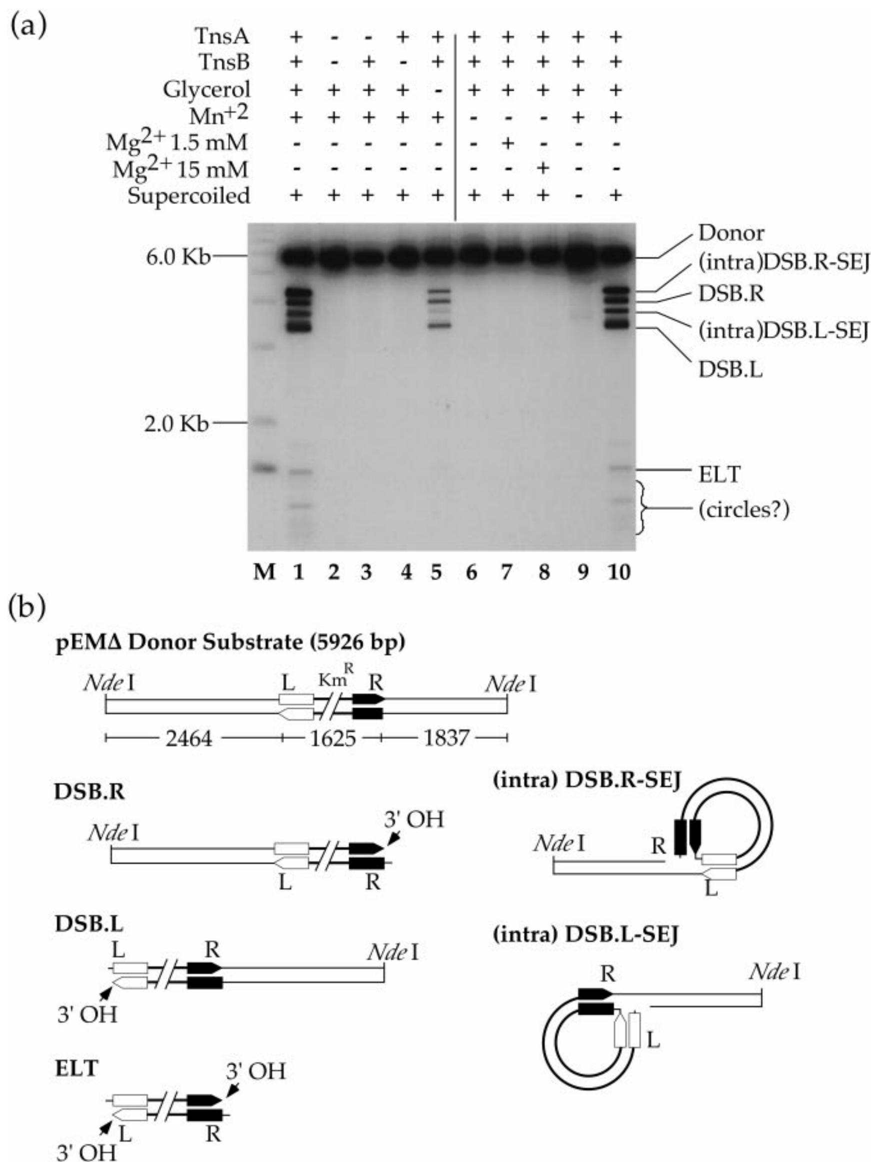

Figure 1. TnsAB can execute breakage and joining in the presence of Mn^{2+}. (a) Recombination reactions were performed using a supercoiled or relaxed miniTn7 plasmid (pEMΔ) as a substrate with TnsAB and various combinations of Mg^{2+}, Mn^{2+} and glycerol as indicated. The reaction products were digested with *Nde*I, displayed by native agarose gel electrophoresis and visualized by hybridization with a miniTn7-specific probe. (b) The substrate and products of recombination are shown diagrammatically, with Tn7L (open bars) and Tn7R (filled bars) flanking a Km^R segment. A double-strand break (DSB) at Tn7R results in a DSB.R; intramolecular single-end joining of the exposed R end to Tn7L results in (intra) DSB.R-SEJ; a double-strand break at Tn7L results in a DSB.L; intramolecular single-end joining of the exposed L end to Tn7R results in (intra) DSB.L-SEJ; double-strand breaks at both L and R result in an excised linear transposon (ELT). The species labeled circles are likely formed by end to end joining of the transposon with concomitant release from the flanking donor backbone.

Recombination promoted by TnsAB alone specifically requires the presence of Mn^{2+}; no recombination can be observed in the presence of Mg^{2+} (lanes 7 and 8) or in the presence of Ca^{2+} or Zn^{2+} (data not shown). The profound effect of Mn^{2+} on recombination is consistent with the view that a divalent metal ion (usually $Mg^{2+)}$ is located at or near the active site(s) that executes the DNA processing reactions (Mizuuchi, 1997). Although some TnsAB recombination can be observed at very low (0.4 %) levels of glycerol (lane 5), recombination is significantly stimulated by the addition of glycerol to 20 % (lanes 1 and 10).

We have examined the effect of TnsC addition on TnsAB recombination in the presence of Mn^{2+}, finding that recombination is stimulated and yields the same products as TnsAB recombination (data not shown).

Another feature of TnsAB recombination is that this reaction specifically requires that the donor DNA substrate be supercoiled; no recombination is observed with a linearized donor substrate (lane

9). In TnsABC recombination (data not shown) and the complete TnsABC + D system, recombination can efficiently occur with both relaxed and supercoiled substrates (Bainton *et al.*, 1993; Gary *et al.*, 1996). One explanation for the supercoiling requirement is that supercoiling promotes interactions between the transposon ends.

Intramolecular recombination requires two Tn*7* ends

The finding that one end of Tn7 often joins to the other Tn7 end in a single donor substrate during intramolecular TnsAB recombination suggests that the ends interact. We examined the Tn7 end requirements in intramolecular TnsAB recombination by analyzing DNA substrates containing only a single end segment (Figure 2). (Note that the orientation of the miniTn7 elements in different substrates varies, changing the relative migrations of the recombination products). No DNA breakage or joining with TnsAB is observed with these single-end substrates, suggesting that interactions between the ends are key to recombination. Thus although the use of Mn^{2+} does alter the protein recombinase requirements, the fundamental substrate requirement for two Tn7 ends to promote recombination (Gary *et al.*, 1996) is not bypassed in the minimal system.

