The EMBO Journal vol.15 no.22 pp.6348–6361, 1996

The Tn*7* transposase is a heteromeric complex in which DNA breakage and joining activities are distributed between different gene products

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The bacterial transposon Tn*7* translocates by a cut and paste mechanism: excision from the donor site results from double-strand breaks at each end of Tn*7* and target insertion results from joining of the exposed 3′ Tn*7* tips to the target DNA. Through site-directed mutagenesis of the Tn*7*-encoded transposition proteins TnsA and TnsB, we demonstrate that the Tn*7* transposase is a heteromeric complex of these proteins, each protein executing different DNA processing reactions. TnsA mediates DNA cleavage reactions at the 5′ ends of Tn*7*, and TnsB mediates DNA breakage and joining reactions at the 3′ ends of Tn*7*. Thus the double-strand breaks that underlie Tn*7* excision result from a collaboration between two active sites, one in TnsA and one in TnsB; the same (or a closely related) active site in TnsB also mediates the subsequent joining of the 3′ ends to the target. Both TnsA and TnsB appear to be members of the retroviral integrase superfamily: mutation of their putative DD(35)E motifs blocks catalytic activity. Recombinases of this class require a divalent metal cofactor that is thought to interact with these acidic residues. Through analysis of the metal ion specificity of a TnsA mutant containing a sulfur (cysteine) substitution, we provide evidence that a divalent metal actually interacts with these acidic amino acids.

Keywords: active site/DD(35)E motif/DNA transposition/protein–DNA complex/transposase

Introduction

The movement of transposable elements between non-homologous insertion sites results from DNA breakage and joining reactions that disconnect the element from flanking DNA at the donor site and then join the ends of the element to the target site. Central to the translocation of all elements examined in biochemical detail are DNA strand cleavage reactions that expose the 3′ termini of the transposon, and strand transfer reactions that join these exposed 3′ termini to staggered positions on the target DNA (Mizuuchi, 1992a,b). Repair of the resulting gaps that flank the newly inserted element generates the target site duplications characteristic of transposition. With some elements, the 5′ transposon ends are also cleaved; whether or not 5′ end cleavage occurs has a profound impact on the nature of the recombination product. When both the 3′ and 5′ ends of the transposon are broken, the element is excised from the donor site and inserted into the target site to form directly a simple insertion through a 'cut and paste' mechanism. When only 3′ end cleavage occurs, i.e. in the absence of cleavage at the 5′ ends, the transposon remains attached to the donor site and is also attached to the target site. Processing of this joint molecule, called a Shapiro intermediate or strand transfer product (Arthur and Sherratt, 1979; Shapiro, 1979; Craigie and Mizuuchi, 1985), by host replication functions (Craigie and Mizuuchi, 1985; Kruklitis and Nakai, 1994) generates a structure called a co-integrate in which two copies of the transposon fuse the donor backbone and the target DNAs.

The breakage and joining reactions that underlie transposition are mediated by transposases that bind specifically to the ends of the transposon (Berg and Howe, 1989; Mizuuchi, 1992b; Craig, 1996a). The transposase is usually encoded by the mobile element itself, although host proteins may also participate in recombination. In many cases, a single element-encoded gene product executes both the breakage and joining steps. For example, a tetramer of a single gene product forms the active transposase of bacteriophage Mu (Lavoie and Chaconas, 1996). Analysis of transposase mutants defective in catalysis but active in other recombination steps such as DNA recognition and assembly into higher order recombination complexes has revealed that the individual breakage and joining events at the two 3′ ends of the transposon are distributed among the transposase protomers, with the same (or closely related) active site in each protomer being involved in a DNA breakage or joining reaction (Baker and Mizuuchi, 1992; Baker *et al.*, 1994; Kim *et al.*, 1995; Yang *et al.*, 1995; Aldaz *et al.*, 1996; Savilahti and Mizuuchi, 1996). Similarly, a single gene product (integrase) executes the 3′ end breakage and joining reactions that underlie retroviral DNA integration (Katz and Skalka, 1994). In other cases, a single gene product transposase can execute all the steps of cut and paste transposition, i.e. both the 3′ end breakage and joining reactions and also 5′ end breakage reactions. The bacterial transposon Tn*10* is a particularly well studied element of this type (Kleckner *et al.*, 1996). It has been shown that the same (or closely related) active sites in the single gene product Tn*10* transposase, which also functions as an oligomer, promote both the 3′ and 5′ transposon end DNA processing reactions (Bolland and Kleckner, 1996). The translocation of many other cut and paste elements, for example the Tc1 and Tc3 elements of *Caenorhabditis elegans* and other members of the widespread *mariner* family of elements, is also probably mediated by a single gene product transposase (van Luenen *et al.*, 1994; Vos *et al.*, 1996).

Although many transposition systems involve a single gene product transposase, there are reactions in which multiple gene products are intimately involved in the breakage and joining process. In the case of the P element of *Drosophila*, the element-encoded recombinase binds to internal sites which are not adjacent to the element termini where breakage and joining will occur; rather, a host protein binds to this most terminal region (Kaufman and Rio, 1992; Beall *et al.*, 1994). Specific roles in DNA breakage and joining have not yet been assigned to these proteins. In V(D)J recombination, a reaction mechanistically related to transposition (Craig, 1996b; van Gent *et al.*, 1996), two proteins (Rag-1 and Rag-2) collaborate to execute the double-strand breaks which initiate recombination (McBlane *et al.*, 1995). In a distinct reaction, conservative site-specific recombination, the highly related XerC and XerD proteins execute related but distinct steps in strand exchange (Blakely *et al.*, 1993). In the case of phage Mu, it is likely that a host-encoded activity distinct from the MuA transposase itself can provoke cleavage at the 5′ ends of Mu following the MuA-mediated 3′ end breakage and joining reactions (Craigie and Mizuuchi, 1985; Mizuuchi, 1992b). However, this 5′ cleavage activity is not essential to recombination, since the 3′ end breakage and joining reactions that result in transposition can occur efficiently in its absence.

The bacterial transposon Tn*7* (Barth *et al.*, 1976; Craig, 1996c) moves through a cut and paste mechanism involving double-strand breaks at both ends of Tn*7* and joining of the 3′ ends to the target DNA (Bainton *et al.*, 1991, 1993) (Figure 1). Tn*7* transposition involves multiple Tn*7*-encoded proteins: TnsA, TnsB, TnsC, TnsD and TnsE. Different subsets of Tns proteins promote insertion into different target sites: TnsABC+D promote Tn*7* insertion into a specific site in the *Escherichia coli* chromosome, *attTn7*, and TnsABC+E promote Tn*7* insertion into many non-*attTn7* sites (Rogers *et al.*, 1986; Waddell and Craig, 1988; Kubo and Craig, 1990). Molecular analysis of Tn*7* transposition into *attTn7* revealed that all components of this reaction (TnsA, TnsB, TnsC, TnsD, the donor transposon DNA and the target *attTn7* DNA) must be present for the initiation of recombination (Bainton *et al.*, 1991, 1993). This requirement suggests that a prerequisite for recombination is the formation of an elaborate nucleoprotein complex involving specific recognition of the substrate DNAs, and also involving synapsis of the transposon ends with each other and with the target DNA; multiple specific protein–DNA and protein–protein interactions are surely necessary to form this active complex. Which Tn*7*-encoded protein(s) in such a complex forms the actual transposase and executes the DNA strand breakage and joining reactions? We have been unable to detect any breakage and joining activity by any Tns protein individually, probably reflecting the requirement for proper complex assembly to 'activate' the breakage and joining activities. However, we have found that TnsA+TnsB, in the absence of other Tns proteins and under non-standard reaction conditions *in vitro*, can together execute double-strand breakage and intramolecular joining reactions that are related to but distinct from standard intermolecular transposition (M.Biery, M.Lopata and N.L.Craig, in preparation). This result focuses attention on TnsA and TnsB as candidates for the Tn*7* transposase. Other work has established that TnsB plays a key role in substrate recognition, being a sequence-specific DNA binding protein which interacts with multiple sites at each Tn*7* end (Arciszewska and Craig, 1991; Arciszewska *et al.*, 1991; Tang *et al.*, 1991). TnsA has no detectable DNA binding activity (Bainton *et al.*, 1993). The other Tns proteins, TnsC, TnsD and TnsE, are likely to play key roles in selecting the element insertion site (Bainton *et al.*, 1993; Craig, 1996c).

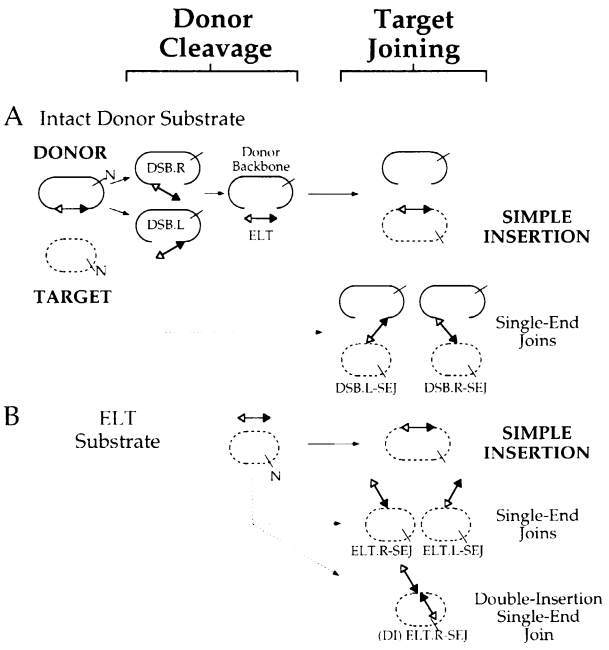

Fig. 1. Tn*7* transposition reactions. Tn*7* transposition proceeds through two distinct steps, initiating with donor cleavage followed by target joining. Various substrates and products are shown, the substrates differing in whether or not they have already undergone donor cleavage (adapted from Gary *et al.*, 1996). The *cis*-acting recombination sequences at the Tn*7* ends which include and extend beyond Tn*7*'s terminal 30 bp inverted repeats are designated by an open triangle (Tn*7*L) and a closed triangle (Tn*7*R); the target DNA is shown as a dashed line. (**A**) An intact donor substrate in which the transposon is flanked by donor backbone DNA. The position of *Nde*I (N) sites in the donor and target plasmids are indicated. DSBs result from a double-strand break at either Tn*7*L (DSB.L) or Tn*7*R (DSB.R); a simple insertion results from joining the two transposon ends of a single Tn*7* element to a target DNA; a single-end join (SEJ) results from the joining of a single Tn*7* end to the target DNA. Both DSB–SEJs and ELT–SEJs can form. (**B**) An excised linear transposon (ELT) substrate which can form a simple insertion in which both Tn*7* ends join to a single target, or a double-insertion single-end join [(DI) ELT.R–SEJ] when two Tn*7*R ends from two different ELTs join to a single target.

