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Analysis of Gain-of-Function Mutants of an ATP-dependent Regulator of Tn*7* Transposition

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The bacterial transposon Tn*7* is distinguished by its unusal discrimination among targets, being particularly attracted to certain target DNA and actively avoiding other DNA. Tn*7* transposition is mediated by the interaction of two alternative transposon-encoded target selection proteins, TnsD and TnsE, with a common core transposition machinery composed of the transposase (TnsAB) and an ATP-dependent DNA-binding protein TnsC. No transposition is observed with wild-type TnsABC. Here, we analyze the properties of two gain-of-function TnsC mutants that allow transposition in the absence of TnsD or TnsE. We find that these TnsC mutants have altered interactions with ATP and DNA that can account for their gain-of-function phenotype. We also show that TnsC is an ATPase and that it directly interacts with the TnsAB transposase. This work provides strong support to the view that TnsC and its ATP state are central to the control of Tn*7* transposition.

Keywords: transposon; ATP hydrolysis; control of transposase; transposase activation

Introduction

The transposition of mobile DNA elements is executed by protein-DNA machines whose assembly is a carefully regulated process. Transposase proteins form the catalytic core of these machines, binding to the ends of the transposable element and executing the DNA breakage and joining reactions that move the element from one DNA site to another (Mizuuchi, 1992). Some transposition machines also contain ATP-dependent proteins, with the best studied examples being the bacteriophage Mu (Lavoie & Chaconas, 1996) and the bacterial transposon Tn*7* (Craig, 1996). Here, we have investigated the role of the ATP-binding protein TnsC in Tn*7* transposition.

Tn*7* exerts exceptional control over the selection of targets for new insertion events. Whereas most transposons insert into a wide variety of target DNAs, Tn*7* preferentially inserts into certain types of target DNA and preferentially avoids others. Tn*7* transposition occurs at high frequency into a unique site in the *Escherichia coli* chromosome called *attTn7* (Craig, 1996); transposition is also preferentially directed into plasmids undergoing conjugation (Wolkow *et al.*, 1996). By contrast, transposition into DNA molecules that already contain a Tn*7* element is strongly discouraged (Hauer & Shapiro, 1984; Arciszewska *et al.*, 1989); these DNA molecules are described as being ''immune'' to Tn*7* transposition. Thus, Tn*7* can evaluate multiple features of a potential target DNA and insert into the most favorable target available.

Tn*7* encodes five proteins that are used in different combinations to direct insertions into these different types of target DNAs. TnsA, TnsB, TnsC and TnsD (TnsABC + D) promote transposition into *attTn7*, whereas TnsA, TnsB, TnsC and TnsE (TnsABC + E) promote transposition into chromosomal sites unrelated to *attTn7* and also into conjugating plasmids (Craig, 1996). The TnsABC + D transposition reaction has been reconstituted *in vitro* (Bainton *et al.*, 1991, 1993), allowing the roles of the individual Tns proteins to be elucidated. TnsA and TnsB act interdependently to execute the catalytic steps of Tn*7* transposition; therefore TnsAB serves as the Tn*7* transposase (May & Craig, 1996; Sarnovsky *et al.*, 1996; Biery *et al.*, 2000a; Lu & Craig, 2000). TnsC is an ATP-dependent DNA-binding protein (Gamas & Craig, 1992) and TnsD is an *attTn7*-specific DNA-binding protein (Bainton *et al.*, 1993). The Tns proteins assemble with the transposon and the *attTn7* DNAs to form a functional transposition complex

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whose assembly appears to precede the initiation of the chemical steps of Tn7 transposition (Bainton *et al*., 1993).

TnsC is a central player in the selection of an appropriate target DNA. Alone, TnsC binds DNA without any obvious sequence specificity (Gamas & Craig, 1992). However, its distribution among potential target DNAs matches the specificity of Tn7 transposition. For example, TnsC is recruited to *attTn7* sites through interactions with TnsD (Bainton *et al*., 1993); TnsC may be similarly recruited to conjugating plasmids by TnsE (Wolkow *et al*., 1996). By contrast, TnsC appears to be actively removed from immune targets through interactions with TnsB (Bainton *et al*., 1993; Stellwagen & Craig, 1997a,b). Thus, the presence or absence of TnsC provides a molecular reflection of the positive and negative features of a potential target DNA.

ATP hydrolysis appears to be required for TnsC to achieve this selective distribution. When non-hydrolyzable ATP analogs such as ATPγS or AMP-PNP are substituted for ATP in the *in vitro* transposition reaction, Tn7 transposition will occur but target selectivity is lost. Under this condition, TnsABC alone is sufficient to promote transposition; TnsD is no longer required and transposition events are no longer directed into *attTn7* sites or away from immune targets (Bainton *et al*., 1993). An attractive hypothesis is that when ATP hydrolysis is blocked, TnsC is constitutively able to interact with target DNAs and promote transposition.

Here, we have probed the mechanisms by which TnsC influences Tn7 transposition. We have identified several new biochemical activities of TnsC: we provide direct evidence that TnsC is an ATPase and demonstrate that TnsC interacts both physically and functionally with the TnsAB transposase. We have also characterized two gain-of-function TnsC mutants that relax some or all of Tn7's target specificity *in vivo* (Stellwagen & Craig, 1997b). Interestingly, the distinct transposition phenotype of each mutant can be correlated with altered interactions with ATP and DNA. This work provides experimental support for the view that TnsC can stimulate transposition when it is in an ATP bound form and cannot activate transposition in the absence of ATP (Bainton *et al*., 1993; Stellwagen & Craig, 1998).

Results

TnsC mutants promote Tn*7* transposition *in vitro*

Although TnsA, TnsB and TnsC form the core of the Tn7 transposition machinery, virtually no transposition is seen with TnsABC alone. Inputs from either TnsD or TnsE are required to activate the TnsABC machinery as well as to select a target site. However, gain-of-function TnsC mutants have been isolated that enable Tn7 transposition to occur in the absence of TnsD or TnsE (Stellwagen & Craig, 1997b). *In vivo* analysis revealed that the TnsC mutants fall into two phenotypic classes. Transposition promoted by one class of TnsC mutants, represented by $TnsC^{A225V}$, can still respond to signals from different target DNAs. For example, immune target molecules are still recognized and avoided by the $TnsABC^{A225V}$ transposition machinery; TnsD and TnsE can also influence the targeting of $TnsC^{A225V}$-mediated events. The A225V mutation lies just a few amino acid residues upstream of the Walker B portion of the ATP-binding motif in TnsC. A second class of TnsC mutants, represented by $TnsC^{S401Y\Delta402}$, does not appear to respond to target DNA signals. Immune and non-immune target DNAs are used with equal frequency by the $TnsABC^{S401Y\Delta402}$ machinery, and those transposition events are not influenced by TnsD or TnsE.

