Avoiding self: two Tn7-encoded proteins mediate target immunity in Tn7 transposition

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The bacterial transposon Tn7 exhibits target immunity, a process that prevents Tn7 from transposing into target DNAs that already contain a copy of the transposon. This work investigates the mechanism of target immunity in vitro. We demonstrate that two Tn7-encoded proteins—TnsB, which binds specifically to the ends of Tn7, and TnsC, the ATP-dependent DNA binding protein—act as a molecular switch to impose immunity on target DNAs containing Tn7 (or just Tn7 ends). TnsC binds to target DNA molecules and communicates with the Tn7 transposition machinery; here we show that target DNAs containing Tn7 ends are also bound and subsequently inactivated by TnsB. Protein–protein interactions between TnsB and TnsC appear to be responsible for this inactivation; the target DNA promotes these interactions by tethering TnsB and TnsC in high local concentration. An attractive model that emerges from this work is that TnsB triggers the dissociation of TnsC from the Tn7 end-containing target DNA; that dissociation depends on TnsC’s ability to hydrolyze ATP. We propose that these interactions between TnsB and TnsC not only prevent Tn7 from inserting into itself, but also facilitate the selection of preferred target sites that is the hallmark of Tn7 transposition.

Keywords: ATP binding protein/DNA binding/molecular switch/protein–DNA interaction/transposition

Introduction

DNA molecules are the substrates for a variety of processes, including replication, transcription and recombination. Many positive regulatory mechanisms have been described which favor the selection of certain DNAs as substrates for one of these processes. In some situations, however, the positive features of a DNA can be overridden by negative regulatory mechanisms. For example, some transcriptional units are ‘silenced’ by being packaged within specialized chromatin structures which prevent interactions with RNA polymerase (Loo and Rine, 1995). Eukaryotic origins of replication also become ‘silent’ after initiating DNA replication, as part of the cell cycle control that permits an origin to fire once and only once during S phase (Muzi-Falconi et al., 1996; Wuarin and Nurse, 1996).

Negative regulatory mechanisms can also influence the selection of target sites in transposition. The bacterial transposons Tn5 and Tn7 and the bacteriophage Mu are sensitive to a process called target immunity, in which a target molecule that already contains a copy of one of these elements is prevented from receiving further insertions of that element (Robinson et al., 1977; Hauer and Shapiro, 1984; Reyes et al., 1987). Thus, the target DNA becomes ‘silent’ or ‘immune’ to the transposition machinery.

The signal that confers immunity to a target DNA is provided by the ends of the resident Tn3, Tn7 or Mu element. Transposon ends contain special sequences that are the substrates for the DNA breakage and joining reactions that move the element from one DNA molecule to another; transposon ends also contain binding sites for transposase, the enzyme which executes the DNA breakage and joining reactions (reviewed by Mizuuchi, 1992). The presence of Tn3, Tn7 or Mu ends in a target plasmid reduces the frequency of transposition into that plasmid 100- to 1000-fold in vivo; however, transposition into other target molecules which do not contain transposon ends is not inhibited (Lee et al., 1983; Darzins et al., 1988; Arciszewska et al., 1989). Therefore, target immunity is essentially a cis-acting phenomenon that prevents new insertions from occurring ‘close’ to transposon ends.

How close is ‘close’? In the case of Tn7, large (60 kb) derivatives of the Escherichia coli F plasmid are protected from Tn7 transposition when the plasmid contains Tn7 end sequences (Arciszewska et al., 1989). Transposition is inhibited over even larger distances in the E.coli chromosome: the presence of Tn7 ends was shown to reduce insertions into chromosomal sites 190 kb away (DeBoy and Craig, 1996). However, transposition into a target site 1.9 Mb from the Tn7 ends was not affected, demonstrating that the Tn7 ends do not cause a global inhibition of transposition (DeBoy and Craig, 1996).

The ability to discriminate between targets that are ‘close’ and ‘far’ from Tn7 ends may be useful in promoting the spread and survival of Tn7. Short-range transposition events would be discouraged; instead, the spread of the transposon to distant sites in the chromosome and new plasmids would be favored. Immunity would also discourage events that could potentially destroy Tn7, such as intramolecular transposition events or the hopping of one copy of Tn7 into another. Thus, target immunity plays a key role in determining what target sites the Tn7 transposition machinery will select.