To probe the specific roles of TnsA and TnsB in the actual strand breakage and joining reactions, our strategy here has been to generate and examine the properties of site-directed 'catalytic' mutants of TnsA and TnsB, i.e. mutants that are blocked in particular steps of strand breakage and joining but which remain active in the many other steps necessary for the formation of the multiprotein Tn*7* transposition complex. We have reported previously that a mutation in TnsA can block DNA breakage at the 5′ ends of Tn*7* but does not alter the breakage and joining reactions at the 3′ ends of Tn*7* (May and Craig, 1996). These experiments established that these 3′ and 5′ DNA processing reactions can be separated. Indeed, their separ-

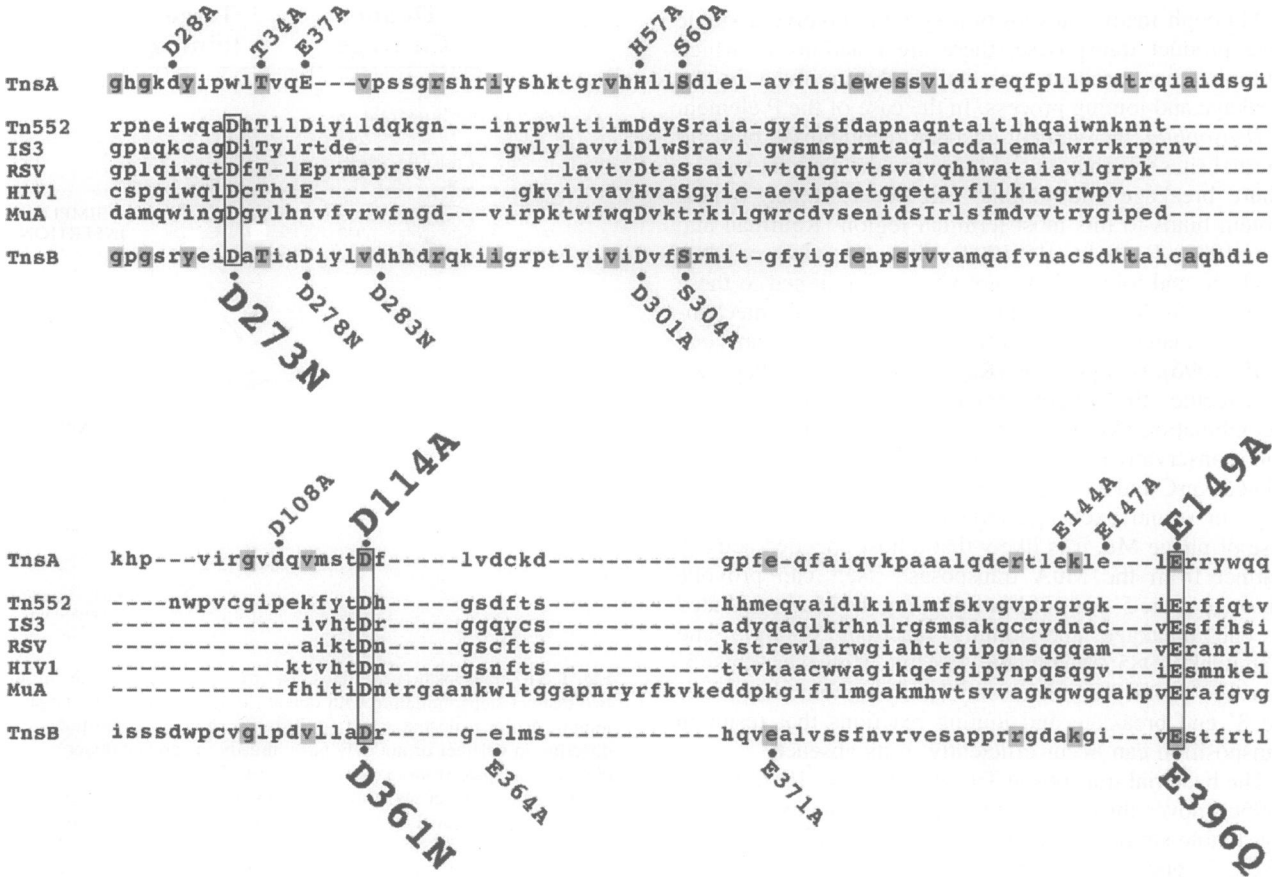

Fig. 2. Working alignment of DD(35)E family members. Representative members of the DD(35)E superfamily of transposases and retroviral integrases are aligned with TnsA and TnsB. The highly conserved DD(35)E residues are boxed; positions of identity between TnsA and TnsB are shaded. Mutagenized TnsA and TnsB residues are indicated except for E424A TnsB. Identical amino acids between TnsA (Flores *et al.*, 1990; Orle and Craig, 1991) and TnsB (Flores *et al.*, 1990) are shaded. The other proteins are: the p480 gene product of the Tn*552* TnpA gene (Rowland and Dyke, 1990); the IS3 transposase (Fayet *et al.*, 1990); the RSV and HIV-1 integrases (Engelman and Craigie, 1992; Kulkosky *et al.*, 1992); and the bacteriophage Mu transposase (Baker and Luo, 1994; Radstrom *et al.*, 1994; Kim *et al.*, 1995). Note that the spacing between the second and third conserved acidic residues of the Mu transposase is 55 amino acids, rather than 35. In this region, the alignment was directed by the 2° structure similarities of HIV integrase and the Mu transposase (Dyda *et al.*, 1994; Rice and Mizuuchi, 1995).

ation has an interesting biological consequence in that Tn*7* is converted, through specific blockage of only the 5′ end cleavage reactions, from a cut and paste element, like Tn*10*, into a replicative element, like phage Mu. These experiments did not, however, reveal which Tns protein is the transposase: are all the active sites for DNA processing reactions in one of the two candidate proteins, TnsA or TnsB, with the other protein acting as a 'modulator' of activity, or are the active sites for the DNA processing reactions disributed between TnsA and TnsB? The work presented here reveals that the Tn*7* transposase is a heteromeric complex in which TnsB and TnsA interdependently contribute active sites for the DNA processing reactions; TnsA contains the active site for 5′ end cleavage and TnsB contains the active site for 3′ end breakage and joining. This separation of active sites between different gene products that act together to form a transposase has not been described previously, although it is not unreasonable to suggest that other recombination reactions will also involve such collaborations.

Our analyses also suggest that TnsA and TnsB are likely to be members of the retroviral integrase superfamily of recombinases. These Tns proteins, like the transposases of retroviruses, retrotransposons, IS3 elements and bacteriophage Mu, contain a limited homology to a highly conserved array of acidic amino acids, the DD(35)E motif, in which the last D and E are generally separated by 35 amino acids (Radstrom *et al.*, 1994; Craig, 1996c; Polard and Chandler, 1995) (Figure 2). An attractive view, supported by genetic, biochemical and structural studies, is that these conserved amino acids lie at (or at least are intimately related to) the active sites which mediate the breakage and strand transfer reactions during recombination (reviewed in Craig, 1995; Grindley and Leschziner, 1995). The DD(35)E motif is thought to provide a binding site for an essential metal ion, usually Mg^{2+}, at or near these active sites (Baker and Luo, 1994; Dyda *et al.*, 1994; Kim *et al.*, 1995; Rice and Mizuuchi, 1995; Bujacz *et al.*, 1996). We provide functional evidence here that TnsA does indeed interact with a metal ion. We show that when an aspartic acid, i.e. an oxygen-containing carboxylate, within the proposed TnsA DD(35)E motif is substituted with a cysteine, i.e. containing sulfur, the metal specificity of the 5′ processing reaction switches from Mg^{2+} to Mn^{2+}. The strategy of analyzing metal ion specificity in the presence of sulfur-substituted reaction components has been exploited previously to show an interaction between an essential metal ion and the substrate

in RNA self-splicing (Dahm and Uhlenbeck, 1991; Piccirilli *et al.*, 1993).

A view that has emerged from the analysis of many recombination systems is that these DNA breakage and joining reactions are highly controlled and coordinated, ensuring that such potentially lethal reactions are limited to particular chromosomal positions and occur at biologically appropriate times. A powerful strategy to impose such coordination is to link the ability to execute recombination to the proper assembly of a nucleoprotein complex containing recombination proteins and DNA substrates, a process involving multiple protein–DNA and protein–protein interactions (Baker, 1993). The identification of a multi-gene product Tn*7* transposase provides a new and distinctive example of a 'division of labor' among recombination proteins and provides a new scenario for the control of recombination reactions.

Results

TnsA mutations block breakage at the 5′ ends of Tn7 but do not alter 3′ end processing reactions

Which Tns protein(s) forms the Tn*7* transposase? It has not been possible to detect any DNA breakage or joining activity with either TnsB or TnsA alone or when either of these proteins is simply omitted from recombination reactions (Bainton *et al.*, 1993; Gary *et al.*, 1996); thus it has not been possible to assign unique roles in DNA processing to either of these proteins. To dissect their roles in transposition, we generated mutant versions of these proteins specifically defective in the DNA breakage and joining steps but still active in the many other steps essential for recombination, such as specific recognition of the substrate DNA and the ability to assemble into a recombination-competent nucleoprotein complex.