To understand the alterations responsible for these gain-of-function phenotypes, biochemical characterization of $TnsC^{A225V}$ and $TnsC^{S401Y\Delta402}$ is required. We have previously shown that these TnsC mutants do promote transposition *in vitro* in the presence of only TnsAB and that insertions in the $TnsABC^{mutant}$ reactions occur at many different sites (Biery *et al*., 2000b) (see also below).

To verify that the distinct *in vivo* properties of $TnsC^{A225V}$ and $TnsC^{S401Y\Delta402}$ are being recapitulated in the *in vitro* transposition system, we examined whether TnsD could influence the mutant transposition reactions. The *in vitro* Tn7 transposition reaction proceeds *via* a cut-and-paste mechanism, in which a miniTn7 (mTn7) element is cleaved from a donor plasmid and transferred into an *attTn7*-containing target plasmid (Bainton *et al*., 1993) (Figure 1(a)). When TnsA, TnsB, $TnsC^{wt}$ and TnsD are present, as well as the essential cofactors ATP and Mg^{2+}, efficient transposition is seen and virtually all of the insertions are located in *attTn7* (Figure 1(b)). TnsD had no apparent impact on the target site selection of the $TnsABC^{S401Y\Delta402}$ transposition reactions; insertions into *attTn7* were not observed in either the presence or the absence of TnsD. However, TnsD strongly influenced the $TnsABC^{A225V}$ machinery: the percentage of insertions into the *attTn7* fragment increased from 4% to 65% when TnsD was present. These results suggest that *in vitro*, as had previously been observed *in vivo*, the $TnsABC^{A225V}$ machinery retains the ability to respond to TnsD, whereas the $TnsABC^{S401Y\Delta402}$ machinery does not.

$TnsC^{A225V}$ has an altered ATP hydrolysis activity

What is the biochemical basis of the gain-of-function phenotypes of $TnsC^{A225V}$ and $TnsC^{S401Y\Delta402}$? An attractive hypothesis is that the mutants have altered interactions with ATP. The transposition activities of $TnsC^{wt}$ are affected by the type of adenine nucleotide present in the reaction: $TnsC^{wt}$ binds to target DNA and promotes

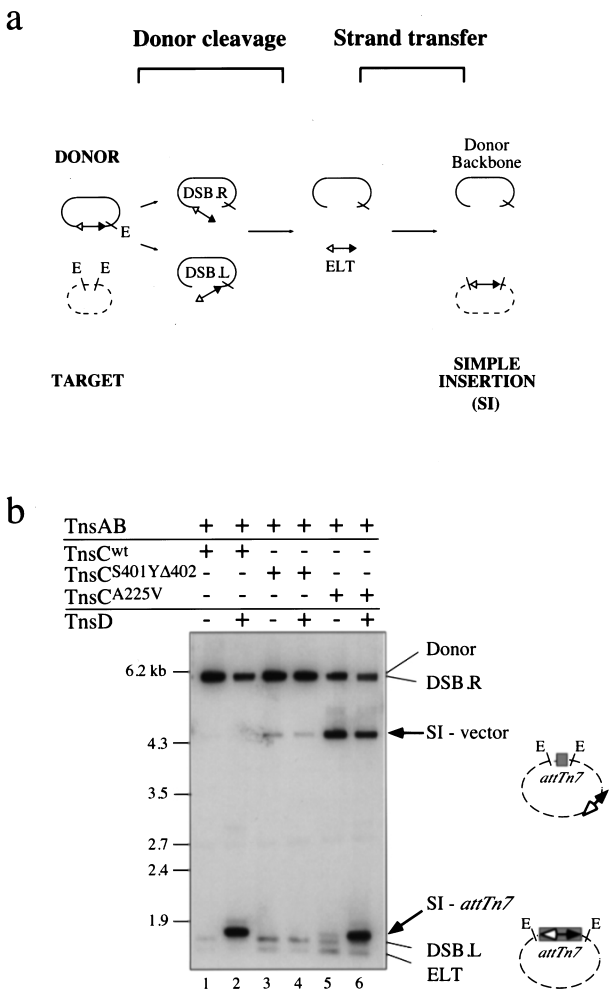

Figure 1. In vitro transposition activities of wild-type and mutant TnsC proteins in the presence and absence of TnsD. (a) The Tn*7* transposition pathway. The miniTn*7* element is excised from the donor DNA (continuous line) by double-strand breaks at the transposon ends (arrowheads), and then transferred into the target DNA (broken line) to generate a simple insertion (SI). Donor molecules that have a double-strand break at the left or right end (DSB.L or DSB.R) appear transiently as intermediates in the reaction, as does the fully excised linear transposon (ELT). (b) *Eco*RI analysis. Insertions into the small *attTn7* fragment and the vector backbone are differentiated. The illustrations depict an insertion into *attTn7* and a representative insertion elsewhere in the vector backbone. The positions of *Eco*RI (RI) sites are indicated in the donor and target plasmids; the *attTn7* site in the target plasmid is represented as a grey box.

transposition in the presence of ATP, whereas TnsC^{wt} exhibits neither of these activities in the presence of ADP (Gamas & Craig, 1992; Bainton *et al.*, 1993). Non-hydrolyzable ATP analogs also impact the activity of TnsC^{wt}, enabling it to promote transposition into any target DNA without the need for TnsD (Bainton *et al.*, 1993). These observations suggest that TnsC is an ATPase, and that ATP hydrolysis may be important in switching the protein from an active (ATP-bound) to an inactive (ADP-bound) conformation. Below, we demonstrate that TnsC^{wt} is indeed an ATPase and we investigate whether that ATPase activity is altered in the TnsC mutants.

A modest ATP hydrolysis activity ($3.1(\pm 0.4) \times 10^{-2}$ mol ATP hydrolyzed/mol TnsC per minute) is present in our TnsC^{wt} preparations that copurifies with the TnsC^{wt} polypeptide in size-exclusion chromatography (Figure 2(a)). TnsC^{wt} elutes as a single peak at the position expected of a TnsC dimer (data not shown). Fractions were collected across the column and assayed for the ability to hydrolyze ATP; the elution profiles of the TnsC^{wt} protein and the ATPase activity were coincident. SDS-PAGE confirmed the presence of TnsC^{wt} in these fractions and transposition assays verified that the TnsC^{wt} protein was functional (data not shown). Thus, TnsC^{wt} appears to be an ATPase.