Double-strand breaks and intramolecular joining are blocked by alterations at the transposon termini

To further probe the substrate requirements of TnsAB recombination, we analyzed reactions using DNA substrates containing mutations at the transposon termini. The extreme termini of Tn7 are

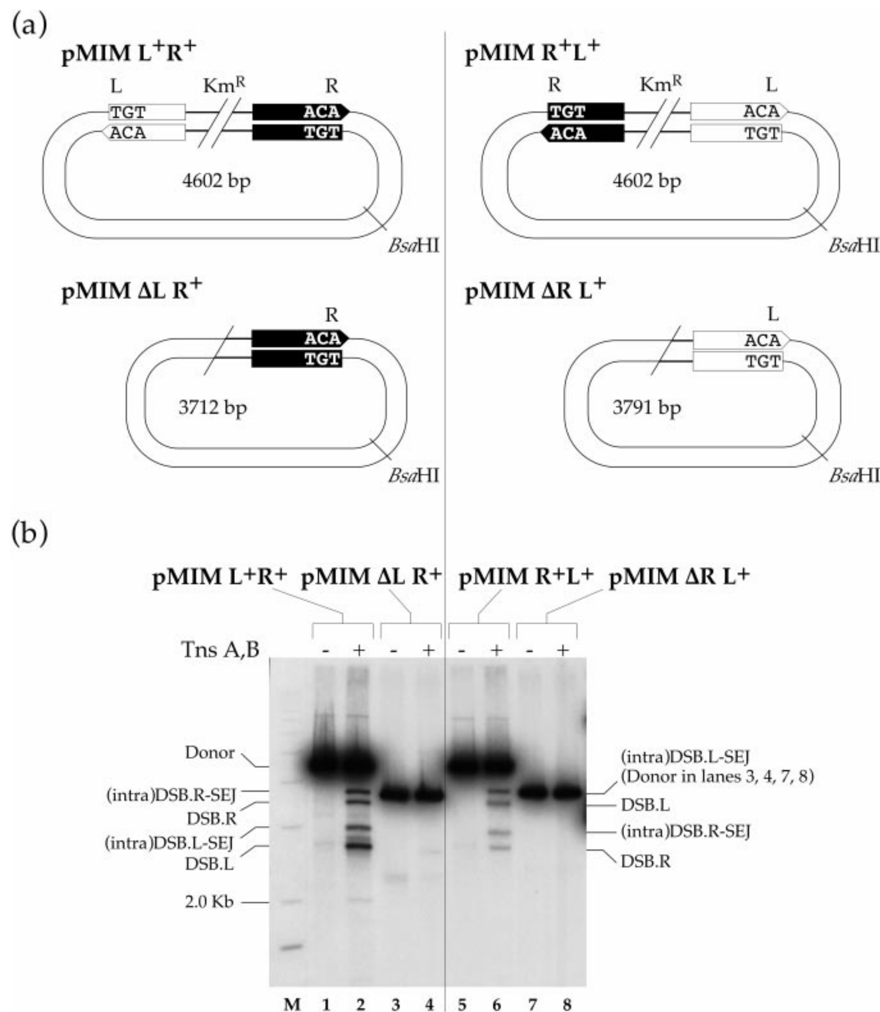

Figure 2. TnsAB recombination requires two Tn7 ends. (a) Recombination substrates containing various Tn7 end segments are shown that contain both Tn7L and Tn7R (pMIM L^+R^+ and pMIM R^+L^+), contain only Tn7R (pMIM R^+ ΔL) or contain only Tn7L (pMIM ΔR L^+). Note that the orientation of the miniTn7 element varies with respect to the donor backbone, so that the sizes of the various DSB.L and DSB.R species also vary as indicated in (b). (b) Recombination reactions were performed using various supercoiled miniTn7 plasmids as substrates. The recombination reaction products were digested with *Bsa*HI, displayed by native agarose gel electrophoresis and visualized by hybridization with a miniTn7-specific probe.

CA-3′, a dinucleotide highly conserved at the ends of many transposable elements, including retroviruses (Polard & Chandler, 1995a). Mutation of the Tn7 termini from CA-3′ to GT-3′ blocks the 3′ breakage and joining events in the TnsABC + D system (Gary *et al.*, 1996). These end mutations affect recombination in the TnsAB system (Figure 3): no DSB breakage or joining is observed with a substrate in which both Tn7 ends are changed from CA-3′ to GT-3′ (pSIM L^-R^-; lane 8). (Note that the orientation of the miniTn7 element in different substrates varies, changing the relative sizes of the Tn7L and Tn7R products.) With a chimeric substrate, one that contains one wild-type and one mutant end, considerable DSB formation and joining does occur with the wild-type end but little DSB breakage at or joining of the mutant end is observed. For example, with the Chi L^+R^- substrate, which contains a wild-type $Tn7L^+$ end and a mutant $Tn7R^-$ end, we observe DSBs at the L^+ end and joining of the L^+ end to the R^- end but no DSBs are observed at the mutant R^- end (lane 6).

We examined the DNA strands of these chimeric substrates individually using denaturing gel electrophoresis and strand-specific hybridization probes (data not shown). Although DSBs are blocked at these mutant ends in the Mn^{2+} TnsAB reactions, cleavage at the 5′ end still occurs, i.e. only 3′ end cleavage is blocked. These terminal mutations also specifically block 3′ end breakage in the TnsABC + D system (Gary *et al.*, 1996). The positions of the 5′ cleavage are the same in both these systems, occurring several nucleotides outside the actual transposon termini.