We have reported previously that mutation of TnsA D114 to alanine (D114A) blocks cleavage at the 5′ ends, but 3′ end breakage and joining still proceed, forming a fusion product (FP) in which the donor and target plasmids are joined through a copy of the transposon, (May and Craig, 1996) (Figure 3A). Thus the D114A mutant has the hallmarks of a 'catalytic' mutant, i.e. only 5′ end breakage is blocked and the other steps key to transposition, such as recognition of DNA substrates and formation of an appropriate transposition complex, must still occur. This result does not, however, reveal the actual location of the 5′ cleavage active site; this active site could be in TnsA, or it might be in TnsB and regulated in some way by TnsA. Our experiments here suggest that TnsA actually contains the active site for 5′ end processing. A weak homology is observable between a region of TnsA and the active site region of other transposases, the conserved DD(35)E triad of amino acids, in which the last D and E are generally separated by 35 amino acids (Figure 2). This putative DD(35)E motif in TnsA is not convincing. Indeed, a match was only possible after the identification of homology between TnsA and TnsB in this region of the protein; the motif in TnsB is far more compelling (see below). Most notably, TnsA lacks an acidic amino acid that obviously corresponds to the first D of the canonical DD(35)E motif. However, the D114A mutation which blocks 5′ end processing is altered in the second D of TnsA's version of the motif.

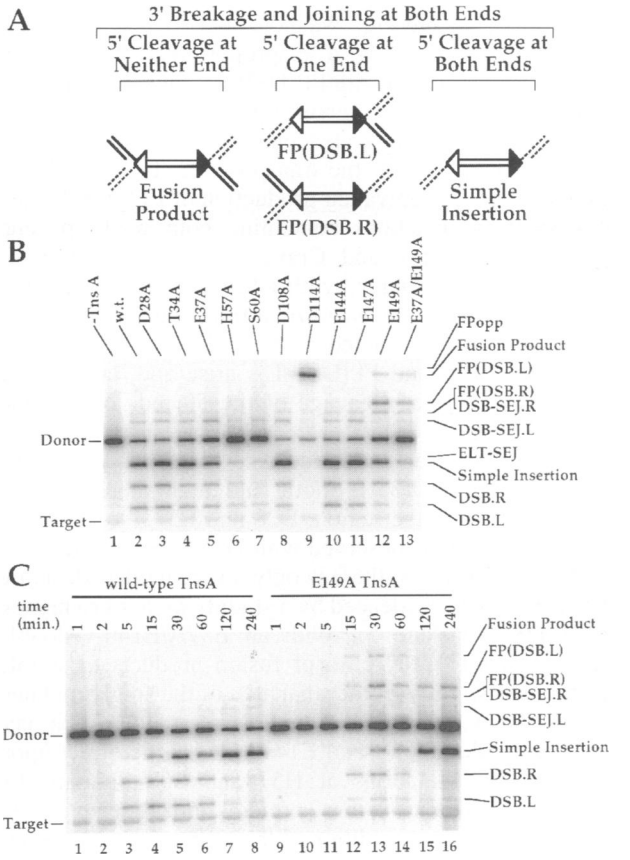

Fig. 3. TnsA mutations block double-strand breaks and result in transposon-mediated fusions between the donor and target plasmids. (**A**) Mutation of TnsA can block 5′ end cleavage and result in the formation of donor–target plasmid fusions. When 5′ cleavage at both transposon ends does not occur, a fusion product (FP) is formed; FP_{opp} (not shown) containing Tn*7* inserted in the opposite orientation in *attTn7*, can also form. When 5′ end cleavage occurs at one Tn*7* end but not the other, two different fusions containing DSBs (FP.DSB) are formed. (**B**) *In vitro* Tn*7* transposition reactions were carried out with wild-type or mutant TnsA proteins using supercoiled donor plasmid DNA; the products were displayed on native agarose gels. Substrates and other products are labeled as in (A) and Figure 1. (**C**) Time courses of recombination with wild-type and E149A TnsA. This time course was performed at 22°C instead of the standard 30°C, slowing the reaction rate and allowing analysis of intermediates (Bainton *et al.*, 1991).

To probe further the role of TnsA's putative DD(35)E motif in recombination, we evaluated other mutants in *in vitro* reactions using purified Tns proteins and supercoiled plasmid donor substrates (Figure 3B). As previously reported, D114A TnsA blocks cleavage at the 5′ ends of Tn*7* but does not affect breakage and joining at the 3′ ends (May and Craig, 1996). E149A TnsA, altered in the E of TnsA's version of the DD(35)E motif, also results in reduction of 5′ end cleavage (lane 12). In contrast to the complete block imposed by D114A TnsA, 5′ cleavage is only partially reduced with E149A TnsA; however, 5′ cleavage with E149A TnsA *in vivo* is as reduced as D114A TnsA (see below). Two species resulting from reduced 5′ end cleavage are observed, the FP and a partial fusion product, FP(double-strand break), in which 5′ cleavage does not occur at one end of Tn*7* but does occur at the other end. Considerable amounts of simple insertions and donor molecules broken by a double-strand break

(DSB) are also present. We note that in reactions with E149A TnsA, there is a disproportionate amount of FP(DSB.L) compared with FP(DSB.R); thus E149A TnsA displays a bias in left versus right Tn*7* end utilization, hinting that TnsA may also play a role in defining the functional identities of the transposon ends. This bias is in contrast to the equivalent production of FP(DSB.L) and FP(DSB.R) in reactions containing both wild-type and D114A TnsA (May and Craig, 1996). The partial 5′ cleavage defect displayed with E149A reflects a decrease in cleavage rate, as revealed by a time course analysis of the E149A TnsA-mediated reaction (Figure 3C); at early time points, FPs and FP(DSB.L)s arise and, later in the time course, further cleavage of the 5′ ends occurs and the amount of FP decreases.

TnsA lacks an acidic amino acid corresponding to the first D of the DD(35)E motif, although there are two acidic residues in the region, D28 and E37. No alteration of recombination is observed with D28A (Figure 3B, lane 3). With E37A TnsA, there is only a very modest decrease in 5′ cleavage as evidenced by a slightly higher production of FP(DSB.L) (lane 5). With an E37A/E149A double mutant (lane 13), the ratio of fusion products to simple insertions is slightly greater than that of the singly mutated E149A TnsA (lane 12). Other TnsA point mutations, T34A, D108A, E144A and E147A, had little effect upon transposition. Mutations at H57 and S60 dramatically reduced recombination; these proteins appear to have more extensive defects, as addition of these proteins to reactions containing limiting wild-type TnsA had little effect (data not shown).

These experiments reveal that TnsA has an intimate role in 5′ end cleavage: in certain TnsA mutants, 5′ end cleavage is altered while 3′ end activities persist. It is notable that these TnsA mutants display a catalytic phenotype; although a specific chemical step fails to occur, i.e. 5′ end cleavage, many other steps in recombination do occur to allow the formation of a nucleoprotein complex competent to execute the 3′ end processing reactions. Assignment of the active site to TnsA on the basis of these results alone cannot be firmly made, in particular because of the limited homology between TnsA and the DD(35)E motif. However, we provide functional evidence below that TnsA interacts directly with the metal cofactor, supporting both the view that the active site for 5′ end cleavage is in TnsA, and the hypothesis that TnsA is a member of the DD(35)E family.

We have also evaluated the effects of these TnsA mutations on Tn*7* recombination *in vivo* (data not shown). We previously reported that with D114A TnsA, Tn*7* makes predominantly (80%) co-integrate products rather than the simple insertions obtained with wild-type Tn*7* (May and Craig, 1996). Co-integrates are the *in vivo* consequence of a failure in 5′ end cleavage; the Shapiro intermediate formed by breakage and joining at exclusively the 3′ ends of the transposon is the substrate for DNA replication which generates the co-integrate. E149A TnsA also generates a very high proportion of co-integrate molecules *in vivo* (77%). The observation of co-integrate formation with D114A and E149A TnsA supports the view that these mutant proteins are defective specifically in 5′ end cleavage; with wild-type TnsA and with the other TnsA mutants examined, the recombination products are simple inserts, consistent with double-strand breaks involving both 3′ and 5′ end cleavage. Moreover, all TnsA mutants examined supported overall transposition, i.e. joining of the 3′ ends of the transposon to the target plasmid, at a level comparable with that of wild-type (data not shown). We also note that while *in vitro* recombination using H57A and S60A TnsA was reduced markedly, transposition *in vivo* using these two mutants was unaffected (data not shown); the nature of this suppression is not understood.

Evidence for a direct interaction between metal and TnsA

The DD(35)E motif is thought to provide a binding site for a divalent metal at or near an active site(s) for DNA hydrolysis and strand transfer (Baker and Luo, 1994; Dyda *et al.*, 1994; Kim *et al.*, 1995; Polard and Chandler, 1995; Rice and Mizuuchi, 1995; Bujacz *et al.*, 1996). Mg^{2+} is a critical cofactor in Tn*7* recombination *in vitro*; no recombination is observed in the absence of divalent metal (Bainton *et al.*, 1993). A reasonable hypothesis is that this metal ion is a cofactor for TnsA (and/or TnsB, see below). However, the homology between TnsA and the DD(35)E motif is limited; establishing that TnsA does contain a metal-dependent active site requires other evidence. We have used the differential chemistry of metal–sulfur and metal–oxygen interactions (Pecoraro *et al.*, 1984) to provide evidence that an essential metal acts in close proximity to a specific aspartate in the DD(35)E motif of TnsA. Similar metal ion specificity experiments have been done with ribozymes in which the metal requirements of thiol-containing substrates were explored (Dahm and Uhlenbeck, 1991; Piccirilli *et al.*, 1993). The finding that Mn^{2+} was able to support the cleavage of the thiol-substituted RNA substrate far better than could Mg^{2+}, i.e. that a change in metal ion specificity was observed, suggested an interaction between the metal and the substrate in the active sites of these enzymes.