The Tn7 transposition machinery also evaluates a potential target DNA for positive features. Tn7 transposition occurs at high frequency into a single site in the E.coli chromosome called attTn7 (Barth et al., 1976; Lichtenstein and Brenner, 1982). Plasmids undergoing conjugation are also preferred targets for Tn7 transposition (Wolkow et al., 1996). Thus, attTn7 and conjugating plasmids contain positive signals that attract the transposition machinery to
these target DNAs. Different combinations of the Tn7-encoded proteins TnsA, TnsB, TnsC, TnsD and TnsE are used to select these different targets: TnsABC+D promotes transposition into \textit{attTn7}, whereas TnsABC+E promotes transposition into conjugating plasmids (Rogers et al., 1986; Waddell and Craig, 1988). Target immunity is observed in both the TnsABC+D and TnsABC+E transposition pathways (Arciszewska et al., 1989), suggesting that the negative signal provided by a Tn7 end is dominant to the positive signals which might also be contained on a potential target molecule.

Tn7 transposition into \textit{attTn7} has been reconstituted \textit{in vitro} using purified proteins (Bainton et al., 1993), and the roles of the Tns proteins in executing Tn7 transposition have been investigated. TnsA and TnsB act interdependently to catalyze the chemical steps of Tn7 transposition, thus TnsA+B constitutes the Tn7 transposase (May and Craig, 1996; Sarnovsky et al., 1996). TnsB binds specifically to the transposon ends (Arciszewska and Craig, 1991; Arciszewska et al., 1991; Tang et al., 1991), while TnsA is likely recruited to the transposon ends through protein–protein interactions with TnsB.

The TnsA+B transposase by itself is not catalytically active; TnsC, TnsD and an appropriate target DNA are also required (Bainton et al., 1993; Gary et al., 1996). TnsD is an \textit{attTn7}-specific DNA-binding protein which recruits TnsC, an ATPase that is also an ATP-dependent DNA-binding protein, to \textit{attTn7} targets. TnsC–TnsD–\textit{attTn7} complexes, in turn, interact with the TnsA+B transposase and activate its breakage and joining activities (Gamas and Craig, 1992; Bainton et al., 1993; A.Stellwagen and N.L.Craig, in preparation). TnsC has been proposed to be a key connector between the target site and the TnsA+B transposase, and the ATP state of TnsC is hypothesized to regulate its ability to forge that connection (Bainton et al., 1993, Stellwagen and Craig, 1997).

Tn7 transposition occurs by a cut-and-paste mechanism, in which the element is first excised from a donor site and then inserted into a target DNA (Bainton et al., 1991). The nature of the target DNA regulates both of these steps \textit{in vitro}: if an \textit{attTn7} target molecule is omitted from the reaction, virtually no transposition intermediates or products are seen. Target immunity is reproduced in the \textit{in vitro} Tn7 transposition reaction (Bainton et al., 1991, 1993); the evaluation of Tn7 end-containing targets also occurs early in the course of the reaction. No transposition products or intermediates are observed when the target DNA contains \textit{attTn7} but also carries a Tn7 right end. Therefore, Tn7 end-containing target DNAs are immune to Tn7 transposition not because they fail to capture excised transposons, but because they fail to provoke the excision of the transposon in the first place.

\textit{In vitro} approaches have been previously used to investigate target immunity in Mu transposition. Adzuma and Mizuuchi (1988, 1989) demonstrated that Mu target immunity results from the redistribution of the regulatory protein MuB from target DNAs containing Mu ends to target DNAs without ends. This redistribution is promoted by the MuA transposase, and requires ATP hydrolysis. However, it has been unclear whether this mechanism would be unique to Mu or whether it would apply to other transposons. In particular, it has been unclear how Tn7—

\begin{figure}[h]
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\caption{The Tn7 transposition pathway. The mTn7 element is excised from the donor substrate (solid line) by double-strand breaks and then joined to the target DNA (dashed line) to generate a simple insertion. Donor molecules which 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). The \textit{cis}-acting recombination sequences at the transposon ends are indicated by an open triangle (left end) and a closed triangle (right end). The positions of Ndel (N) sites in the donor and target plasmids are also indicated.}
\end{figure}

with its multiple proteins and multiple target selection pathways—might adapt this immunity mechanism.