Thus, although the presence of Mn^{2+} does alter some requirements for Tn7 transposition, the iden-

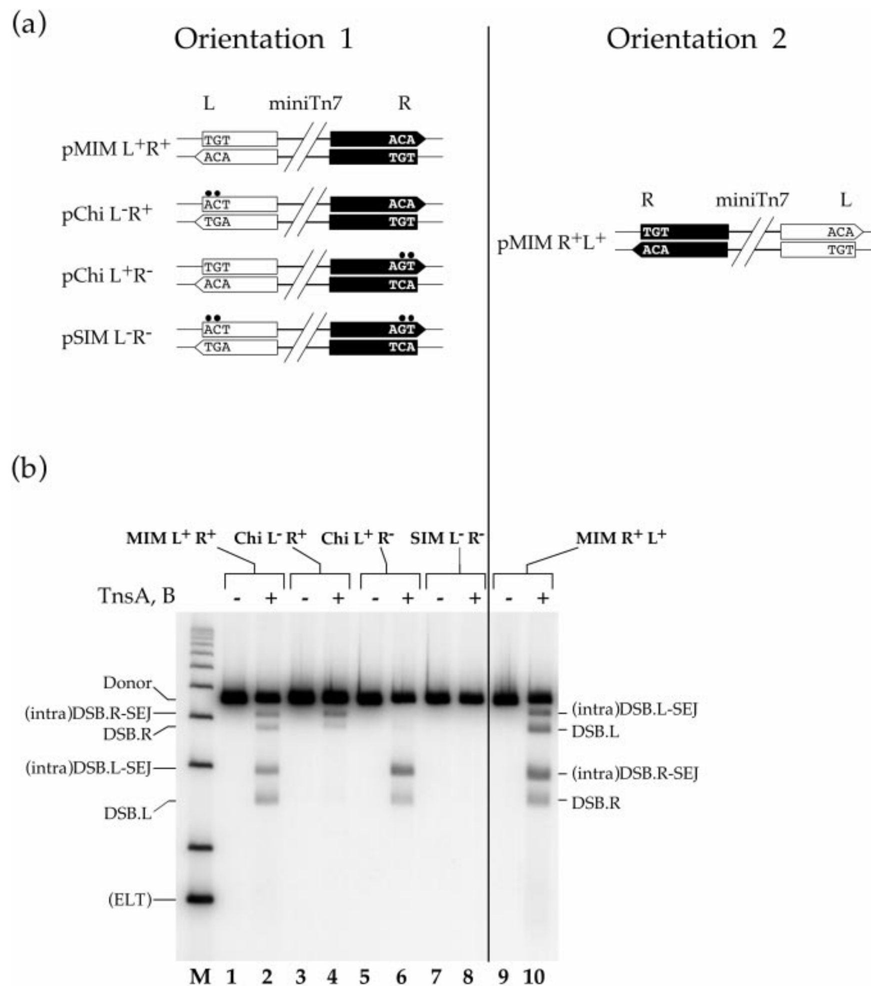

Figure 3. Mutations at the termini of Tn7 can block TnsAB recombination. (a) Recombination substrates containing wild-type (-CA-3′) and/or mutant (-GT-3′; black dots) Tn7 termini are shown. Plasmids with two wild-type ends are shown, pMIM L^+R^+ and pMIM R^+L^+; note that the orientation of the miniTn7 element with respect to the vector backbone varies, so the sizes of the DSB species vary as indicated in (b); in pSIM L^-R^-, both L and R are mutant; in Chi L^-R^+, L is mutant and R is wild-type and in Chi L^+R^-, L is wild-type and R is mutant. (b) Recombination reactions were performed using supercoiled miniTn7 plasmid substrates with wild-type and mutant termini. The reaction products were digested with AlwNI, displayed by native agarose gel electrophoresis and visualized by hybridization with a miniTn7-specific probe.

tity of the sequences at the transposon termini that are most intimately involved in breakage and joining remains critical.

Analysis of TnsAB recombination products by electron microscopy

We used rotary shadowing and transmission electron microscopy (EM) to analyze non-restricted recombination mixtures (Figure 4(a)). We observed a variety of species: linear molecules that likely are DSBs; linear molecules with various sized loops at one end that likely result from double-strand breaks followed by intramolecular joining; and both small and large relaxed and supertwisted circles, which likely represent circularized transposons and unrecombined substrate.

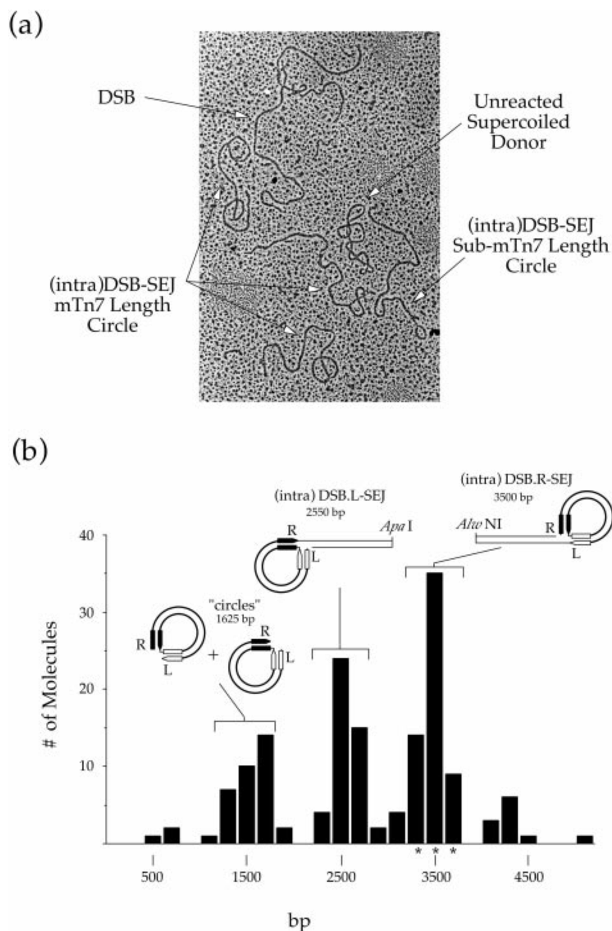

Figure 4. Analysis of TnsAB recombination products by electron microscopy. A substrate plasmid (pEMΔ) was subjected to TnsAB recombination, the DNA recovered by extraction with phenol and precipitation with ethanol, applied to grids and examined by electron microscopy as described in Materials and Methods. (b) A substrate plasmid (pEMΔ) was subjected to TnsAB recombination, the DNA recovered by extraction with phenol and precipitation with ethanol, digested with *Alw*NI and *Apa*I, recovered by precipitation in ethanol, applied to grids and examined by electron microscopy. The lengths of looped molecules were determined as described in Materials and Methods; the average of the measured lengths of the indicated (intra) DSB.R-SEJ species (asterisks) was calculated to be 3230 bp/μm and this value was used to determine the size in base-pairs of the other species observed.