We have found that a cysteine substitution at the D114 position of TnsA changes the metal ion specificity of the 5′ end cleavage reaction. To focus directly on the involvement of the divalent metal in the 5′ end cleavage activity, we temporally staged the reaction (Figure 4A). In the first step, we used D114 mutants of TnsA, either D114A or D114C, in a Mg^{2+}-supported reaction to generate high levels of FP, then added an additional metal and continued the incubation. Production of FP(DSB) or simple insertions, in which double-strand breaks have been introduced at either or both transposon ends, are the signatures of a suppression of the 5′ end cleavage defect.

Strikingly, we observe a Mn^{2+}-specific suppression of the D114C TnsA 5′ cleavage defect (Figure 4B, lanes 10–12). In the presence of Mn^{2+} and D114C TnsA, specific 5′ end cleavage of the FP occurs. As the concentration of Mn^{2+} increases, more FP(DSB)s and simple insertion products are generated. It is interesting that 5′ cleavage at Tn*7*L appears to occur preferentially, as indicated by the disproportionately high level of FP(DSB.L). It should be noted that Mn^{2+} can introduce a degree of permissiveness to many *in vitro* reactions (Vermote and Halford, 1992; Polard and Chandler, 1995). However, with D114A TnsA, we see no such permissive phenotype; no Mn^{2+}-supported 5′ cleavage with D114A TnsA is observed (lanes

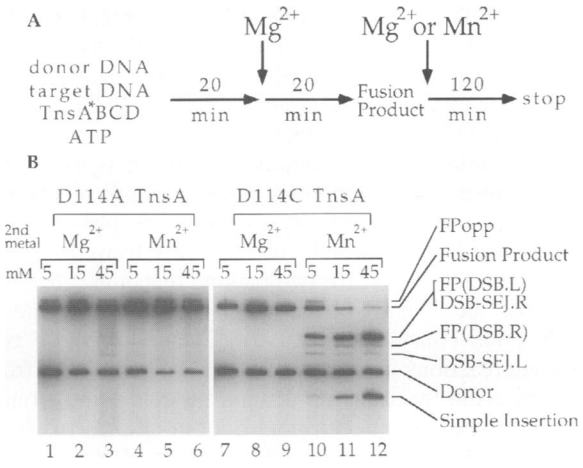

Fig. 4. TnsA is in contact with divalent metal: introduction of a sulfur (cysteine) into TnsA changes the metal ion specificity of 5′ cleavage from Mg^{2+} to Mn^{2+}. *In vitro* Tn*7* transposition reactions were staged differently [(**A**) see text] using D114A or D114C TnsA in the presence of Mg^{2+} or Mn^{2+}; the products are displayed on native agarose gels. Substrates and other products are labeled as in Figures 1 and 6.

4–6). Moreover, no 5′ cleavage products are generated by any other combination of D114 mutations and Ca^{2+} or Zn^{2+} (data not shown).

The Mn^{2+}-specific suppression of the sulfur-containing D114C mutant implies that this acidic residue in wild-type TnsA is, in fact, in a metal binding site, strongly implicating a metal-dependent active site in TnsA for the hydrolysis of the 5′ end during Tn*7* transposition. This novel use of sulfur (cysteine) substitutions may prove useful in the study of the role of metals in other protein–nucleic acid interactions. Indeed, a similar suppression has been observed recently within the Mu transposase active site (E.Krementsova and T.Baker, personal communication).

TnsB mutations can block the joining of 3′ Tn7 ends to target DNA but do not alter 5′ end processing reactions

The results presented above provide evidence that the active site for 5′ end cleavage lies in TnsA. Where is the active site(s) for the 3′ end processing reactions of breakage and joining? Also in TnsA? In TnsB? A modest homology to the DD(35)E motif is also present in TnsB (Figure 2). We also note the limited homologies between TnsA and TnsB other than the DD(35)E motif (Figure 2); indeed, this alignment of TnsA with TnsB was key to the recognition of the degenerate DD(35)E motif of TnsA. We generated TnsB mutants and analyzed their activities in recombination. As observed with TnsA DD(35)E mutants, these TnsB mutants also appear to display a catalytic defect; 3′ end breakage and joining reactions are blocked specifically without dramatically affecting other aspects of the reaction, such as the ability to enter into the nucleoprotein complex and support the 5′ end cleavage activity of TnsA. Thus, the experiments described below provide evidence that the 3′ end processing reactions in Tn*7* transposition are mediated by active sites in TnsB.

To evaluate directly the 3′ end joining step in Tn*7* transposition apart from DNA breakage activities, we used an excised linear Tn*7* element (ELT) as a substrate DNA.

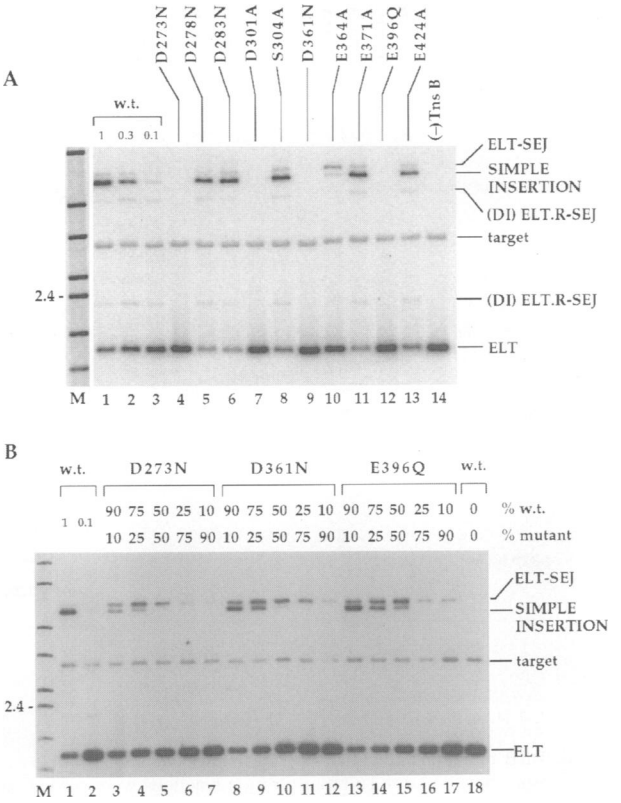

Fig. 5. TnsB mutations block strand transfer of exposed 3′ Tn*7* ends to the target DNA. *In vitro* Tn*7* transposition reactions were carried out with either wild-type or mutant TnsB (**A**) or a mixture of wild-type and mutant TnsB (**B**) using ELTs as a donor DNA substrate; the products are displayed on native agarose gels. Substrates and products are labeled as in Figure 1. In addition to a simple insertion, in which both ends join to a single target, other recombination products are particularly notable with ELT substrates: a single-end join (SEJ) in which only one transposon end is joined to one strand of the target, and a double-insertion single-end join [(DI) ELT.R–SEJ] in which two different ELTs join to a single *attTn7* (Bainton *et al.*, 1991; Gary *et al.*, 1996).

The ELT has been disconnected from the donor DNA by restriction of specialized donor molecules (Bainton *et al.*, 1991; Gary *et al.*, 1996) (Figure 1). We note that TnsA is required to support target joining with an ELT substrate, although the chemical step executed by TnsA, 5′ end cleavage, is not actually required in this reaction (Gary *et al.*, 1996). This result reveals that the 3′ end joining activity is dependent on the presence of TnsA in the nucleoprotein complex.

With an ELT substrate, no target joining can be detected with D273N, D301A, D361N or E396Q TnsB (Figure 5A). D273, D361 and E396 are the signature amino acids of the TnsB DD(35)E motif, suggesting a critical role for this motif in joining of the 3′ Tn*7* ends to the target DNA. With E364A TnsB, target joining does occur but the major products are single-end joins (SEJs), in which only one end of the ELT has been joined successfully to the target. Target joining using D278N, D283N, S304A, E371A or E424A TnsB is not significantly different from that seen with wild-type TnsB.

In ELT reactions using mixtures of wild-type and the joining-defective TnsBs D273N, D361N or E396Q TnsB, the addition of the mutant protein dramatically changes

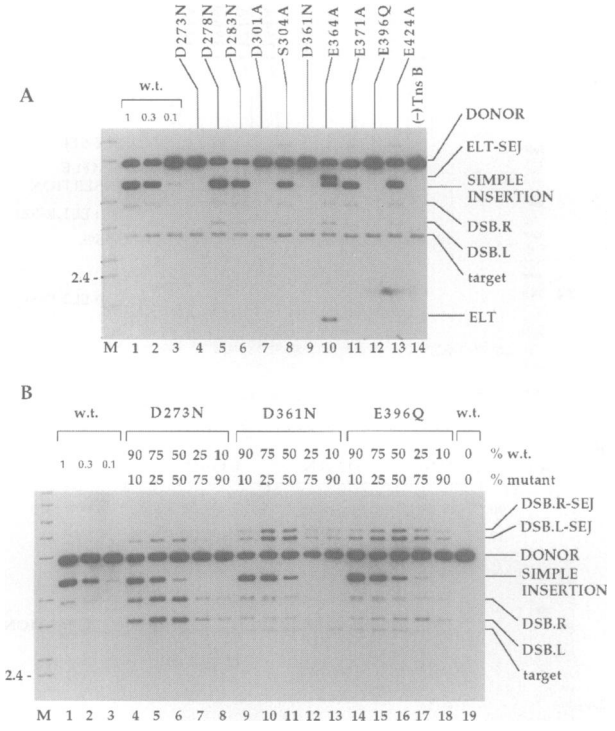

Fig. 6. TnsB mutations block the double-strand breaks that initiate Tn*7* transposition. *In vitro* Tn*7* transposition reactions were carried out with wild-type or mutant TnsB (**A**), or a mixture of wild-type and mutant TnsB (**B**), using supercoiled donor plasmid as substrate; the products are displayed on native agarose gels. Joining of the exposed 3′ end of a DSB to *attTn7* results in a double-strand break–single-end join (DSB–SEJ). Substrates and products are labeled as in Figure 1.

the pattern of the ELT recombination products (Figure 5B). Whereas the major products with wild-type TnsB are simple insertions formed by the joining of both transposon ends to the target, addition of these mutant proteins results in the accumulation of SEJs formed by the joining of a single transposon end. We interpret these results to indicate that strand transfer at both ends of Tn*7* to form a simple insertion requires multiple TnsBs in the nucleoprotein complex active in transposition, and that these TnsB mutants have catalytic defects; they do not promote end joining but are active in other stages of recombination, such as substrate recognition and formation of a multiprotein complex. By contrast, addition of D301A TnsB had little effect on wild-type recombination (data not shown), suggesting that this mutant has more than a simple catalytic defect. No recombination was observed with pairwise combinations of D273N, D361N and E396Q TnsB, nor with a mixture of all three mutants (data not shown).