The ATPase activities of the TnsC mutants were also examined. TnsC^{A225V} does have ATPase activity, but TnsC^{A225V} hydrolyzes ATP less efficiently than TnsC^{wt} (Figure 2(b)); TnsC^{A225V} hydrolyzes ATP at $1.0(\pm 0.1) \times 10^{-2}$ mol ATP hydrolyzed/mol TnsC per minute, about threefold less than TnsC^{wt}. We speculate that this reduced ATPase activity might contribute to the gain-of-function activity of TnsC^{A225V}, by enabling the mutant protein to spend more time in the active (ATP-bound) conformation than TnsC^{wt}.

An ATPase activity has also been detected in the TnsC$^{S401Y\Delta402}$ preparations (data not shown), but it has not yet been possible to unambiguously tie that activity to the TnsC$^{S401Y\Delta402}$ polypeptide. However, as described below, the interactions of TnsC$^{S401Y\Delta402}$ with ATP and ADP appear to be quite different from those of TnsC^{wt} and TnsC^{A225V}.

ADP does not inhibit TnsC$^{S401Y\Delta402}$ transposition reactions

Tn*7* transposition requires ATP. ADP does not support the TnsABC^{wt} + D transposition reaction (Bainton *et al.*, 1993), and experiments with mixed ADP and ATP nucleotides have demonstrated that ADP is, in fact, a potent inhibitor of this reaction (A.E.S. and N.L.C., unpublished observations). We examined the transposition activity of the TnsC mutants in the presence of ADP (Figure 3). As is true of TnsABC + D transposition, TnsABC^{A225V} transposition is also strongly inhibited by the addition of ADP. Strikingly, however, ADP does not inhibit the TnsABC$^{S401Y\Delta402}$ reaction. Instead, similar levels of TnsABC$^{S401Y\Delta402}$ transposition are observed in the presence of ADP, ATP or AMP-PNP, or in the absence of exogenously added nucleotide (data not shown).

Why are TnsC$^{S401Y\Delta402}$ reactions not inhibited by ADP? An interesting possibility is that

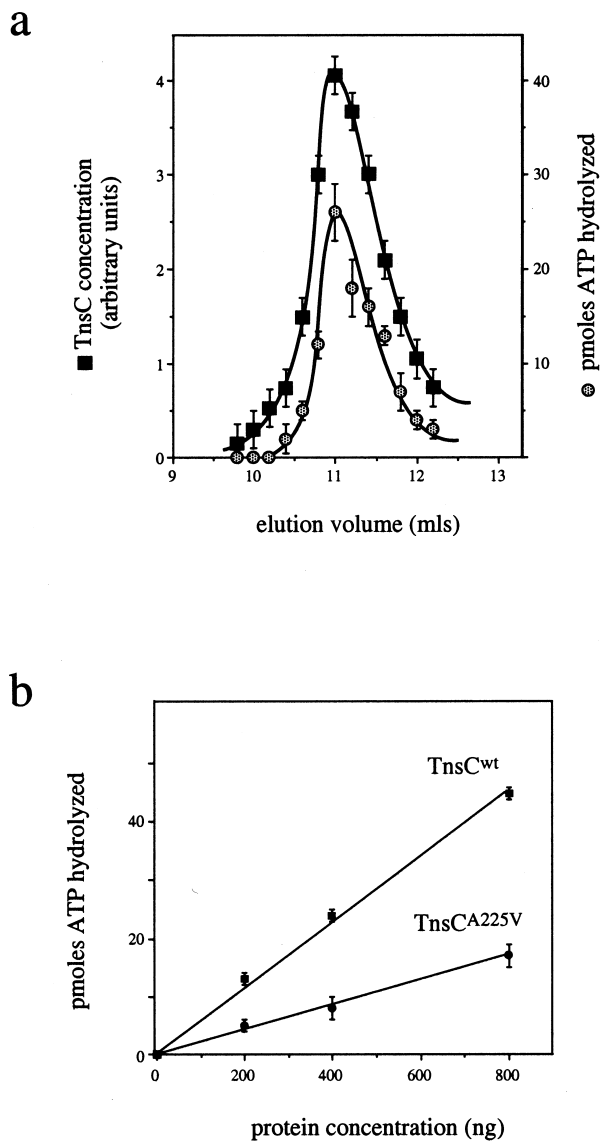

Figure 2. ATP hydrolysis activities of TnsC^{wt} and TnsC^{A225V}. (a) ATPase activity of TnsC^{wt}. TnsC was fractionated over a size-exclusion column and the protein concentration (squares) and ATPase activity (circles) of each fraction were determined. The averages of two experiments are presented. (b) Comparison of the ATPase activities of TnsC^{wt} and TnsC^{A225V}. Each value was measured in triplicate.

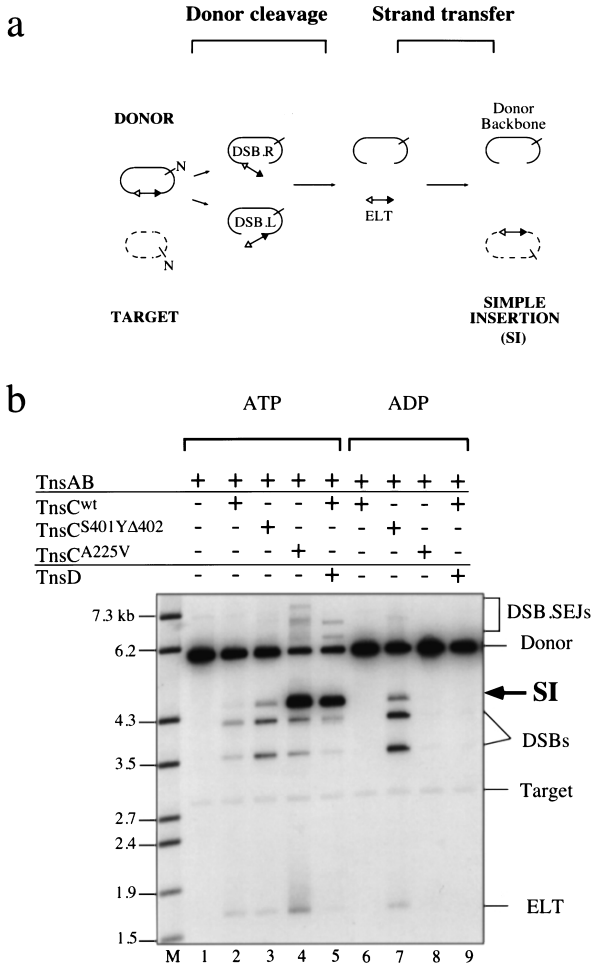

Figure 3. TnsC mutants can promote Tn*7* transposition in the presence of ADP. (a) The Tn*7* transposition pathway is portrayed as described in the legend to Figure 1(a). The positions of *Nde*I (N) sites in the donor and target plasmids are indicated. (b) Transposition in the presence of ATP and ADP. An autoradiogram displaying the products of Tn*7* transposition reactions is shown. Tns proteins present in each reaction are indicated above each lane. DSB.SEJs (double-strand break/single-end joins) are transposition products in which one end of the transposon has been cleaved and inserted into the target DNA, while the other end remains attached to the donor molecule. Reactions in lanes 1-5 were carried out under standard reaction conditions. Reactions in lanes 6-9 contained 2 mM ADP (added exogenously) and 10 μM ATP (contributed by the TnsC storage buffer).