We examined recombination products after restriction with an enzyme that cuts asymmetrically within the donor backbone. By projection and tracing, we measured the lengths of the species containing loops, i.e. the small circles and linears with tail loops (lariats). Data for 155 independent traces are displayed by size increments in Figure 4(b)). The majority of the structures analyzed can be readily assigned to one of three distinct groups based on their relative sizes: (1) transposon circles; (2) (intra) DSB.R-SEJ; and (3) (intra) DSB.L-SEJ. We estimated the size in base-pairs for all molecules analyzed by assuming that all the molecules in the largest length cluster (marked with * in Figure 4(b)) were DSB.R-SEJ species of 3500 bp and then used their average measured length to convert all molecules from measured length to base-pairs. If we assume an average length of 3.4 Å/nucleotide for standard Watson-Crick *B*-form DNA, we arrive at a conversion factor of 2941 bp/μm. The conversion value we determined was 3200 bp/μm, in good agreement with this factor.

We separately analyzed the sizes of the loops and stems of these molecules, finding that about 50 % of the molecules in each class have loops that are approximately transposon-sized, i.e. result from end-to-end joining of the transposon segment in each molecule (data not shown). The remaining molecules had loops that were heterogeneous in size, resulting from the joining of a broken transposon end to another (non-Tn*7* end) position in the donor molecule. These other loops were predominantly shorter than transposon length, indicating that joining occurred at many different positions within the transposon segment; we were unable to discern a preferred position of joining. These EM analyses support the view that the species designated (intra) DSB-SEJ indeed result from DSB at the transposon ends and intramolecular joining of the exposed end. These studies reveal that although Tn7 end to Tn7 end joining is preferred, joining can occur at other target positions inside the element.

The EM analysis also revealed the presence of transposon circles in reaction mixtures; these species are likely the ''circles'' noted in Figure 1(a). These circles may be formed directly during TnsAB recombination and are heterogeneous because of differing topology (knotted *versus* unknotted) and/or from size heterogeneity. Alternatively, these species may result from breakage of the stem segment of a single-end join during sample processing.

Analysis of recombination products at the nucleotide level

Where do the TnsAB DNA breakage and joining reactions occur? We analyzed reaction products on denaturing gels using strand-specific oligonucleotide hybridization probes, following digestion of the recombination substrates and products with a restriction enzyme (*Sal*I) to generate conveniently sized DNA fragments. Shown in Figure 5 are analyses of events involving the top (5′) end of Tn7L (a), lanes 1-3) and of analysis of the bottom (3′) strand of Tn7L ((b), lanes 1-3). In each case, unreacted substrate DNA, DNA broken at the transposon ends, and DNAs where one transposon end has joined to the other transposon end are evident. Analysis with similar probes specific for Tn7R yielded similar results (data not shown).

Consider first the analysis of events at the 3′ (bottom strand) Tn7L end using a probe specific

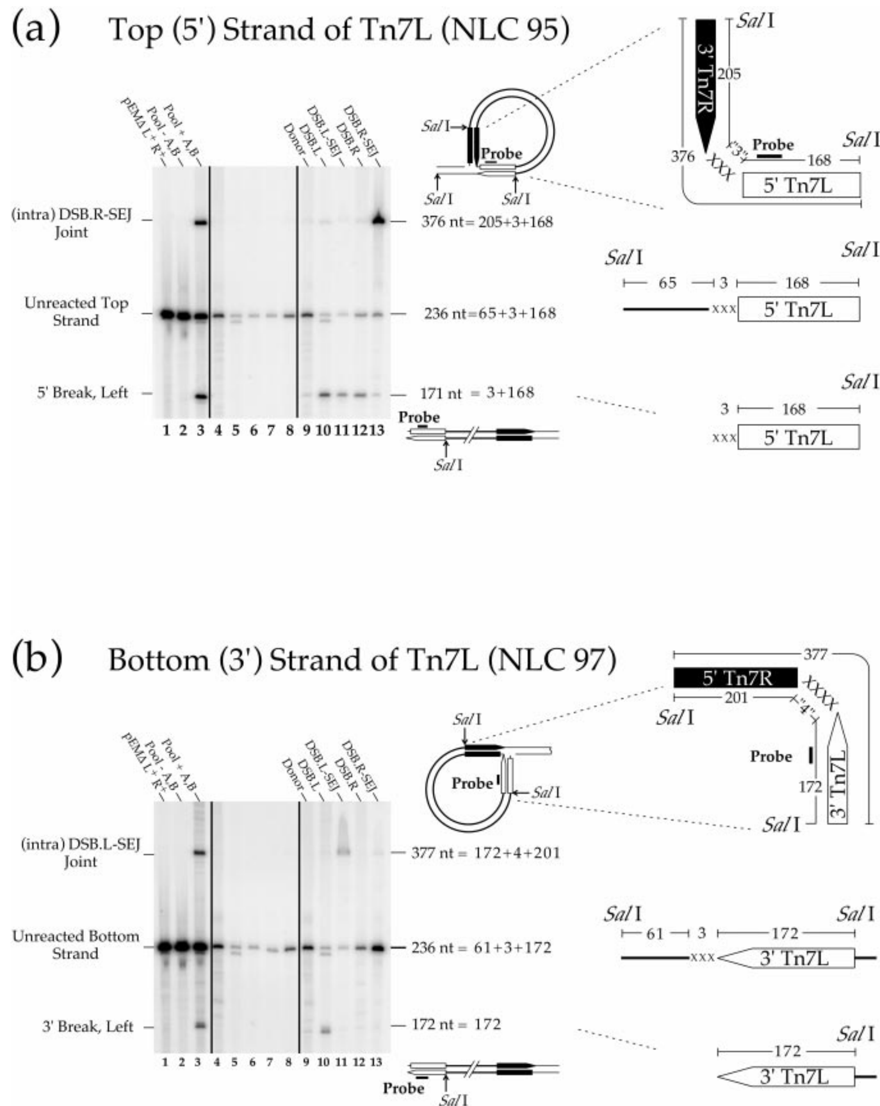

Figure 5. Analysis of TnsAB recombination products by denaturing gel electrophoresis. (a) TnsAB recombination reactions were performed using a supercoiled miniTn7 plasmid (pEMΔ) as a substrate, displayed on a denaturing gel and detected by oligonucleotide strand-specific and end-specific hybridization probes. In lanes 1-3, recombination reaction mixtures were directly digested with *Sal*I and run on the denaturing gel. In lanes 4-13, recombination products were isolated by preparative gel electrophoresis and gel extraction (see Figure 7), digested with *Sal*I and run on the denaturing gel; lanes 4-8 are species isolated from a preparative control lane incubated without TnsAB; and lanes 9-13 are isolated recombination products from a TnsAB-containing reaction. DNA strand breaks and joins at Tn7L are analyzed with NLC 95, which hybridizes to the top (5′) Tn7L end and with NLC 97, which hybridizes to the bottom (3′) Tn7L end; the various species are illustrated. The unreacted donor top (5′) strand is 236 nt; simple breakage to expose the 5′ end results in a 171 nt species, and joining of the 3′ end of Tn7R to the 5′ end of Tn7L results in a 376 nt species. The unreacted donor bottom (3′) strand is 236 nt, simple breakage to expose the 3′ end results in a 172 nt species and joining of the 3′ end of Tn7L to the 5′ end of Tn7R results predominantly in a 377 nt species.