TnsB mutations block breakage at the 3′ ends of Tn7 but do not alter 5′ end processing reactions

To probe the effects of TnsB mutations on the initiating double-strand break step, we examined the TnsB mutants in reactions using supercoiled plasmid donor substrates (Figure 6A). Most notably, no DNA breakage or joining reactions were detected with this assay using D273N, D301A, D361N or E396Q TnsB. Therefore, these TnsB mutant derivatives are not only altered in 3′ end target joining activity, as shown above with ELT substrates, but are also altered in the preceding donor breakage reactions. E364A TnsB is able to execute donor breakage, but appears altered in target joining, as indicated by the accumulation of ELTs and ELT–SEJs. The patterns displayed with other TnsB mutants (D278N, D283N, S304A, E371A, E424A) resemble that of wild-type.

The examination of recombination products using this non-denaturing electrophoresis assay does not reveal any single-strand nicking activity that may be present. The double-strand breaks which cut Tn*7* away from the donor backbone actually involve two nicking reactions: cleavage to expose the 3′ ends of the transposon and cleavage of the 5′ ends (Gary *et al.*, 1996). To analyze these two cleavage reactions individually, we examined DNAs from recombination reactions on denaturing gels using strand-specific probes for the top and bottom strands of Tn*7*L and Tn*7*R (Figure 7). With wild-type TnsB (lanes 1–3), 3′ end breaks (Tn*7*L = 172 nt, probe B; Tn*7*R = 204 nt, probe C) are observed only at low levels because most of the processed 3′ ends are joined to the target DNA (Tn*7*L = 530 nt, probe B; Tn*7*R = 400 nt, probe C); the major 5′ strand products are cleavages at these ends (Tn*7*L = 171 nt, probe A; Tn*7*R = 203 nt, probe D). No DNA cleavage is seen in the absence of TnsB (lane 14). Strikingly, wild-type levels of 5′ end cleavage are observed with D273N, D361N and E396Q TnsB but the 3′ end reactions of breakage and joining are not detectable with these mutants (lanes 4, 9 and 12). Thus, these DD(35)E TnsB mutants appear to be catalytic in nature; they are blocked in their own 3′ end activities but are able to enter into the transposase complex and support other aspects of Tn*7* transposition. Neither 3′ nor 5′ end cleavages are detectable with D301A (lane 7), suggesting again that the effects of this mutation are more extensive than a simple catalytic defect.

When wild-type TnsB and the breakage-and-joining defective TnsBs (D273N, D361N or E396Q) are mixed in reactions using supercoiled plasmid donor substrates, addition of the mutant protein results in the accumulation of DSBs and DSB–SEJs (Figure 6B). These results indicate that the nucleoprotein complex which executes Tn*7* recombination contains multiple TnsB proteins, i.e. that multiple active sites for 3′ end processing are involved; similar results were obtained with ELT substrates (Figure 5B). The patterns of recombination products obtained in such mixing experiments can provide information about the actual number of 3′ end processing sites, i.e. the likely number of transposase protomers, involved in a recombination reaction (Baker *et al.*, 1994; Kleckner *et al.*, 1996; Bolland and Kleckner, 1996). However, the mutant proteins may impose biases in the assembly and stability of recombination complexes (Baker *et al.*, 1994; Baker and Luo, 1994; Kim *et al.*, 1995). Thus while these TnsB mixing experiments do provide strong evidence that multiple TnsB active sites are involved in an active recombination complex, a clear definition of the actual number of active sites in such a complex cannot be inferred easily from only these experiments.

We have also examined the effects of TnsB on Tn*7* transposition *in vivo*, analyzing TnsABC+D transposition to *attTn7* and also transposition mediated by a different ensemble of Tn*7* transposition proteins, TnsABC+E, to non-*attTn7* targets (Rogers *et al.*, 1986; Waddell and Craig, 1988; Kubo and Craig, 1990). TnsB DD(35)E

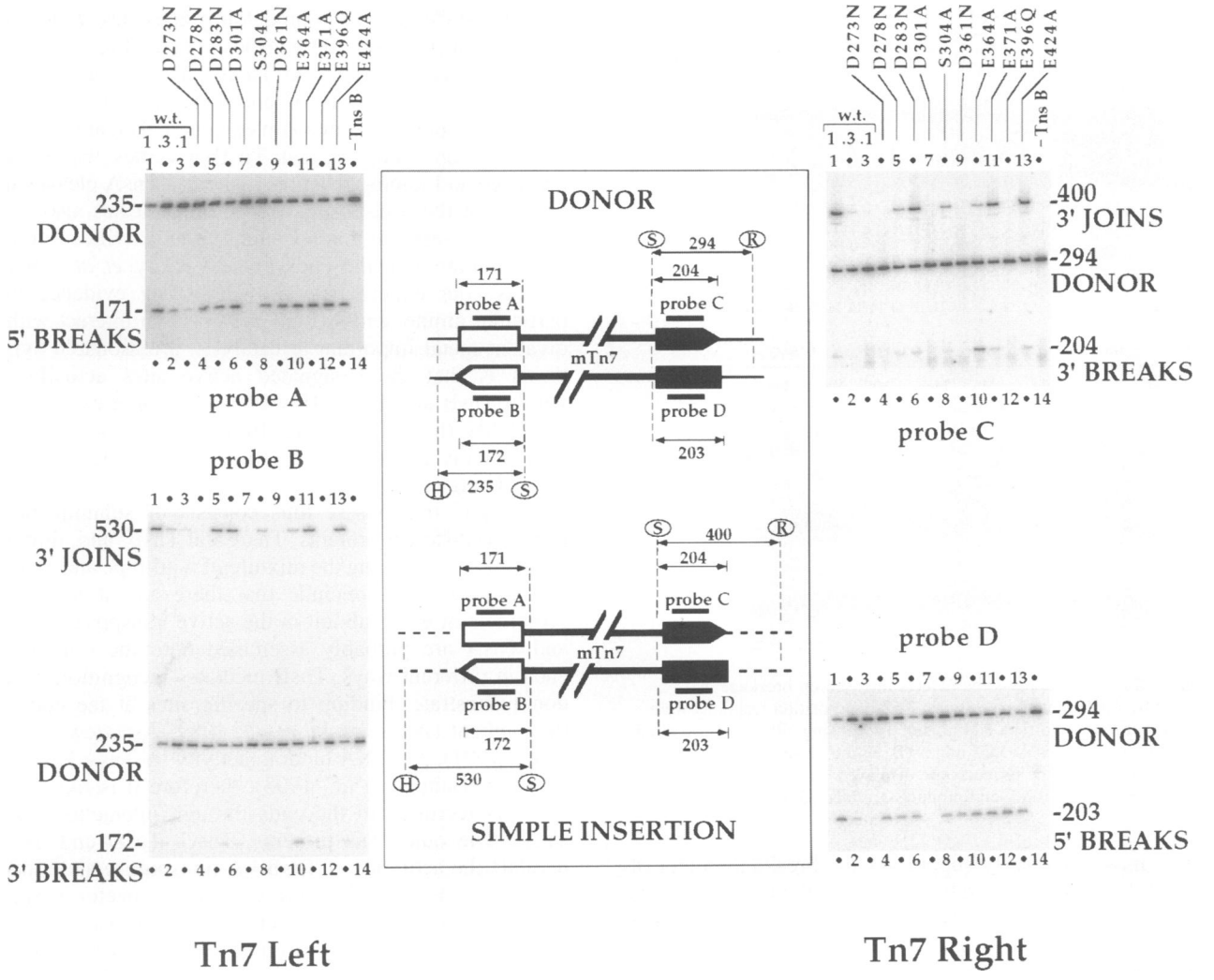

Fig. 7. TnsB mutations block cleavage at the 3′ ends of Tn*7* but do not block 5′ end cleavage. *In vitro* Tn*7* transposition reactions were carried out as in Figure 4, but the products are displayed on denaturing polyacrylamide gels; following transfer to nitrocellulose, the fates of the various strands at the Tn*7*L and Tn*7*R ends were analyzed with strand-specific oligonucleotide probes as indicated (probes A, B, C and D). The diagram shows a substrate donor DNA (top) and the simple insertion product (bottom). When cleavage of the 3′ transposon ends occurs, these species are detected principally as joins to the target DNA, whereas the products of 5′ end cleavage remain as cleaved species. The hybridization positions of the probes are shown and the sizes of the various substrate and product fragments are indicated. S = *Sal*I, R = *Eco*RI, H = *Hin*dIII.

mutants are defective in both transposition pathways (data not shown). Consistent with the view that alteration of the TnsB DD(35)E motif imposes a catalytic block on recombination, these TnsB mutants act as dominant-negative inhibitors of transposition *in vivo* when both wild-type and mutant TnsBs are present (data not shown).

Thus certain TnsB mutants, D273N, D361N and E396Q, are specifically defective in the chemical events that involve the 3′ ends of the transposon: the cleavage reactions which expose the 3′ ends and, as also shown above, the joining of exposed 3′ ends to the target DNA. An attractive hypothesis is that these DD(35)E mutations result in catalytic defects and that the active site for these reactions is contained in TnsB.