In this work, we have investigated the mechanism of Tn7 target immunity. We find that the key proteins responsible for Tn7 target immunity are TnsB, the transposon end-binding protein, and TnsC, the ATP-dependent target DNA-binding protein. When TnsB and TnsC are in high local concentration (i.e. when both are localized to a Tn7 end-containing target DNA), immunity is imposed on that target. An attractive model that emerges from this work is that TnsB promotes the dissociation of TnsC from Tn7 end-containing target DNAs, through an ATP-dependent mechanism. Thus, TnsB appears to impose target immunity by influencing the distribution of TnsC among potential target DNAs. We discuss the similarities between the mechanisms by which Tn7 and Mu achieve target immunity, and we discuss how Tn7 exploits this mechanism not only to avoid immune targets but also to select preferred targets for Tn7 transposition.

\section*{Results}

\textbf{Tns proteins involved in target use and target immunity}

Tn7 transposition has been reconstituted \textit{in vitro} with TnsA, TnsB, TnsC, TnsD and two required cofactors, ATP and Mg\textsuperscript{2+} (Bainton et al., 1993). These Tns proteins and cofactors direct the transposition of a mini-Tn7 element from a donor plasmid to an \textit{attTn7}-containing target plasmid (Figure 1). The mini-Tn7 element (mTn7) contains an antibiotic resistance cassette flanked by functional Tn7 end sequences. Transposition proceeds via a cut-and-paste mechanism, in which the element is excised from the donor site by double-strand breaks at the transposon ends and then joined to the target molecule to generate a simple insertion.

The assembly of Tns proteins with the \textit{attTn7} target DNA is a critical step in Tn7 transposition. Previous work has demonstrated that the \textit{attTn7}-specific DNA-binding protein TnsD and the ATP-dependent DNA-binding protein TnsC form a complex on the target DNA which can then interact with the TnsA+B transposase to trigger the initiation of transposition (Bainton et al., 1993). However,
with and without Tn7 target complexes form very inefficiently in the presence of Mg\(^{2+}\). Therefore, Tn7 transposition reactions are staged: the proteins and DNAs are first incubated under low Mg\(^{2+}\) conditions to promote target complex assembly (a step called the ‘preincubation’), then Mg\(^{2+}\) is added to initiate transposition. No additional target complexes are formed after the addition of Mg\(^{2+}\). Only the components of the target complex—TnsC, TnsD, ATP and the attTn7 target DNA—need to be present during the low Mg\(^{2+}\) preincubation; the presence of the donor DNA, TnsA or TnsB at this step is not required (Bainton et al., 1993).

Having established which Tns proteins are involved in target use, our first goal in this work was to determine which Tns proteins are involved in target immunity. We carried out order-of-addition experiments which were staged somewhat differently than the reactions described above: both the donor and target DNAs were separately preincubated with Tns proteins and then combined to initiate transposition (Figure 2A). The donor DNA was always preincubated with the TnsA+B transposase and Mg\(^{2+}\). By contrast, the proteins present during the target preincubation were varied. Any protein(s) omitted from the target preincubation were added when the donor and target preincubations were combined, so that the final composition of each reaction was the same but the proteins present at the time of target complex assembly were different.

Target immunity was observed when all four Tns proteins were preincubated with the target DNAs: 50-fold fewer insertions occurred into the target DNA containing a Tn7 right end than the target lacking Tn7 ends (Figure 2B, lane 1). However, immunity was severely compromised when TnsC and TnsD were the only proteins present in the target preincubation: a 20-fold increase in insertions into the Tn7 end-containing target was seen (Figure 2B, lane 2). Including TnsB in the target preincubation fully restored the discrimination between targets with and without Tn7 ends (lane 4); the presence of TnsA in the target preincubation was not required (lane 3). Consistent with the results of Bainton et al. (1993), omitting TnsC or TnsD from the target preincubation eliminated all insertion events (Figure 2B, lanes 5 and 6). Similarly, omitting the target DNA without ends from the target preincubation also blocked transposition; this target could not be utilized when it was added after the preincubations were combined (lane 7). Taken together, these results suggest that although TnsC and TnsD are important for target use, they are not sufficient to discriminate between Tn7 DNAs with and without Tn7 ends. TnsB, the transposon end-binding protein, must also be present during the assembly of target complexes for Tn7 end-containing targets to be recognized and avoided.