TnsC$^{S401Y\Delta402}$ might be locked in a constitutively active conformation, capable of promoting transposition regardless of the type of adenine cofactor present in the reaction. However, it should be noted that TnsC$^{S401Y\Delta402}$, like all TnsC proteins, is purified and stored in buffers containing ATP, and some fraction of these proteins is likely introduced into the transposition reaction in an ATP-bound state. Therefore, the failure to observe ADP inhibition could also result from an altered ability of TnsC$^{S401Y\Delta402}$ to exchange nucleotides. For example, TnsC$^{S401Y\Delta402}$ might bind ATP with increased affinity, such that it is unable to release the ATP molecule with which it was purified and bind the exogenously added ADP. To address this possibility, we purified TnsC$^{S401Y\Delta402}$ in buffers containing ADP rather than ATP; the ADP-purified protein retains the ability to promote transposition (data not shown).

TnsC mutants bind DNA with increased affinity

What other properties of TnsC might be influenced by the altered ATP interactions of $TnsC^{S401Y\Delta402}$ and $TnsC^{A225V}$? One possibility is that the DNA-binding activities of the mutant proteins are affected, since the ability of $TnsC^{wt}$ to bind DNA is ATP-dependent (Gamas & Craig, 1992).

A filter-binding assay was used to directly compare the DNA-binding activities of wild-type and mutant TnsC proteins. Binding of each protein was evaluated in increasing concentrations of Mg^{2+} (Figure 4(a)) as we have previously observed that binding of wild-type TnsC to DNA decreases in increasing concentrations of Mg^{2+} (Gamas & Craig, 1992). $TnsC^{S401Y\Delta402}$ and $TnsC^{A225V}$ retained considerable DNA-binding activity even in 12 mM Mg^{2+}, whereas $TnsC^{wt}$'s ability to bind DNA was greatly reduced. We have also observed that *in vitro* transposition promoted by $TnsC^{A225V}$ and $TnsC^{S401Y\Delta402}$ will occur in high concentrations of Mg^{2+} which do not support transposition by wild-type TnsC (Figure 4(b)).

We also investigated the stability of the various TnsC-DNA complexes in pulse-chase experiments (Figure 4(c)). $TnsC^{wt}$ displayed less than 30 % of its original DNA-binding activity after a 30 minute incubation with cold competitor DNA. By contrast, $TnsC^{S401Y\Delta402}$ and $TnsC^{A225V}$ retained more than 80 % binding after the same time, suggesting that these TnsC mutants have significantly slower rates of dissociation from DNA than $TnsC^{wt}$. Interestingly, $TnsC^{wt}$ was able to match the stability of the TnsC mutants in TnsC-DNA complexes when the non-hydrolyzable analog AMP-PNP was substituted for ATP in the $TnsC^{wt}$ DNA-binding reactions.

Thus, the TnsC mutants not only have altered interactions with ATP but also (and perhaps consequently) have enhanced DNA-binding activities. Both of these properties likely contribute to the gain-of-function phenotypes of the TnsC mutants.

TnsC stimulates the catalytic activity of the TnsAB transposase

TnsC has been proposed to receive signals from potential target DNA, as well as to communicate that information to the TnsAB transposase (Bainton *et al.*, 1993; Stellwagen & Craig, 1997b). The simplest model would be for TnsC to directly interact with TnsAB (i.e. through protein-protein interactions) but TnsC could also influence TnsAB through indirect means (i.e. by distorting the target DNA backbone in a way that gives TnsAB greater access to the insertion site). Here, we provide evidence that $TnsC^{wt}$ can directly and functionally interact with the TnsAB transposase in the absence of target DNA, and we identify a domain of $TnsC^{wt}$ that appears to be important for these interactions.

To uncover a functional interaction between TnsC and TnsAB, the conditions of the *in vitro* transposition reaction were modified to uncouple the donor cleavage step from its usual dependence on TnsD + *attTn7*. The target DNA was omitted from these reactions, as was the low Mg^{2+} preincubation step that facilitates $TnsC^{wt}$ binding to DNA, and the concentration of glycerol was increased. Under these conditions, no donor cleavage was seen with TnsAB alone. However, $TnsC^{wt}$ was able to stimulate the donor cleavage activity of the TnsAB transposase in a concentration-dependent manner (Figure 5(b), lanes 1-5). These results suggest that, under these modified reaction conditions, $TnsC^{wt}$ can interact with the TnsAB transposase and that these interactions can occur in the absence of an explicit target DNA.

We also examined the ability of a truncated TnsC protein to stimulate donor cleavage. Previous work had identified a TnsC deletion mutant ($TnsC^{\Delta1\text{-}293}$) that enabled TnsAB to promote a limited amount of Tn7 transposition *in vivo* (Stellwagen & Craig, 1997b). Therefore we speculated that the carboxy-terminal half of $TnsC^{wt}$ might be responsible for the interactions of $TnsC^{wt}$ with TnsAB. We purified $TnsC^{\Delta1\text{-}293}$ as a fusion protein with the glutathione-*S*-transferase (GST) domain and examined its activity in the donor cleavage assay (Figure 5(b), lanes 9-11). Like $TnsC^{wt}$, GST-$TnsC^{\Delta1\text{-}293}$ was able to stimulate the donor cleavage reaction, although the specific activity of the truncated protein was approximately 50-fold lower than that of intact protein. Thus, at least a part of the ''TnsAB activation domain'' of $TnsC^{wt}$ appears to lie within the carboxy-terminal half of the protein.

GST-$TnsC^{\Delta1\text{-}293}$ has no detectable DNA-binding activity (data not shown). This may indicate that some or all of the residues that comprise the DNA-binding domain lie within the amino-terminal half of $TnsC^{wt}$; alternatively the DNA-binding domain may be unable to fold properly in GST-$TnsC^{\Delta1\text{-}293}$. Nevertheless, the ability of GST-$TnsC^{\Delta1\text{-}293}$ to promote donor cleavage supports the view that TnsC can interact with the TnsAB transposase independent of its interactions with the target DNA.