Mn^{2+} can suppress the breakage and joining defects imposed by TnsB mutations

To challenge further the hypothesis that TnsB is a member of the retroviral integrase family of metal-dependent recombinases, we also examined Tn*7* recombination in the presence of Mn^{2+} (Figure 8). With wild-type TnsB and TnsA, DNA breakage and joining can occur with Mn^{2+}, although Mn^{2+}-promoted recombination is not equivalent to that seen with Mg^{2+} (Bainton *et al.*, 1993; M.Biery, M.Lopata and N.L.Craig, in preparation). We also analyzed the effects of Mn^{2+} on the DD(35)E mutant TnsBs that were shown above to be inactive in the presence of Mg^{2+}. Mn^{2+} can partially suppress the block imposed on double-strand break formation by D361N TnsB (Figure 8A). This partial suppression is in contrast to the complete lack of suppression of the 5′ end cleavage defect of D114A TnsA with Mn^{2+} (Figure 4B, lanes 4–6). The suppressive effects of Mn^{2+} on the 3′ end joining reactions are much more pronounced. With an ELT substrate, considerable Mn^{2+}-supported joining occurs with D273N and E396Q TnsB but virtually no recombination is detectable with D361N TnsB (Figure 8B). Thus, although Mn^{2+} can suppress some aspects of the recombination blocks imposed by the D273N, D361N and E396Q TnsB mutants, the suppressive effects vary:

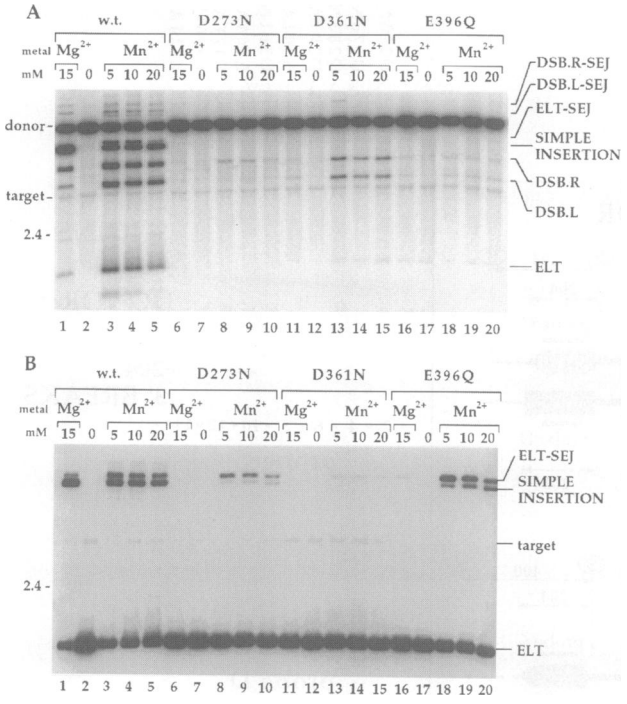

Fig. 8. Mn^{2+} can suppress the defects imposed on breakage and joining by TnsB mutations. *In vitro* Tn*7* transposition reactions were carried out with wild-type and mutant TnsB using either a supercoiled donor plasmid (**A**) or an ELT donor (**B**) in the presence of Mg^{2+} or Mn^{2+} as indicated; the products are displayed on native agarose gels. Recombination substrates and products are labeled as in Figure 1.

Mn^{2+} most effectively suppresses the breakage defect of D361N but most profoundly suppresses the joining defects of D273N and E396Q. No recombination is detectable with Ca^{2+}, Zn^{2+}, Cd^{2+} or Co^{2+}, with wild-type or mutant TnsB using either ELTs or intact supercoiled substrates (data not shown).

The finding that the defects imposed on recombination by TnsB DD(35)E mutations can be partially suppressed by the presence of the alternative metal cofactor Mn^{2+} is consistent with the view that the DD(35)E motif in TnsB provides a metal-dependent active site for the DNA breakage and joining reactions at the 3′ ends of Tn*7*. Furthermore, experiments presented above suggest that a DD(35)E motif in TnsA provides a metal-dependent active site for 5′ end processing. Thus the DNA breakage and joining reactions that underlie the translocation of Tn*7* are mediated by collaborations between metal-dependent active sites in two different gene products: TnsB and TnsA interdependently form the Tn*7* transposase.

Discussion

A functional model for Tn7 transposition

Tn*7* transposition is initiated by double-strand breaks that excise the element from flanking DNA at the donor site; the newly exposed 3′ ends of the excised transposon are joined to the target DNA (Bainton *et al.*, 1991, 1993). The experiments presented here provide evidence that Tn*7* uses a distinctive recombination mechanism. Two element-encoded proteins that appear to be members of the retroviral integrase superfamily, TnsA and TnsB, form a heteromeric Tn*7* transposase; moreover, each gene product provides different DNA processing activities which act on different strands at the transposon ends (Figure 9). Our analyses here of TnsA and TnsB catalytic mutants, i.e. protein mutants capable of entering the recombination-competent nucleoprotein complex but blocked at particular reaction steps, suggest that TnsB executes the 3′ end breakage and joining reactions, whereas TnsA cleaves the 5′ ends of the transposon. We note that it has also been possible to separate these 3′ and 5′ end activities through DNA mutation in the ends of Tn*7* (Gary *et al.*, 1996). Because our experiments provide strong evidence that particular amino acids within each protein interact with a divalent metal important to catalysis, a reasonable hypothesis is that the designated active sites actually lie within TnsB and TnsA. However, we cannot exclude the possibility that amino acids from multiple polypeptides may actually contribute to a common active site (Jayaram, 1994; Craigie, 1996).

The Tn*7* transposase thus consists of subunits of at least two different proteins, TnsA and TnsB, and, through experiments involving the mixture of wild-type and mutant proteins, we can conclude that there are at least two protomers of each subunit in the active transposase. TnsB and TnsA are probably assembled onto the transposon ends in different ways. TnsB mediates recognition of the donor substrate, binding to specific sites at the ends of the element (Arciszewska *et al.*, 1989; Arciszewska and Craig, 1991). No DNA binding activity by TnsA has been detected (Bainton *et al.*, 1993), therefore it is likely that TnsA is recruited to the ends through interactions with TnsB. The other Tns proteins, TnsC, TnsD and TnsE, regulate the activities of TnsB+A transposase and modulate target selection: TnsC serves as the connector between TnsB+A and the target protein TnsD and also probably the alternative target-determining protein TnsE (Bainton *et al.*, 1993; Craig, 1995a; M.Biery, M.Lopata and N.L.Craig, in preparation; A.Stellwagen and N.L.Craig, in preparation). TnsABC+D mediate insertion into Tn*7*'s specific chromosomal site *attTn7*, and TnsABC+E mediate insertion into many different non-*attTn7* sites (Rogers *et al.*, 1986; Waddell and Craig, 1988; Kubo and Craig, 1990; Bainton *et al.*, 1993; Craig, 1996c).

Tn*7* uses a novel mechanism to catalyze double-strand breaks: two proteins interdependently conduct cleavage reactions on different strands at the transposon ends. This mechanism is distinctive not only among transposition systems (Haniford *et al.*, 1989, 1991; Mizuuchi, 1992b; Chalmers and Kleckner, 1994; Kleckner *et al.*, 1995), but also among more general endonucleolytic processes (Linn *et al.*, 1993). Although many DNA processing reactions do involve homo-oligomers in which different protomers act on different DNA strands, the strategy of using a collaboration between two different gene products to execute a double-strand break in DNA is uncommon. It is important to note that although TnsA and TnsB execute distinct chemical steps, they are dependent on each other for their own activities; no reaction is observed with either protein alone (Bainton *et al.*, 1993; Gary *et al.*, 1996). Two different proteins also comprise the recombinase in the Xer conservative site-specific recombination system (Blakely *et al.*, 1993). Like TnsA and TnsB, XerC and XerD work interdependently to execute recombination; however, unlike TnsA and TnsB, the Xer proteins are

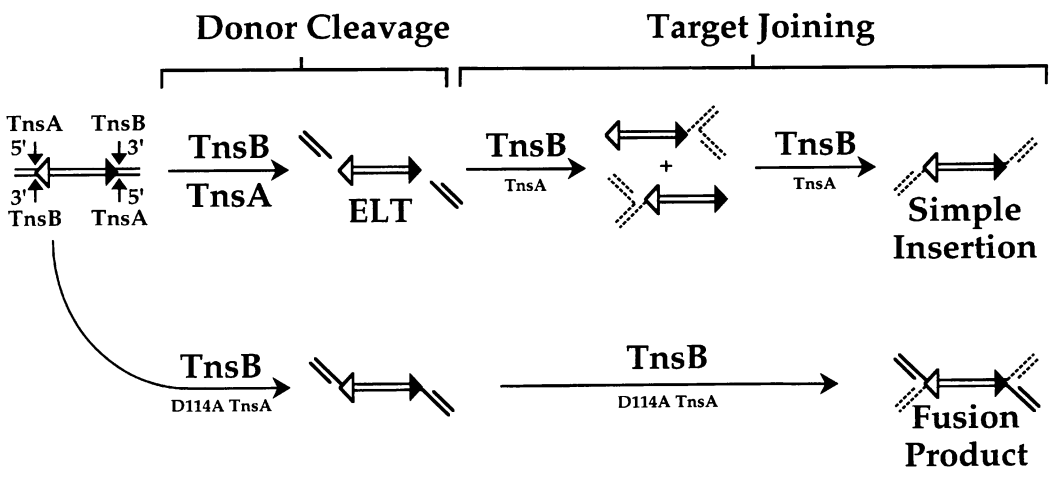

Fig. 9. A model of Tn7 transposition. TnsB and TnsA, two proteins of the retroviral integrase superfamily, execute the chemical steps in Tn7 recombination; the presence of both proteins is required for all chemical steps. Recombination initiates with donor cleavage in which double-strand breaks disconnect the element from the donor backbone: TnsB executes the cleavages which expose the 3′ ends of Tn7, and TnsA mediates the 5′ end cleavage reactions. When both 3′ and 5′ end cleavage occur, an ELT intermediate is formed. When 3′ end cleavage proceeds, but 5′ end cleavage is blocked through a mutation in TnsA, a nicked donor intermediate is formed. In the strand transfer step, TnsB joins the newly exposed 3′ ends to the target DNA. Target joining occurs in two steps, each 3′ transposon end joining to one target strand, forming a single-end join as a recombination intermediate. A simple insertion is formed when the ELT joins to the target DNA; a fusion product is formed when the nicked donor joins to the target DNA.

closely related and conduct parallel chemical steps (Arciszewska and Sherratt, 1995; Colloms *et al.*, 1996).