for this strand (Figure 5(b)). One DNA species is the unreacted donor DNA (236 nt) containing the Tn7L end segment (172 nt) joined to flanking donor sequences (64 nt); this fragment is generated by digestion at *Sal*I sites, one in the flanking donor DNA and one inside the transposon. The smaller 172 nt fragment is generated by TnsAB cleavage at the Tn7L end, exposing the 3′OH terminus and *Sal*I digestion within the element. The larger species (377 nt), intra DSB.L-SEJ, results from the joining of the Tn7L 3′OH terminus to a position adjacent to the 5′ (bottom) end of Tn7R and *Sal*I digestion at positions inside both the Tn7L and Tn7R segments. The major species results from an attack of the Tn7L 3′OH end to a position 4 nt from the 5′ Tn7R termini (see below). Thus the larger species is 377 nt contains the 172 nt Tn7L segment, 4 nt of donor DNA flanking the right end and 201 nt from the Tn7R end.

Related species are seen with the top 5′ strand at Tn7L (Figure 5(a)). The unbroken substrate DNA is 236 nt in length, being composed of the 168 nt of Tn7L and flanking donor DNA of 68 nt (65 + 3nt). (The Tn7L segments are different lengths in Figure 5(a) and (b) because of the staggered breaks introduced by digestion with *Sal*I). The smaller 171 nt fragment derives from cleavage near the 5′ end of the transposon by TnsAB; this cleavage occurs such that 3 nt of flanking donor DNA is attached to the transposon terminus (Bainton *et al*., 1991). The longer 376 nt species results from the attack of the 3′ end of Tn7R at a position 3 nt outside the terminus of the 5′ end of Tn7L and *Sal*I digestion.

These analyses reveal that the fundamental chemistry of TnsAB-promoted recombination is the same as that of TnsABC + D recombination: DNA breakage reactions at the 3′ and 5′ ends of Tn7 and joining of the 3′ ends of Tn7 to the target DNA (Bainton *et al*., 1993).

Mapping the positions of intramolecular DNA strand joining

Where exactly do the joins between the Tn7L and the Tn7R ends occur? To determine at nucleotide resolution where these novel intramolecular joining reactions occur, we examined recombination products on high-resolution denaturing gels, using strand-specific hybridization probes (Figure 6). When the joining of the 3′ end of Tn7L to the 5′ strand of Tn7R in the (intra) DSB.L-SEJ is analyzed, multiple joining positions can be observed (Figure 6(b)). The most prominent joint species is 105 nt in length, consistent with a preferred position of the L end joining 4 nt outside the actual 5′ terminus of Tn7R. When the joining of the 3′ end of Tn7R to the top 5′ strand of Tn7L in an (intra) DSB.R-SEJ. is examined, two species, 105 nt and 106 nt in length, are evident. These sizes are consistent with the 3′ end of Tn7R joining 3 nt and 4 nt from the actual 5′ terminus of Tn7L.

Analysis of isolated recombination products

We have isolated and characterized the various TnsAB recombination products individually. Following incubations for recombination and restriction, reaction mixtures were displayed on a preparative agarose gel; DNAs from a reaction that contained and, as a control, one that lacked recombination proteins were similarly displayed (Figure 7). Individual gel bands, both observed recombination products and similar positions in a control lane, were excised and the DNA extracted. The extracted recombination products were displayed on a second native agarose gel to assess their purity; each extracted species corresponded to the expected species (Figure 7).

The extracted DNA samples were digested with *Sal*I, separated on a high-resolution denaturing gel, and visualized with a Tn7L 5′ end strand-specific probe (Figure 5(a), lanes 9-12) and with a Tn7L 3′ end-specific probe (Figure 5(b), lanes 9-12). The distinctive species observed by such analysis using Tn7L probes and others with Tn7R probes (data not shown) for each recombination product band, i.e. (intra) DSB.R-SEJ etc., is consistent with the assigned identity of that band (see above). One additional piece of information is that some 5′ nicking occurs at Tn7L in the DSB.R species, as the 5′ probe detects both intact (236 nt) and cleaved (172 nt) species.

An unexpected species was present in all extracted fractions: a 236 nt fragment representing unbroken donor DNA. Since this donor species is present in all the gel slices extracted from parallel positions in the control lane that were not incubated with TnsAB (lanes 4-8), this species simply represents an unexpectedly wide dispersion of the unrecombined donor DNA throughout the preparative gel.

These analyses by electron microscopy and denaturing gel electrophoresis of Tn7 TnsAB recombination products provides direct evidence for the structures of the DSB and (intra) DSB-SEJ species.

Discussion

This work has revealed that TnsA and TnsB together can execute DNA strand breakage and joining reactions when Mn^{2+} is substituted for Mg^{2+}, the usual cofactor for Tn7 transposition. It is not surprising that TnsB is required in this minimal Tn7 system: TnsB plays a key role in substrate recognition as a sequence-specific, DNA-binding protein that recognizes sequences necessary for recombination at both the left and right ends of Tn7 (Arciszewska *et al*., 1991; Tang *et al*., 1991; DeBoy & Craig, 1996). No specific DNA binding has been detected with TnsA (Bainton *et al*., 1993), and an attractive view is that TnsA interacts and is thereby also positioned at the ends of Tn7 though protein-protein interactions with TnsB (Sarnovsky