The interactions between GST-$TnsC^{\Delta1\text{-}293}$ and TnsAB are also likely ATP-independent: the ATP-binding motifs of $TnsC^{wt}$ are not present in the truncated protein (Figure 5(a)), and ATP is not present in the $TnsC^{\Delta1\text{-}293}$ donor cleavage reactions. We speculate that the structure and/or the accessibility of the TnsAB activation domain within $TnsC^{wt}$ is regulated by the ATP state of the protein.

We also examined the abilities of $TnsC^{S401Y\Delta402}$ and $TnsC^{A225V}$ to stimulate donor cleavage; their activity was essentially equivalent to $TnsC^{wt}$ (data not shown). In addition, a GST-$TnsC^{\Delta1\text{-}293}$ was constructed containing the $TnsC^{S401Y\Delta402}$ mutation; its behavior was indistinguishable from wild-type GST-$TnsC^{\Delta1\text{-}293}$. These results may indicate that the gain-of-function activity of these TnsC mutants is not due to enhanced interactions with the TnsAB

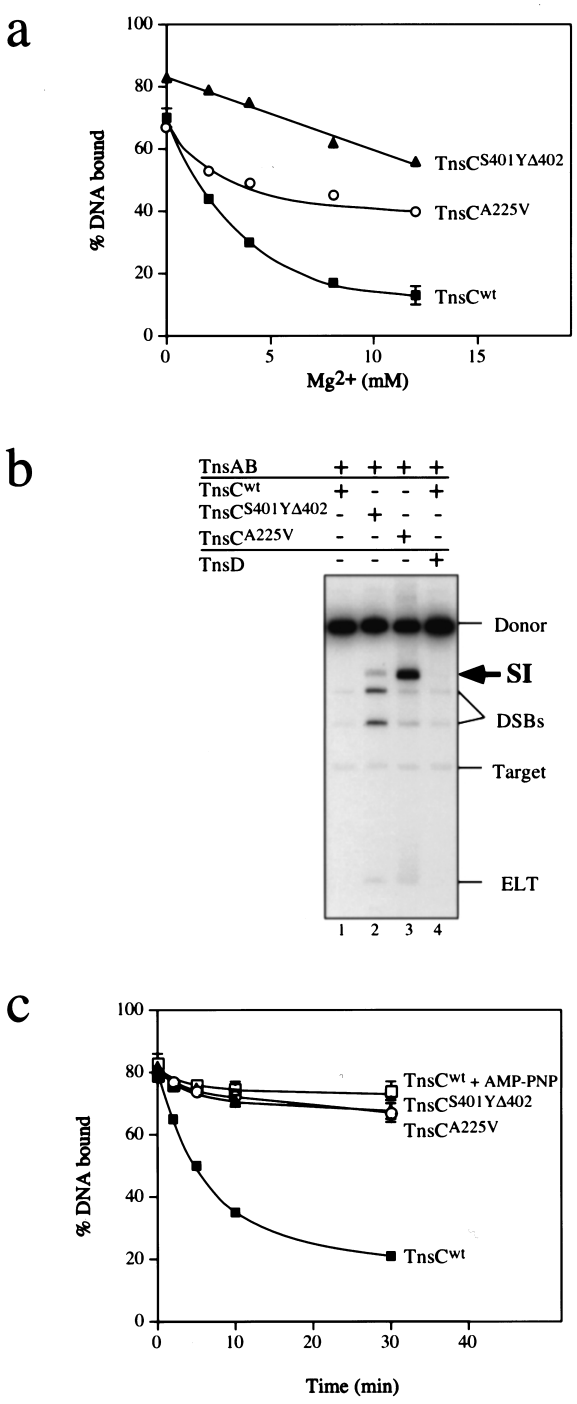

Figure 4. DNA binding and transposition activities of the wild-type and mutant TnsC proteins. (a) Effects of Mg^{2+}. The DNA-binding activity of each TnsC protein at various concentrations of Mg^{2+} is presented. All measurements were done in triplicate and the average is plotted. Unless otherwise indicated, the error bars for each value are smaller than the size of the symbol. (b) *In vitro* transposition assays. In contrast to standard reactions in which a target preincubation step is done and then Mg^{2+} is added for the reaction incubation, 15 mM Mg^{2+} was present in the target preincubation step and throughout the course of these reactions. (c) Pulse-chase assays. The DNA-binding activity of each TnsC protein prior to the addition of cold competitor was determined and then measured over time in the presence of an excess of non-specific DNA. $TnsC^{wt}$'s activity was determined in the presence of ATP and AMP-PNP, while $TnsC^{S401Y\Delta402}$ and $TnsC^{A225V}$ were evaluated in the presence of ATP only.

transposase. Alternatively, authentic differences in the activities of the TnsC mutants may be masked by the conditions of the donor cleavage assay.

Protein-protein interactions between TnsC and TnsA

Physical interactions between GST-$TnsC^{\Delta1\text{-}293}$ and the components of the TnsAB transposase were investigated in pulldown assays (Figure 5(c)). TnsA co-precipitated with GST-$TnsC^{\Delta1\text{-}293}$ under all conditions examined. This interaction was dependent on the $TnsC^{\Delta1\text{-}293}$ domain, since GST alone was unable to precipitate TnsA. In reciprocal experiments, a GST-TnsA fusion protein was able to precipitate $TnsC^{wt}$ (J. Feng and N.L.C., unpublished observations; Lu & Craig, 2000). Together, these results suggest that TnsA and TnsC physically interact, a view supported by the finding that TnsC can protect TnsA from proteolytic attack (Lu & Craig, 2000).

Co-precipitation of GST-$TnsC^{\Delta1\text{-}293}$ and TnsB was not observed under any of the conditions examined. We have postulated, however, that an interaction between TnsC and TnsB is important for Tn7 target immunity, i.e. the active avoidance by Tn7 of a target DNA already containing Tn7 (Stellwagen & Craig, 1997a). Other experiments will be required to determine if the TnsC-TnsB interaction is mediated by the N terminus of TnsC or whether an interaction between $TnsC^{\Delta1\text{-}293}$ and TnsB will be revealed under other conditions.

Discussion

Here, we have demonstrated that TnsC is an ATPase, and therefore has the potential to switch between the ATP- and ADP-bound states. We have characterized two TnsC gain-of-function mutants and we find that their altered transposition activities can be correlated with altered interactions with ATP and DNA. We have also shown that TnsC directly interacts with the TnsAB transposase and can stimulate its catalytic activity. Below, we discuss $TnsC^{wt}$ and the TnsC mutants in terms of a model in which TnsC controls Tn7 transposition by switching between an ATP-bound state that promotes transposition and an ADP-bound (or nucleotide free) state that does not promote transposition. We also compare the mechanism of ATP-dependent control of Tn7 transposition and bacteriophage Mu transposition.