These observations reveal that TnsB plays multiple roles in Tn7 transposition. As part of the TnsA+B transposase, TnsB is involved in promoting insertions into target DNAs. In fact, mutational analysis suggests that the active site responsible for target joining is likely in TnsB (Sarnovsky et al., 1996). However, the experiment above suggests that TnsB is also involved in preventing insertions from occurring into target DNAs which contain Tn7 ends. This ‘regulatory’ activity of TnsB does not appear to require TnsA, in contrast to the ‘catalytic’ activity of TnsB which is dependent on the presence of TnsA. We will argue below that TnsB confers immunity to a target DNA by promoting the removal of TnsC from the target and thus reducing the target’s ability to interact with the transposase.

The data in Figure 2B suggest that the time at which TnsB is introduced into the transposition reaction influences whether TnsB’s regulatory or catalytic role predominates. Strong target immunity was established when TnsB was able to exert its regulatory effects prior to the initiation of Tn7 transposition. By contrast, when TnsB’s
addition was simultaneous with the initiation of transposition, considerable insertion occurred into Tn7 end-containing targets, suggesting that TnsB was interacting with many of these targets catalytically. Even under these conditions, however, the targets with and without Tn7 ends were not used equivalently: 2- to 3-fold fewer insertions were seen into targets containing Tn7 ends than into targets lacking Tn7 ends. This unequal target use may indicate that even as some Tn7 end-containing targets were being utilized by TnsA + B, others were being inactivated by TnsB. Additional experiments probing the time requirements for establishing target immunity are described below.

**TnsD is not essential for Tn7 target immunity**

Tn7 transposition in vitro is dependent on TnsD for successful interactions with target DNA. Previous work has demonstrated that TnsD not only provides the attTn7 specificity of the Tn7 transposition reaction but also activates the wild-type TnsABC machinery (Bainton et al., 1993). Recently, gain-of-function mutations in TnsC have been identified which enable the TnsABC machinery to execute transposition in the absence of TnsD (Stellwagen and Craig, 1997). In this work, we have exploited the gain-of-function mutant TnsC<sub>A225V</sub>. Transposition promoted by TnsABC<sub>A225V</sub> occurs into many different target sites in vitro, yet responds to target signals and target binding proteins much like wild-type Tn7 transposition (Stellwagen and Craig, 1997).

We examined target discrimination in reactions containing TnsC<sub>A225V</sub> in vitro (Figure 2B, lanes 8–11). Although these reactions lacked TnsD, target immunity was still observed: 30-fold fewer insertions were seen into target DNAs containing Tn<sub>7</sub> ends than into target DNAs lacking Tn<sub>7</sub> ends when TnsA, TnsB and TnsC<sub>A225V</sub> were preincubated with the target DNAs (lane 8). Once again, target immunity was not effectively imposed when TnsB was omitted from the target preincubation (Figure 2B, lanes 9 and 10). However, preincubating the target DNAs with TnsB and TnsC<sub>A225V</sub> was sufficient to allow target immunity to be established (lane 11). These experiments suggest that TnsC<sub>A225V</sub> promotes transposition into target DNAs in general, and that TnsB specifically discourages transposition from occurring into target DNAs containing Tn<sub>7</sub> ends. TnsD, therefore, is not essential for Tn<sub>7</sub> end-containing target DNAs to be recognized and avoided. While it is possible that TnsD contributes to the immunity response in the wild-type Tn<sub>7</sub> transposition reaction, we propose that TnsB and TnsC—the transposon end-binding protein and the ATP-dependent target DNA-binding protein—undergo the primary interactions that are responsible for Tn<sub>7</sub> target immunity.

The insertions generated in TnsABC<sub>A225V</sub> reactions without TnsB in the target preincubation were located in many different positions in the Tn<sub>7</sub> end-containing target plasmid (data not shown). Including TnsB in the target preincubation reduced the level of insertions into all of those positions. Thus, Tn<sub>7</sub> target immunity is not restricted to attTn<sub>7</sub> sites in vitro, but is conferred to the entire Tn<sub>7</sub> end-containing plasmid.