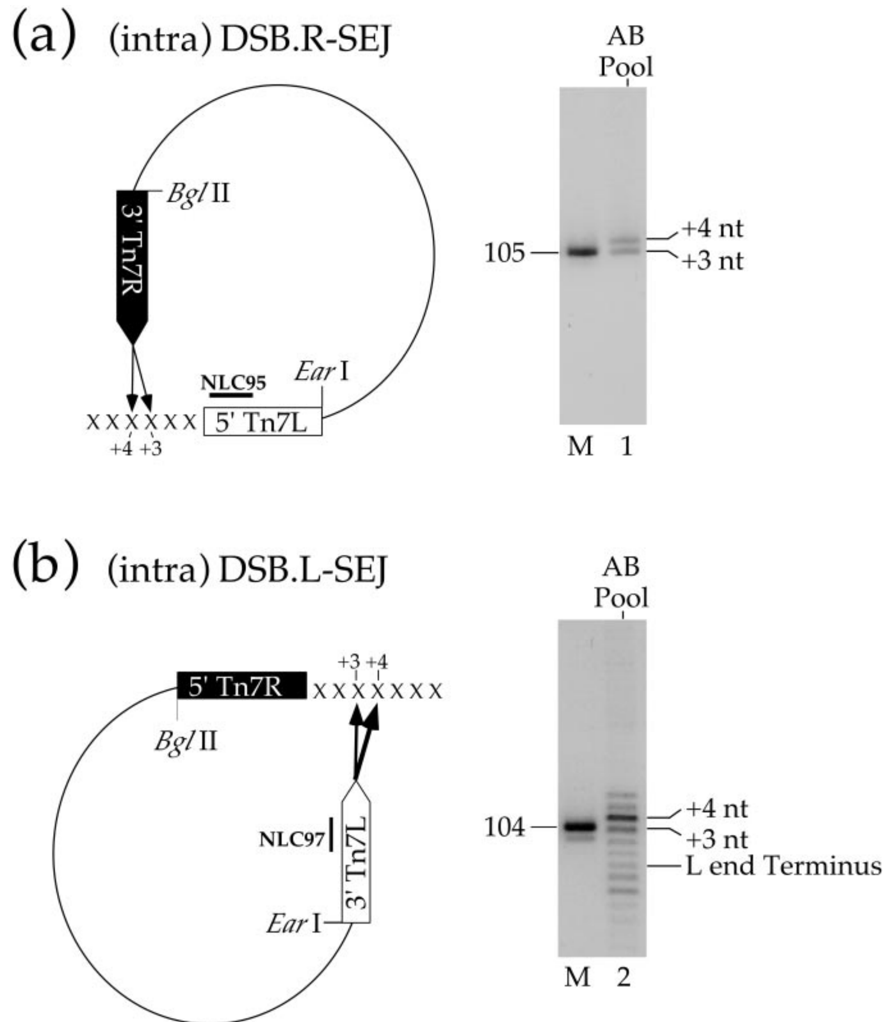

Figure 6. Analysis of TnsAB recombination joints at the nucleotide level. (a) The positions of the joining of the 3′ end of Tn7R to the 5′ end of Tn7L in the (intra) DSB.R-SEJ (which results in circularization of the top strand of Tn7) are shown diagrammatically as determined by analysis by denaturing, high-resolution gel electrophoresis. The x are donor backbone nucleotides that flank the termini of Tn7 and are sites of end joining during intramolecular recombination. After recombination, pEMΔ (lane 1) was digested with *Bgl*II and *Ear*I, displayed on a denaturing gel, and hybridized with the Tn7L top strand-specific probe NLC95. Lane M contains a marker 3′ Tn7R - 5′ Tn7L fragment 105 nt in length from pMLP1. The 3′ Tn7R end joining occurs predominantly within the flanking donor DNA either 3 nt or 4 nt from the Tn7 terminus. (b) The positions of the joining of the 3′ end of Tn7L to the 5′ end of Tn7R in the (intra) DSB.L-SEJ (which results in circularization of the bottom strand of Tn7). After recombination, pEMΔ (lane 1) was digested with *Bgl*II and *Ear*I, displayed on a denaturing gel, and hybridized with the Tn7L bottom strand-specific probe NLC97. Lane M contains a marker 3′ Tn7L - 5′ Tn7R fragment 104 nt in length from pMLP1. The 3′ Tn7L end joining is heterogeneous, with the most pronounced joining position 4 nt from the Tn7R terminus.

et al., 1996). From previous mutational analysis of TnsB and TnsA, we have argued elsewhere that active sites for the DNA processing reactions actually lie in both TnsB and TnsA, TnsB executing the 3′ end breakage and joining reactions, and TnsA executing the 5′ cleavage reactions (May & Craig, 1996; Sarnovsky *et al.*, 1996). TnsB is a member of the retroviral integrase superfamily, which can execute Mg^{2+}-dependent breakage and joining (Craig, 1995b; Grindley & Leschziner, 1995; Sarnovsky *et al.*, 1996) and TnsA mostly closely resembles a restriction enzyme (unpublished results). The finding here that TnsAB alone can execute recombination under alternative reaction conditions supports the view that the active sites for DNA processing lie entirely in these two Tns proteins.

Recombination in the minimal TnsAB system proceeds in the absence of ATP, a necessary cofactor in the TnsABC and TnsABC + D systems (Bainton *et al.*, 1993). Thus ATP plays a regulatory capacity role in transposition. We have shown elsewhere that TnsC is the ATP-utilizing protein (Gamas & Craig, 1992). ATP plays a regulatory role in other recombination systems, most notably bacteriophage Mu, in which the ATP-utilizing protein MuB plays a key role in directing the MuA transposase to an appropriate target DNA (Lavoie & Chaconas, 1996). The fact that TnsAB