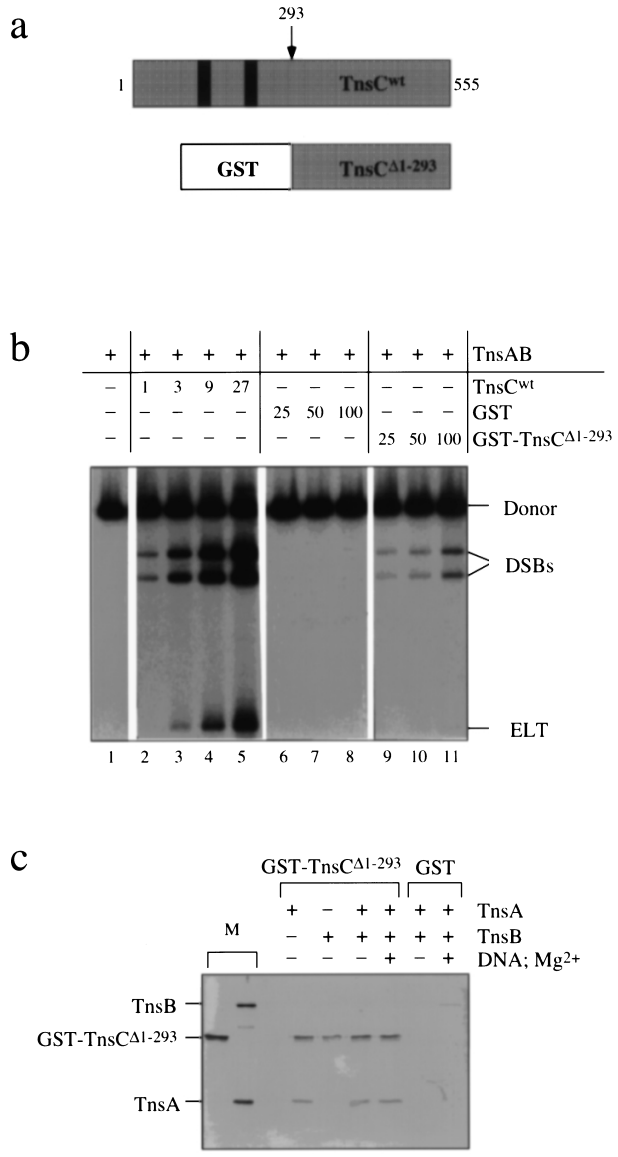

Figure 5. TnsC interacts with the TnsAB transposase. (a) The primary structures of TnsC^{wt} (555 amino acid residues) and GST-TnsC$^{\Delta 1\text{-}293}$ are shown. To construct GST-TnsC$^{\Delta 1\text{-}293}$, the amino-terminal half of TnsC^{wt}, including the ATP-binding motifs (black boxes) was deleted and the remaining carboxy-terminal half of TnsC was fused to a glutathione-*S*-transferase (GST) domain. (b) Donor cleavage activity of intact and truncated TnsC. The conditions of the *in vitro* transposition reaction were modified to uncouple the donor cleavage step from the strand transfer step. TnsAB was present in all reactions; the identities and concentrations of other proteins are indicated above each lane. (c) Protein-protein interactions of GST-TnsC$^{\Delta 1\text{-}293}$. Proteins that coprecipitated with GST-TnsC$^{\Delta 1\text{-}293}$ are identified by SDS-PAGE and Western blotting. Size markers for each protein are included on the gel. The proteins present in each reaction are indicated above each lane; the inclusion of donor DNA and Mg^{2+} is also indicated.

Control of Tn*7* transposition *via* ATP

Tn7 transposition requires ATP: no transposition is observed in the absence of ATP. As TnsC is an ATP-dependent DNA-binding protein, we have hypothesized that TnsC bound to ATP and target DNA exists in an active form that can stimulate transposition, whereas TnsC bound to ADP (or lacking nucleotide) cannot (Gamas & Craig, 1992; Bainton *et al.*, 1993; Stellwagen & Craig, 1997a, 1998). We have shown here that TnsC has an ATPase activity. Thus, ATP binding and hydrolysis may provide a mechanism to switch TnsC between conformations that are active and inactive in promoting transposition. We have also identified a domain of TnsC that can physically and functionally interact with the TnsAB transposase but lacks wild-type TnsC's ATP-binding motifs and DNA-binding activity. We speculate that this domain may normally be buried within the TnsC^{wt} protein structure, and that the exposure of this domain may be governed by the ATP state of the protein.

We have demonstrated here that several gain-of-function TnsC mutants that can promote transposition in the absence of the target selectors TnsD and TnsE have altered ATP-dependent activities. These findings provide support for the view that the ATP state of TnsC is critical in determining its ability to promote transposition. TnsC^{A225V} hydrolyzes ATP less efficiently than does wild-type TnsC, and we have also observed increased stability of TnsC^{A225V}-DNA complexes. An attractive hypothesis is that this mutant's gain-of-function phenotype follows from a stabilization of the TnsC-ATP state that promotes transposition. The TnsC^{A225V} mutation lies very close to the Walker B portion of TnsC'S ATP-binding motif. Several features of this mutant are, however, consistent with retaining some capacity for ATP hydrolysis. In the presence of TnsD and *attTn7*, this mutant efficiently promotes insertion into *attTn7*, a process that occurs only poorly in the presence of a non-hydrolyzable analogue of ATP. Moreover, this mutant still exhibits transposition immunity, i.e. does not insert into DNAs that already contain a copy of Tn7, a process that we have previously shown requires ATP hydrolysis (Stellwagen & Craig, 1997a).

The gain-of-function mutant TnsC$^{S401Y\Delta 402}$, by contrast, appears to be constitutively activated. TnsC$^{S401Y\Delta 402}$ can promote transposition in the presence of either ATP or ADP, suggesting that TnsC$^{S401Y\Delta 402}$ is not changing conformations in response to ATP binding and hydrolysis. TnsC$^{S401Y\Delta 402}$ is also unresponsive to Tn7's target binding proteins: TnsABC$^{S401Y\Delta 402}$ transposition reactions are not targeted to *attTn7* and are not sensitive to target immunity (Stellwagen & Craig, 1997b). Interestingly, the TnsC$^{S401Y\Delta 402}$ mutation lies far from TnsC's ATP-binding motif. We speculate that the amino acid changes in TnsC$^{S401Y\Delta 402}$ enable the protein to assume an active conformation independent of its ATP state.