TnsB is the protein which executes the critical breakage and joining reactions at the 3′ ends of Tn7 that actually underlie transposition (Figure 9). We have demonstrated here that changing single amino acids in TnsB can block both the cleavages that separate the 3′ ends from flanking donor DNA and the joining of these 3′ ends to the target; these changes do not block 5′ end cleavage. The transposition-defective TnsBs do retain the ability to recognize other components of the recombination machinery, i.e. can enter into a nucleoprotein complex, and thus appear to be catalysis mutants. It is attractive to suppose that in Tn7, as in other DD(35)E transposases, the 3′ end cleavage and target joining steps are profoundly related (Engelman *et al.*, 1991; Mizuuchi and Adzuma, 1991; Vink *et al.*, 1991; Mizuuchi, 1992a,b).

Our results suggest that TnsB executes the DNA processing reactions involving *only* the 3′ ends of Tn7; however, Tn7 recombination also involves 5′ end cleavage. We have reported elsewhere that a mutation in TnsA can block the 5′ end, but not 3′ end processing reactions (May and Craig, 1996). The work presented here provides evidence that TnsA actually contains the active site for 5′ end cleavage and is thus distinct from the active site for 3′ end processing in TnsB. In virtually all other systems that have been analyzed, the protein which binds specifically to the element ends, and thus mediates element recognition, also executes the DNA breakage and joining steps (Mizuuchi, 1992b). Although TnsB can specifically recognize a Tn7 substrate and contains the active sites for 3′ end processing, the presence of TnsA is required to promote these TnsB activities, even when 5′ end cleavage does not occur. Thus TnsA actually has at least two roles in Tn7 recombination: executing the 5′ end cleavage activities and modulating TnsB 3′ end processing. Similarly, the presence of TnsB is required for TnsA to execute 5′ end cleavage; TnsB probably positions TnsA at the ends of Tn7 and also modulates the ability of TnsA to execute the cleavages. This interdependence of TnsA and TnsB is likely to play a central and key role in controlling Tn7 transposition. Moreover, the requirement for multiple TnsB protomers in recombination, as shown here, and the requirement for multiple TnsA protomers (May and Craig, 1996) is also consistent with the view that a critical step in Tn7 transposition, as in other recombination systems, is the assembly of higher order complexes both to promote synapsis of the DNA substrates and to form an active recombinase. The requirement for such nucleoprotein complex assembly provides a critical checkpoint in the transposition mechanism, assuring that all the critical components in transposition are present before a potentially lethal double-strand break is introduced into the bacterial chromosome or an inappropriate single-end join product is formed (Bainton *et al.*, 1993).

Tn7's strategy of dividing the active sites for transposition among multiple gene products has not been described previously, but multiple gene products are known to be intimately involved in several other recombination systems, for example P element transposition, Xer recombination and V(D)J rearrangement. Indeed, there are several resemblances between Tn7 transposition and V(D)J recombination (Craig, 1996b; van Gent *et al.*, 1996). The critical chemical steps in these reactions involve direct transesterification reactions. Moreover, the key and initiating event in V(D)J recombination is the introduction of a single-strand nick at the 5′ end of the excisable DNA segment containing the signal sequences located between the immunoglobulin gene segments to be joined. In V(D)J rearrangement, this single-strand nick is converted to a double-strand break by an intramolecular attack of the exposed 3′ end. TnsA cleavage occurs with the same chemical polarity and position, i.e. at the 5′ end of an excisable segment. It will be interesting to see if the 5′ nicks introduced by TnsA can be converted similarly into double-strand breaks. It will also be interesting to see if

in the V(D)J system, as occurs with TnsA, the 5′ cleavage activity is positioned on the DNA substrate through protein–protein interactions with another protein that specifically binds to the signal sequences.

TnsB and TnsA probably belong to the DD(35)E transposase superfamily

Our amino acid changes in TnsB and TnsA were guided by comparison of their sequences with those of other recombinases containing the DD(35)E motif, a signature cluster of acidic amino acids present in many other recombinases including retroviral integrases (reviewed in Craig, 1995; Grindley and Leschziner, 1995; Polard and Chandler, 1995). Studies of other members of this superfamily have provided strong evidence that these amino acids play a critical role in the DNA strand cleavage and strand transfer reactions underlying the translocation of many mobile elements (Engelman and Craigie, 1992; Kulkosky *et al.*, 1992; van Gent *et al.*, 1992; Leavitt *et al.*, 1993; Baker and Luo, 1994; Vos and Plasterk, 1994; Kim *et al.*, 1995). Indeed, alteration of this motif in many other recombinases imposes catalytic blocks, as we have also observed with TnsB and TnsA mutants. The DD(35)E motif is probably a site of action of the divalent metal cofactor essential in these recombination reactions (Baker and Luo, 1994; Dyda *et al.*, 1994; Kim *et al.*, 1995; Rice and Mizuuchi, 1995; Bujacz *et al.*, 1996). We have presented two kinds of experiments, suppression by Mn^{2+} of TnsB DD(35)E mutations and alteration of the metal ion specificity with D114C TnsA, which support the view that metal does interact with the DD(35)E motif. Most critically, these TnsA metal ion specificity experiments also provide functional evidence that TnsA interacts with a divalent metal which is essential for execution of the 5′ processing reactions, probably acting at or near the active site.

It is interesting that TnsA does not have a canonical DD(35)E motif; it lacks an obvious candidate corresponding to the first D position. Although there are acidic amino acids in an appropriate region of TnsA, only the E37A TnsA mutant shows at most a mild defect in catalysis, suggesting that this position is not critical in TnsA endonucleolytic cleavage. While it remains possible that an acidic residue elsewhere in TnsA substitutes for this position in the motif, it is also possible that the TnsA DD(35)E region has a structure that obviates the role of this particular residue in the motif function. Recall that the Tn*7* 3′ strand transfer activity is supplied by TnsB, perhaps alleviating the necessity for an aspartate at this position. Indeed, the first acidic residue of other DD(35)E family members may not be as critical to the endonucleolytic step; mutation of this residue in the DD(35)E motif of HIV-1 integrase can have a much stronger effect on target joining than on 3′ end processing (Leavitt *et al.*, 1993).

The consequences of separation of the 3′ and 5′ end DNA processing activities of Tn7

The separation of the 3′ and 5′ end cleavage activities into different Tn*7* proteins may allow their differential execution and result in different transposition lifestyles. Although direct formation of a simple insertion requires both 3′ and 5′ end DNA processing reactions, transposition can also result when just 3′ end reactions occur, forming a co-integrate product as in the replication of bacteriophage Mu (Craigie and Mizuuchi, 1985; Mizuuchi, 1992b; Lavoie and Chaconas, 1996). Although co-integrates have not been detected with wild-type Tn*7* transposition proteins (Waddell and Craig, 1988; Arciszewska *et al.*, 1989), we have shown elsewhere that Tn*7* co-integration can occur *in vivo* when 5′ end cleavage is eliminated by mutations in TnsA (May and Craig, 1996). It is intriguing that the Tn*552* element, which encodes a transposase containing limited homology not only to the DD(35)E portion of TnsB but across the entire protein, naturally generates co-integrate products (Rowland and Dyke, 1989). While this transposon does not carry a TnsA homolog, it does encode Tn*3*-like co-integrate resolvase functions to generate simple insertion through a replicative mechanism (Rowland and Dyke, 1990). It should be noted that Mu transposition can also involve both 3′ and 5′ end processing; 5′ end cleavage of the strand transfer intermediate apparently requires host proteins, i.e. not just the transposase itself, to form Mu simple insertions (Ohtsubo *et al.*, 1981; Craigie and Mizuuchi, 1985; Mizuuchi, 1992b; Lavoie and Chaconas, 1996). In contrast, Tn*7* transposition occurs by reactions mediated by Tns proteins to form either simple insertions (Bainton *et al.*, 1993) or co-integrates (May and Craig, 1996). The experiments here show that the breakage and joining reactions are mediated by a Tn*7* transposase containing multiple gene products. Other transposons which move by a cut and paste mechanism, notably Tn*10*, use a transposase containing a single gene product both to execute the double-strand breaks that result in excision from the donor, and to execute target joining (Haniford *et al.*, 1989; Chalmers and Kleckner, 1994; Kleckner *et al.*, 1996; Bolland and Kleckner, 1996). It will be interesting to compare in detail the double-strand break reactions executed by single gene product (Tn*10*) and multiple gene product (Tn*7*) transposases.

Concluding remarks

We have suggested previously that a key element in the control of Tn*7* transposition is the assembly of a nucleoprotein complex containing the DNA substrates and Tns proteins (Bainton *et al.*, 1993), such a complex actually being required for the double-strand breaks which initiate recombination. A more detailed view of the Tn*7* transposase has now emerged. It is an oligomeric nucleoprotein complex consisting of multiple subunits of different protein protomers; TnsB specifically recognizes the ends of Tn*7* and executes the breakage and joining events at the 3′ ends, and TnsA mediates the breakage reactions at the 5′ ends. Both TnsB and TnsA appear to be members of the retroviral integrase superfamily in which a Mg^{2+}-dependent phosphoryl transfer reaction can result in either DNA breakage or strand transfer, depending on the nucleophile: H_2O in the strand breakage step or the 3′ hydroxyl of the transposon end in the strand transfer step. We have here identified TnsB as the protein which executes the most central and fundamental of these breakage and joining reactions and whose activity must, therefore, be determined carefully by this nucleoprotein assembly. It will be especially interesting to determine how the 3′ end breakage and joining activities of TnsB are coordinated

interdependently with the TnsA 5′ breakage activity to effect the double-strand break and strand transfer reactions underlying Tn7 translocation.