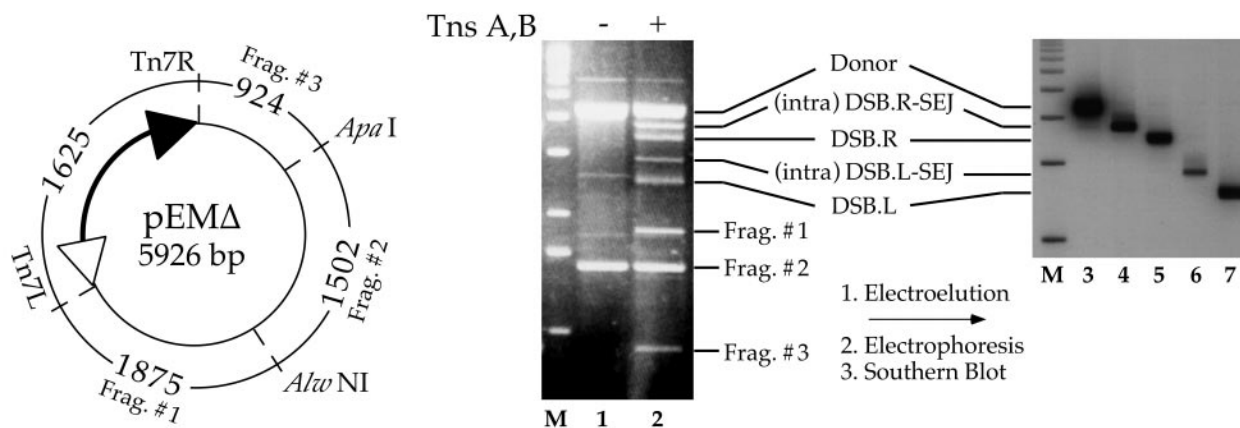

Figure 7. Analysis of isolated recombination products. TnsAB recombination products were isolated from a preparative native agarose gel. pEMΔ (left) was incubated with (lane 2) or without (lane 1) TnsAB, digested with *Alw*NI and *Apa*I, displayed on a native agarose gel, visualized with ethidium bromide staining (middle) and recombinant species or equivalent control gel positions extracted. To assess the purity of the extracted DNAs, they were re-electrophoresed on a native agarose gel and visualized by hybridization with a miniTn7-specific probe (right). These isolated species were then digested with *Sal*I and analyzed (Figure 5).

recombination can proceed without ATP suggests that the critical strand transfer step occurs by a phosphoryl transfer mechanism in which the exposed 3′ end of the transposon acts as an attacking nucleophile on the target DNA strand, in a mechanism related to that described for other recombinases that, like TnsB, are members of the retroviral integrase superfamily (Mizuuchi, 1992a).

Why does TnsAB recombination occur in the presence of Mn^{2+} but not in the presence of Mg^{2+}? It is likely that Mg^{2+} acts at or near the active sites for the DNA processing reactions, for 3′ end breakage and joining in TnsB and for 5′ end breakage in TnsA. Mg^{2+} may play several roles, perhaps promoting activation of a reactive nucleophile and/or coordinating with a DNA substrate. We imagine that when Mn^{2+} is used, the active site(s) for DNA processing can assume an alternative conformation that is competent to execute DNA processing and does not need an ''activating'' signal that is usually provided by the other Tns proteins such as TnsC + D. This hypothesis is reminiscent of the role(s) proposed for Mn^{2+} in relaxing/altering the cleavage specificity of certain restriction enzymes (Hsu & Berg, 1978; Vermote & Halford, 1992). Alteration of recombinase activity by Mn^{2+} has been observed in other recombination systems, including Mu transposition (Baker & Luo, 1994; Kim *et al*., 1995) and retroviral integration (Katzman *et al*., 1989; Drelich *et al*., 1992).

Although Mn^{2+} has profound effects on TnsAB recombination, we have been unable, even in the presence of Mn^{2+}, to detect any DNA breakage activity by TnsB alone (data not shown), emphasizing the interdependence of TnsB and TnsA in promoting DNA breakage and joining (Sarnovsky *et al*., 1996). We have identified two other components that can influence TnsAB recombination, glycerol and the topological state of the DNA substrate. Recombination is significantly increased with higher concentration of glycerol, which may influence the structure of the TnsAB nucleoprotein complex by affecting protein-protein and/or protein-DNA interaction or act as a concentrating agent by excluding water. Alternatively, glycerol may act as a nucleophile in the strand breakage step, and thereby influence the initiation of recombination (Craigie *et al*., 1990; Engelman *et al*., 1991; van Gent *et al*., 1993). TnsAB recombination requires a supercoiled DNA substrate, whereas a relaxed donor DNA is an effective substrate in the TnsABC + D system (Gary *et al*., 1996). Substrate supercoiling may influence several stages in recombination, including the interaction of recombination proteins with each Tn7 end and interactions between the Tn7 ends.

Comparison of Tn*7* circularization to circularization reactions in other recombination systems

We have shown here that Tn7 recombination can promote interactions between the two transposon ends in a single donor plasmid that can lead to their covalent linkage, i.e. to formation of a circular transposon species. The major circular form that we have detected is a lariat, in which the circularized Tn7 remains attached to the donor backbone through a connection at one end of the element; and we have observed some Tn7 circles that have been entirely disconnected from the donor backbone but it remains to be established if these result from specific, i.e. TnsAB-promoted processing, or reflect non-specific degradation of lariat species. At the heart of the Tn7 circularization reaction are DNA breakage and joining reactions using one 3′ transposon end as a substrate for breakage and joining, and the 5′ end of the same transposon

strand as the target site of joining. Breakage and joining reactions involving the 3′ ends of mobile elements are central to all elements that have been studied in biochemical detail (Mizuuchi, 1992b). IS*911* (Polard & Chandler, 1995b) has been shown to execute an intramolecular end to end joining reaction. The products of the IS*911* transposition are related to the Tn7 structures in that all reactions involve 3′ end joining but the Tn7 system is different, in that the cleavage reaction that exposes the reactive 3′ end is actually a double-strand break generating free 3′ and 5′ ends. Thus the Tn7 TnsAB product is a lariat (tail + loop) form rather than the figure-of-eight seen with IS*911*. Under conditions where cleavage at the 5′ ends of Tn7 is blocked by a mutation in TnsA, we have observed figure-of-eight species in Tn7 recombination (unpublished results).

Actual transposon circles that are disconnected from a donor backbone are prominent features of IS*911* recombination; Polard and Chandler have suggested that these may derive from IS*911* figures-of-eight but a mechanism for this conversion remains to be established. As originally discussed by Polard and Chandler, a distinct feature of circularization reactions is that they need not result from the complete excision of the element from the donor backbone followed by end joining; rather, juxtaposition of the ends and their initial covalent linkage can occur while still embedded in the donor backbone.

The Tn*7* recombination machine

The work presented here has sharpened our view of the Tn7 recombination machinery and mechanism. This work and our mutational analysis of TnsA and TnsB has revealed that these proteins execute the most fundamental reactions that underlie transposition. TnsB plays an essential role in identification of the transposon through specific binding to the ends of the transposon, and TnsA and TnsB together execute the chemical steps of DNA strand breakage and joining. The activity of this central breakage and joining machine is modulated and positioned appropriately on the target DNA by the other Tns proteins.