An attractive hypothesis is that the ATP state of TnsC is not simply stochastically changed, but rather is modulated by the presence of other Tns proteins and particular target DNA. We have suggested elsewhere that a key role of TnsD in recombination may be to stabilize the TnsC-ATP state, leading to the activation of transposition (Stellwagen & Craig, 1998). Moreover, the decreased transposition observed with targets that already contain Tn7, i.e. target immunity, may result from the inactivation of TnsC through ATP hydrolysis prompted by TnsB bound to the Tn7-containing DNA (Stellwagen & Craig, 1997a). The view of the control of transposition *via* a switch between nucleoproteins of different conformation and activity bound to different nucleotides is reminiscient of the GTP binding and hydrolysis switch that controls the conformation of the signal transduction protein Ras (Sprang, 1997). We are attracted to the view that TnsB and TnsD are the functional equivalents of GTPase-activating proteins (GAPs) that stimulate GTP hydrolysis and thus the inactivation of Ras, and GDP-GTP exchange factors (GEFs) that promote the release of GDP and therefore facilitate the return to the GTP-bound conformation (Stellwagen & Craig, 1998). Further work is needeed to probe this model.

Control of Tn*7 versus* Mu transposition

The use of an ATP-utilizing protein to regulate transposition is not unique to Tn*7*. The bacteriophage Mu also encodes an ATPase (MuB) that binds to target DNAs and activates the MuA transposase (Maxwell *et al.*, 1987; Baker *et al.*, 1991; Surette & Chaconas, 1991). Although TnsC and MuB share little primary sequence homology, these proteins have strong functional similarities. MuB, like TnsC, appears to use ATP binding and hydrolysis to bind and release potential target DNAs, thus enabling Mu transposition to achieve some degree of target site specificity (Adzuma & Mizuuchi, 1988). The ability of MuB to switch conformations has also been proposed to facilitate the handoff of the target DNA from MuB to the MuA machinery during the course of the Mu transposition reaction (Yamauchi & Baker, 1998).

The molecular switch of Tn7 is distinct from that of Mu in two important ways. First, the intrinsic activities of the two proteins are different. MuB can activate transposase and direct insertions into a target DNA by itself, whereas $TnsC^{wt}$ needs help from TnsD or TnsE to promote these events. In fact, the gain-of-function mutant $TnsC^{A225V}$ appears to more closely match the activity of MuB than $TnsC^{wt}$.

Second, the number of target DNA inputs processed by the two proteins are different. TnsC and MuB can both respond to the negative signal present on immune targets, but TnsC can uniquely respond to the positive target signals communicated by TnsD and TnsE. As a result, Tn*7* transposition is targeted into *attTn7*, a safe haven in the *E. coli* chromosome (Gringauz *et al.*, 1988), or onto conjugating plasmids, a cell-to-cell transport vehicle (Wolkow *et al.*, 1996). By contrast, Mu transposition events are widely distributed throughout the bacterial chromosome, although suicidal insertions of one copy of Mu into another are avoided. The gain-of-function mutants analyzed in this work demonstrate how easily, by single amino acid residue changes, TnsC can escape the regulation imposed by these target site selectors. The fact that TnsC has not accumulated such mutations over time argues that maintaining Tn7's precise target site selection is advantageous for the spread and the survival of the element.

Materials and Methods

DNA substrates

The donor plasmid pEMΔ contains a 1.6 kb miniTn*7* element (Bainton *et al.*, 1993). The target plasmid pKAO4-3 contains the sequences necessary for *attTn7* target activity, flanked by *Eco*RI sites (McKown *et al.*, 1988). The plasmid pLA11, used in the DNA binding assays, also contains an *attTn7* site (Bainton *et al.*, 1991).

Tns proteins

TnsA was purified as a fusion with glutathione-*S*-transferase (GST) and then released from the GST moiety by thrombin cleavage (May & Craig, 1996). TnsB was TnsB-His_6 (Gary *et al.*, 1996). TnsD was TnsD-His (Sharpe & Craig, 1998).

$TnsC^{wt}$ was expressed and purified essentially as described by Gamas & Craig (1992) with one modification: after ammonium sulfate-precipitation of TnsC, contaminants were removed from the pellet by two extractions with buffer A (25 mM Hepes (pH 7.5), 1 mM EDTA, 2 mM DTT 0.1 M) NaCl + ATP rather than by dialysis. The pellet was then resuspended in TnsC storage buffer (25 mM Hepes (pH 7.5), 1 M NaCl, 2.5 mM DTT, 1 mM ATP, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Chaps and 10 % (v/v) glycerol) as described by Gamas & Craig (1992). The resulting material, designated $TnsC^{wt}$ (fraction IIB), is approximately 95 % pure and appears to be identical in activity with fraction III described by Gamas & Craig (1992) (A.E.S. & N.L.C., unpublished observation). The $TnsC^{A225V}$ and $TnsC^{S401Y\Delta402}$ mutants were purified as described (Biery *et al.*, 2000b).

GST-$TnsC^{\Delta1\text{-}293}$ was constructed by cloning a *Bam*HI-*Eco*RI fragment containing $TnsC^{\Delta1\text{-}293}$ into pGEX1 (Pharmacia). pGEX1 and pGEX1-$TnsC^{\Delta1\text{-}293}$ were each transformed into the protease-deficient strain CAG456 (Baker *et al.*, 1984). Cells were grown at 30 °C in LB plus 100 μg/ml carbenicillin until an A_{600} of 0.3 was reached. IPTG (40 μM) was then added and the cells were allowed to double once more. The cells were harvested by centrifugation and washed once in buffer B (25 mM Hepes (pH 7.5), 1 mM EDTA, 2 mM DTT). The cells were then resuspended in buffer B containing 500 mM NaCl and lysed by sonication. Chaps (10 mM) was then added, the cell debris was removed by centrifugation for 25 minutes at 13,000 rpm, and the resulting cleared lysate was incubated with one-fifth the volume of a 50 % slurry of glutathione/agarose beads (Sigma) for one hour at 4 °C, with rocking. The beads were collected by

centrifugation for three minutes at 1000 rpm and washed twice with buffer B containing 500 mM NaCl and 10 mM Chaps. The beads were then incubated with GEX elution buffer (25 mM Hepes (pH 7.5), 1 mM EDTA, 0.5 mM NaCl, 2.5 mM DTT, 10 mM Chaps, 10 mM $MgCl_2$, 10 % glycerol, 10 mM glutathione) for 30 minutes at 4 °C, with rocking, and the eluted GST proteins were collected by centrifugation. Two rounds of elution were performed, and the supernatants were combined and stored at −80 °C. GST-TnsC$^{\Delta 1\text{-}293}$ is approximately 90 % pure.