Materials and methods

DNA substrates

The donor plasmid pEMΔ (5926 bp) contains a 1.6 kb miniTn7 element comprised of a 166 bp Tn7L segment and a 199 bp Tn7R segment flanking a kanamycin resistance gene (Bainton *et al.*, 1993). ELTs were generated by *Mlu*I digestion of pMIM-L$^+$R$^+$ which exposes the terminal -CA-3′ ends of Tn7 (Bainton *et al.*, 1991); after restriction, ELTs were isolated by TBE agarose gel electrophoresis and Qiaex extraction. The miniTn7 in MIM-L$^+$R$^+$ is identical to that of pEMΔ except that the terminal 19 bp of Tn7L have been converted to the Tn7R sequence, a 2 bp change. The 3290 bp target plasmid pRM2 (McKown *et al.*, 1988) contains a 555 bp *attTn7* segment (–342 to +165) in the *Acc*I site of pUC18.

TnsB

In Figures 3 and 4, TnsB-His$_6$ (Gary *et al.*, 1996) was used. In all other experiments, an alternative protein fusion with an epitope tag was used, TnsB-HSV-His$_6$ which contains amino acids 1–694 of TnsB (702 amino acids) fused to a 15 amino acid linker containing a herpes simplex virus epitope followed by a six residue histidine tag; its properties under standard reaction conditions are indistinguishable from those of authentic TnsB (data not shown). The tagged *tnsB* gene was created by introducing the *Bam*HI–*Alw*NI fragment from pET-25B (Novagen) into the *Bam*HI–*Alw*NI site of pCW12 (Waddell and Craig, 1988) which contains a transcriptional fusion of *tnsB* to the *lac* promoter. Mutant derivatives of TnsB-HSV-His$_6$ were generated by PCR with oligonucleotides containing appropriately altered *tnsB* sequences to generate fragments which were then introduced into the TnsB-HSV-His$_6$ plasmid by cloning; mutant sequences were verified by direct DNA sequencing. The resulting plasmids were introduced into CAG456 (*E.coli* K12 *rpsL lacZ*$_{am}$ *trp*$_{am}$ *pho*$_{am}$ *supC*^{ts} *htpR*165) which also contained a pSC101 derivative carrying *lacI*^{q} (Arciszewska *et al.*, 1991). Cells were grown at 30°C to an OD$_{600}$ = 0.4 in LB medium supplemented with 100 μg/ml carbenicillin and 5 μg/ml tetracycline, IPTG was added to 1 mM and growth continued for 1 h. The cells were harvested by centrifugation and resuspended at 4°C in 40 mM imidazole, 20 mM Tris (pH 7.9) and 500 mM NaCl at 0.2 g cells/ml. The cells were then lysed by sonication, centrifuged at 30 000 *g* for 20 min, and the resulting supernatant passed through a 0.45 μm filter. The filtrate was applied to a Ni^{2+} Sepharose B column (Pharmacia), the column washed with the same buffer and TnsB-HSV-His$_6$ eluted with 150 mM imidazole, 20 mM Tris (pH 7.9) and 500 mM NaCl. Peak fractions were pooled and dialyzed against 25 mM Tris (pH 8.0), 500 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 25% glycerol. About 300 μg of TnsB-HSV-His$_6$ were obtained from 2 g of cells; the final fraction was >95% full-length TnsB. TnsB-HSV-His$_6$ derivatives were stored at –80°C in 25 mM HEPES (pH 8.0), 500 mM NaCl, 2 mM DTT, 1 mM EDTA and 25% glycerol.

Other Tns proteins

TnsA was expressed as a fusion with glutathione *S*-transferase, bound to glutathione–agarose beads, and then freed through a limited proteolytic treatment with thrombin (Bainton *et al.*, 1993; May and Craig, 1996). Site-specific mutations were introduced into the *tnsA* gene using PCR as previously described (May and Craig, 1996). TnsA was stored at –80°C in 25 mM HEPES (pH 8.0), 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 5% glycerol and 0.25 mM phenylmethylsulfonyl fluoride (PMSF).

TnsC was fraction III (Gamas and Craig, 1992) and was stored at –80°C in 25 mM HEPES, 1 M NaCl, 2.5 mM DTT, 1 mM ATP, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM CHAPS and 10% glycerol. TnsD was TnsD-His, a derivative containing a C-terminal polyhistidine tag, and was purified by Ni^{2+} affinity chromatography; its properties are indistinguishable from wild-type TnsD in these assays (P.Sharpe and N.L.Craig, unpublished observation). TnsD-His was stored at –80°C in 50 mM Tris (pH 7.5), 2 mM DTT, 500 mM KCl, 1 mM EDTA and 25% glycerol.

In vitro transposition reactions

Reactions were performed essentially as previously described (Bainton *et al.*, 1993; Gary *et al.*, 1996). Reaction mixtures (100 μl final volume) contained 0.25 nM donor DNA (plasmid or ELT), 2.5 nM pRM2 *attTn7* target plasmid, 26 mM HEPES (pH 8.0), 2.1 mM DTT, 4 mM Tris (pH 7.5), 100 μg/ml tRNA, 50 μg/ml bovine serum albumin (BSA), 2.0 mM ATP, 0.05 mM EDTA, 0.1 mM $MgCl_2$, 0.1 mM CHAPS, 14 mM NaCl, 21 mM KCl, 1.2% glycerol, 4 μM PMSF and 15 mM MgAc unless otherwise indicated. Tns proteins were added as follows, unless otherwise indicated. In Figures 3 and 4: 24 nM TnsA, 3 nM TnsB-His$_6$, 8 nM TnsC, 6 nM TnsD; in all other experiments: 24 nM TnsA, 3 nM TnsB-HSV-His$_6$, 13 nM TnsC, 12 nM TnsD. Pre-incubation reaction mixtures containing all components except donor DNA, TnsA, TnsB and metal were assembled on ice; the concentrations of the components in this mixture varied by <15% from the stated final concentrations. The pre-incubation mixtures were then incubated for 20 min at 30°C, or 20 min at 22°C for time course experiments (Figures 3C); donor DNA, TnsA, TnsB and metal were then added individually and sequentially, and the incubation was continued for an additional 20 min, or for the indicated times (Figures 3C). Reactions were stopped by making them 25 mM EDTA and 300 mM NaAc, extracted with phenol:chloroform (1:1); the DNA was ethanol precipitated, digested with restriction enzymes and analyzed by 0.6% agarose gel electrophoresis. All reaction mixtures except those in Figure 7 were digested with *Nde*I which cuts once in pEMΔ 1837 bp outside the right end, and once in pRM2 577 bp from the *attTn7* insertion point. In Figure 7, the reaction products were digested with *Sal*I and *Hin*dIII for hybridization with probes A and B, or with *Sal*I and *Eco*RI for hybridization with probes C and D.

Reactions with Mn^{2+}

In Figure 8, pre-incubation mixtures containing all components except Mn^{2+} were assembled on ice and then incubated for 20 min at 30°C; Mn^{2+} as indicated (final concentration 5–20 mM) was then added and incubation continued for an additional 20 min. In Figure 4, pre-incubation mixtures containing all components except metal were assembled on ice and then incubated for 20 min at 30°C; Mg^{2+} was then added and incubation continued for another 20 min at 30°C; the second metal (Mg^{2+} or Mn^{2+}) was then added and incubation continued for another 2 h. Metal concentrations at the end of the reaction: 15 mM Mg^{2+} and either 5 mM, 15 mM or 45 mM of the additional metal (either Mg^{2+} or Mn^{2+}).

Gel electrophoresis and product analysis

The restricted DNAs of all reactions except Figure 7 were displayed by electrophoresis on 0.6% agarose TBE gels. The gels were transferred to Gene Screen Plus and analyzed by hybridization; the [^{35}S]DNA ladder was from Amersham. The restricted products of the reactions in Figure 7 were displayed by electrophoresis on a 5% denaturing polyacrylamide gel, electrotransferred to Gene Screen Plus and hybridized with a miniTn7 end-specific oligonucleotide. All blots were analyzed by autoradiography using Kodak X-OMAT-AR film or by Molecular Dynamics Phosphorimager. The miniTn7-specific probe used for all blots except in Figure 7 was the kanamycin gene segment between Tn7L and Tn7R. The relevant gel-purified restriction fragment was labeled by random priming with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I (BMB). Oligonucleotide probes were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by NICK Spin Column chromatography (Pharmacia). Probe A: NLC 95 (5′) ATAATCCTTAAAAACTCCATTTCCACCCCT; probe B: NLC 97 (5′) AGGGGTGGAAATGGAGTTTTTAAGGATTAT; probe C: NLC 163 (5′) GTGAAAAAGCATACTGGACTTTTGTTATGG; probe D: NLC 94 (5′) AAAGTCCAGTATGCTTTTTCACAGCATAAC.

Acknowledgements

The authors wish to thank Tomonobu Kusano, Elena Krementsova and Tania Baker for communication of information prior to publication; Fred Dyda and Phoebe Rice for HIV-1 integrase and Mu transposase crystal coordinates prior to publication; Cynthia Wolberger for help with structure interpretation; Melissa Moore for helpful discussions about the metal ion specificity experiments; Matt Biery for developing several critical methods; Patti Eckhoff for help in the preparation of this manuscript, and also other members of the Craig laboratory for helpful conversation during the work and the critical reading of this manuscript, especially Pat Gary, Pam Sharpe and Anne Stellwagen for supplying useful reagents; and Tania Baker and Howard Nash for comments on earlier drafts of the manuscript. N.L.C. is an investigator of the Howard Hughes Medical Institute.

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Received on June 11, 1996; revised on July 30, 1996