Materials and Methods

DNA substrates

The standard miniTn7 element (L^+R^+) is 1.6 kb in length and is comprised of a Tn7 end containing the Tn7 *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 Δ L^+R^+, the terminal 16 bp of Tn7L have been changed to those of Tn7R, a 2 bp change (Bainton *et al*., 1991; Gary *et al*., 1996) that does not obviously affect recombination. The donor plasmid pEMΔ L^+R^+ contains an *E. coli* chromosomal segment into which a miniTn7 element inserted *via* TnsABC + E recombination ligated into the *Sma*I site of pTRC99 (Bainton *et al*., 1993). In the donor plasmid pMIM L^+R^+ and its derivatives (Bainton *et al*., 1991; Gary *et al*., 1996), PCR was used to generate a miniTn7-containing fragment in which the miniTn7 element has end-specific restriction sites that result in wild-type (-CA-3′) or mutant (-GT-3′) transposon termini flanked by *Bam*HI sites in the *Bam*HI site of Bluescript pKS+™ (Stratagene). In pMIM R^+L^+, the Tn7 element lies in the orientation opposite to that of the plasmid backbone. pMLP1 contains a 3′ Tn7R−5′ Tn7L joint recovered by PCR amplification from an isolated pEMÆ (intra) DSB.R-SEJ using the Tn7R primer NLC 100 and the Tn7L/kanamycin primer NLC 101 inserted by cloning into the TA vector pCRII (Invitrogen). As confirmed by DNA sequencing, the Tn7R-Tn7L joint contains 138 bp Tn7R, three junction nucleotides (from Tn7L flank in pEMΔ), 166 nt of Tn7L and about 350 nt of the kanamycin resistance gene. This plasmid was used as a marker on the denaturing gels, having the same base composition as the products being analyzed. NLC 100 = 5′-CCATAACAAAAGTCCAGTATGCTTTTTCAC-3′ and NLC 101 = 5′-AGTTAAGTCTGACCATCTCATCTGTAA CAT-3′.

Tns proteins

TnsA was obtained by affinity purification of a GST-TnsA fusion protein and the release of TnsA by proteolytic cleavage (Bainton *et al*., 1993; May & Craig, 1996). TnsA was stored at −80 °C in 25 mM Hepes (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.25 mM PMSF, 5% (v/v) glycerol. TnsB was either authentic TnsB (Arciszewska *et al*., 1991) or TnsB-His (Gary *et al*., 1996); no difference in these reactions has been observed with TnsB *versus* TnsB-His. TnsB was stored at −80 °C in 25 mM Hepes (pH 8.0), 500 mM KCl, 2 mM DTT, 1 mg/ml bovine serum albumin, 25% glycerol.

In vitro Tn*7* transposition reactions

TnsAB reaction mixtures (usually 100 μl) contained Minimal buffer (final concentration 20 mM Tris (pH 7.6), 1.5 mM $MnCl_2$, 2.0 mM DTT, 100 μg/ml tRNA, 50 μg/ml bovine serum albumin), 0.25 nM substrate donor plasmid, 1.5 μl of TnsA (75 ng, 30 nM) and 1.0 μl of TnsB (25 ng, 3 nM). After 30 minutes incubation at 30 °C, the reaction mixture was extracted with phenol/chloroform (1:1, v/v), the DNA recovered by precipitation in ethanol, digested with restriction enzymes as indicated and RNase A, and analyzed by electrophoresis or electron microscopy.

Gel electrophoresis and size markers

For analysis by native gel electrophoresis, DNAs from recombination mixtures were digested with restriction enzymes as indicated in the Figure legend and displayed by electrophoresis on 1.2% (w/v) agarose TBE gels. In Figure 7, the DNAs were visualized by staining with ethidium bromide, and DNAs extracted from gel slices by Qiaex (Qiagen). In Figures 1-3, the gels were Southern blotted to Gene Screen Plus (Dupont/NEN) for six hours in 0.4 M NaOH, the membranes UV-crosslinked for 90 mJ in a Stratalinker (Stratagene), hybridized with radioactively labeled miniTn7 probe and visualized by autoradiography. For high-resolution, denaturing gel analysis, recombination reaction mixtures were digested with *Sal*I (Figure 5(a)) or *Bgl*II and *Ear*I (Figure 6), or

isolated recombination products were digested with *Sal*I (Figure 5(a)), displayed on Long Ranger™ (AT Biochem) denaturing 5% (Figure 5) or 8% (Figure 6) polyacrylamide gel, electrotransferred to Gene Screen Plus, hybridized with Tn7 end-specific oligonucleotide probes and examined by autoradiography.

Where indicated in the Figures, 1 kb ladder (Gibco/BRL) phosphatased with calf alkaline phosphatase (Boehringer Mannheim Biochemicals) and 5′ end-labelled with [γ-^{32}P]ATP and phage T4 polynucleotide kinase is included as a standard size marker. In Figure 6, the size markers were generated by digestion of pMLP1 with *Bgl*II and *Ear*I.

Preparation of hybridization probes

The miniTn7-specific probe was the kanamycin gene segment between Tn7L and Tn7R (as in pEMΔ) and was obtained by digestion of plasmid DNA with appropriate restriction enzymes, isolation by electrophoresis on a 0.8% agarose TBE gel, and extraction with Qiaex; DNA fragments were labelled by the random priming reaction using [α-^{32}P] dCTP (Amersham) and the Klenow fragment of DNA polymerase I (BMB) according to the manufacturer's instructions. Strand and Tn7 end-specific oligonucleotides that hybridized to either the 5′ or 3′ strand of Tn7L were: NLC95 (5′) ATAATCCTTAAAAACTCCATTTCCACCCCT; NLC97 (5′) AGGG GTGGAAATGGAGTTTTTAAGGATTAT; Oligonucleotide probes were 5′ end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB) for 45 minutes at 37°C. Labelled DNA was separated from unincorporated nucleotides by elution through a G50 Nick Spin Column (Pharmacia).

Electron microscopy and image analysis

DNAs from TnsAB reactions, either undigested or digested with *Alw*NI and *Apa*I, were spread onto nitrocellulose-covered copper grids as described (Pérez-Morga and Englund, 1993). Images were collected at 12,500× as photographic negatives and then converted to slides. The slide images were projected to a screen for image enlargement and all looped species in each field were traced to paper. The lengths of the traces were measured with a Scale Master Plus® digital plan measure (Calculated Industries, Inc.). The appropriate enlargement factor was calculated using projection of a discrete line of known length.

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