Tn*7* transposition *in vitro*

The standard transposition reaction (100 µl) contained 0.25 nM donor DNA (pEMΔ), 2.5 nM target DNA (pKAO4-3), 26 mM Hepes (pH 7.5), 4.2 mM Tris (pH 7.5), 2.1 mM DTT, 60 µg/ml bovine serum albumin (BSA), 0.05 mM EDTA, 0.1 mM $MgCl_2$, 0.1 mM Chaps, 12 mM NaCl, 22 mM KCl, 1.2 % glycerol, 4 µM PMSF, 2 mM ATP and 15 mM MgAc unless otherwise indicated. Tns protein was added to the following final amounts: 60 ng of TnsA, 25 ng of TnsB, 40 ng of TnsC^{wt} (or TnsC^{A225V} or TnsC$^{S401Y\Delta 402}$) and (when present) 22 ng of TnsD. The standard reaction was staged as follows: TnsC and TnsD were preincubated with the target DNA for 20 minutes at 30 °C. Generally, ATP was present in the preincubation and MgAc was not. The donor DNA, TnsA, TnsB, and MgAc were then added and the incubations were continued for another 20 minutes. The transposition reactions were stopped by the addition of phenol/chloroform; the DNA molecules were extracted and collected by ethanol-precipitation. The DNA molecules were analyzed by restriction enzyme digestion (*Nde*I unless otherwise indicated) and gel electrophoresis, followed by Southern transfer to Gene Screen Plus (NEN Research Products) and hybridization with a mTn*7*-specific probe, as described by Sarnovsky *et al.* (1996). Blots were analyzed by autoradiography using Kodak BioMax MR film or by a Molecular Dynamics Phosphorimager.

In Figure 5, the standard transposition reaction was modified to focus on the donor cleavage step. These reactions (100 µl) contained 0.25 nM donor DNA (pEMΔ), 0.9 mM Hepes (pH 7.5), 20 mM Tris (pH 7.5), 2.1 mM DTT, 60 µg/ml BSA, 0.02 mM EDTA, 0.1 mM $MgCl_2$, 0.1 mM Chaps, 12 mM NaCl, 5 mM KCl, 4 µM PMSF, 5 mM MgAc and 15 % glycerol. The reactions also included 60 ng of TnsA, 50 ng of TnsB, and various amounts of GST, GST-TnsC$^{\Delta 1\text{-}293}$, or TnsC. The GST proteins contributed 0.1 mM glutathione to the final reaction composition, while the TnsC protein brought in 10 µM ATP. These modified transposition reactions were not staged; instead, all components were incubated together for 30 minutes at 30 °C; the reactions were then stopped and analyzed as described above.

DNA-binding assays

DNA-binding assays were adapted from those described by Gamas & Craig (1992). Plasmid pLA11 was linearized with *Afl*III and end-labelled using [^{35}S]dCTPαS and the Klenow fragment. Reactions (100 µl) contained 5 ng of ^{35}S-labelled DNA and 20 ng of TnsC, along with 25 mM Hepes (pH 7.5), 2 mM DTT, 50 µg/ml BSA, 100 mM KCl, 7.5 % (w/v) sucrose and 250 µM ATP (or AMP-PNP). In Figure 4(a), the reactions also contained various concentrations of Mg^{2+}. Reactions were incubated for 15 minutes at 30 °C and then filtered through 0.45 µm nitrocellulose filters (Millipore) using a multiport vacuum filtration device (Hoeffer). The filters were washed three times with 0.33 ml of binding buffer, then dried and subjected to scintillation counting. In Figure 4(c), this protocol was modified to do pulse-chase studies. First, TnsC was incubated with the radiolabelled DNA substrate as described above. After 15 minutes, a 500-fold excess of cold competitor DNA (sonicated herring sperm DNA) was added to the binding reactions and the incubations were continued for various times. The reactions were then filtered as described above.

Size-exclusion chromatography

TnsC was fractionated over a 25 ml Superose 12 size-exclusion column (Pharmacia). The column was equilibrated with TnsC column running buffer (25 mM Hepes (pH 7.5), 0.1 mM EDTA, 1 M NaCl, 2.5 mM DTT, and 10 mM $MgCl_2$) at a flow rate of 0.5 ml/minute. A 100 µl sample containing TnsC (fraction IIB, approximately 1.5 mg/ml) was applied to the column, and 0.2 ml fractions were collected. The protein concentration of each fraction was determined by absorbance; the presence of TnsC was confirmed by SDS-PAGE and silver staining. Fractions were supplemented with 10 mM Chaps and 10 % glycerol prior to analysis or storage at −80 °C.

ATP hydrolysis assay

Reactions (100 µl) contained 25 mM Hepes (pH 7.5), 2 mM DTT, 50 µg/ml BSA, 7.5 % sucrose, 6 nM [α^{32}P]ATP and 2 µM cold ATP. Reactions shown in Figure 2(a) also contained 50 mM NaCl, 0.5 mM $MgCl_2$ and 0.5 mM Chaps, while the reactions in Figure 2(b) contained 10 mM NaCl, 0.1 mM $MgCl_2$ and 0.1 mM Chaps and a total of 10 µM cold ATP. TnsC was added as indicated. Reactions were incubated for two hours at 30 °C and analyzed by thin-layer chromatography, using PEI-cellulose TLC plates (EM Science) and 0.75 M KH_2PO_4 (pH 3.5) as a running buffer. The amount of ATP hydrolysis was quantified by Phosphorimager analysis.

GST-TnsC$^{\Delta 1\text{-}293}$ pulldown assay

Reactions (90 µl) were performed in a buffer similar to the Tn*7* transposition buffer: 26 mM Hepes (pH 7.5), 2.1 mM DTT, 60 µg/ml BSA, 7 mM NaCl, 6 mM KCl, 3 µM PMSF, 0.1 mM $MgCl_2$, 0.1 mM Chaps, 0.5 % Triton X-100, 0.02 mM EDTA and 0.4 % glycerol. Where indicated, reactions also contained 100 ng pEMΔ donor DNA, 5 mM MgAc, 69 µg TnsA, 50 µg of TnsB and 150 µg of GST or GST-TnsC$^{\Delta 1\text{-}293}$. After a 20 minute incubation at 30 °C, glutathione/agarose beads (20 µl of a 50 % slurry) were added and the reactions were incubated for a further 30 minutes at room temperature, with rocking. The beads were collected by centrifugation (2000 rpm for two minutes) and washed once with 50 µl of reaction buffer. Proteins were released by resuspending the beads in 30 µl of urea-SDS gel-loading buffer and boiling for five minutes. Recovered proteins were identified by SDS-PAGE and Western analysis, using polyclonal antisera to TnsA, TnsB and TnsC and alkaline phosphatase-conjugated secondary antibodies (BioRad).

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