**TnsB binding sites comprise the DNA signal that confers immunity to a target plasmid**

The experiments above confirm the observations of Bainton et al. (1991) that a Tn<sub>7</sub> right end contains the DNA signal(s) necessary for immunity to be imposed on a target plasmid. We investigated which features of the Tn<sub>7</sub> right end are responsible for the immunity signal, and whether that signal is also present on the Tn<sub>7</sub> left end. The left and right ends of Tn<sub>7</sub> are structurally distinct (Figure 3A). Approximately 160 nucleotides from the left end and 90 nucleotides from the right end are required for efficient transposition (Arciszewska et al., 1989; DeBoy and Craig, 1996). These sequences contain arrays of TnsB binding sites, with three sites on the left end and four overlapping sites on the right end (Arciszewska and Craig, 1991; Arciszewska et al., 1991; Tang et al., 1991). The terminal eight nucleotides of each end, along with the outermost TnsB binding site, form a 30 bp inverted repeat (Lichtenstein and Brenner, 1982; Arciszewska et al., 1991); the CA-3′-OH dinucleotide at the tip of each end is critical for cleavage and joining events to occur (Gary et al., 1996).
Plasmids containing different segments from either the right or left end of Tn7 were examined in the TnsC^{A225V}
activated transposition reaction (Figure 3B); the same general principles have also been observed in reactions
with TnsC^{+D} (data not shown). The ability of each plasmid to serve as a target for Tn7 transposition was
tested under two conditions: (i) without TnsB in the target preincubation, to evaluate the plasmid’s intrinsic target
activity; and (ii) with TnsB included in the target preincubation, to see whether the target activity changes
when immunity can operate. Thus, controlling the presence or absence of TnsB during the target assembly period
allowed immunity to be switched ON and OFF. A target plasmid without Tn7 end sequences was also included in
each reaction as a control for transposition efficiency.

We found that the presence of either the right end or the left end of Tn7 in a target plasmid enabled immunity
to be imposed on that plasmid: insertions into targets containing R1–199 sequences or L1–166 sequences
decreased 13- to 20-fold when immunity was switched ON by including TnsB in the target preincubation (Figure
3B, compare lanes 1 and 2 with lanes 3 and 4). A truncated right end was also tested, in which the 30 bp terminal
inverted repeat was removed but three TnsB binding sites were retained. Although this R38–199 end would not be
predicted to support transposition when part of a mTn7
donor, R38–199 sequences could nonetheless confer immunity when present in a target DNA (Figure 3B, lanes
5 and 6). Even a single TnsB binding site in a target DNA
was found to affect that target’s activity (lanes 7 and 8). Although the transposition efficiencies of the reactions in
lanes 7 and 8 were not identical, the activity of the target DNA containing the TnsB binding site was relatively
more affected (6-fold rather than 2-fold) by the presence of TnsB than was the target DNA without the TnsB site.
Taken together, these observations suggest that TnsB binding sites provide the critical signal which allows
immunity to be imposed on a target DNA. Additional features of Tn7 ends, such as the inverted repeat sequences
at the tips of the element or the synaptic structure that can form with a pair of Tn7 ends, are thus important for
catalysis but are not required for immunity. Although it remains possible that these features may contribute to the
efficiency with which immunity is imposed, the binding of TnsB to a potential target DNA appears to be the
primary event which renders that target immune.

**Communication between TnsB and TnsC**

Target immunity is an example of ‘action at a distance’: a Tn7 end can inhibit insertion into an **attTn7** site many
kilobases away in the case of plasmid targets (Arciszewska
et al., 1989), or even hundreds of kilobases away in the
bacterial chromosome (DeBoy and Craig, 1996). Our
working hypothesis is that an interaction between TnsB,
bound to the transposon end, and TnsC, bound near a
potential insertion site, is important in establishing target
immunity. Various models have been proposed to account
for long-distance interactions between proteins bound to
different DNA sites, such as enhancers and promoters
(reviewed by Wang and Giaever, 1988); those same models
can be considered to explain how TnsB and TnsC might
find each other on a Tn7 end-containing target DNA. One
class of models would propose that TnsB and TnsC
communicate along the DNA. For example, TnsB could
bind to its recognition site and nucleate an inhibitory
structure that could spread down the DNA, inactivating
or displacing TnsC; alternatively, TnsC could use its
ATPase activity to track along the DNA, scanning for
TnsB molecules. A second class of models would propose
that TnsB and TnsC communicate through space via
protein–protein interactions; those interactions would
occur more frequently when the two proteins are both
bound to a Tn7 end-containing target DNA and thus are
in relatively high local concentration.

A classic experiment to differentiate between commu-
nication through space and communication through the
DNA is to put the binding sites of interest on separate
but tethered DNA molecules, namely catenated circles.
Such an experiment was first done to probe the disposition
of DNA sites required for target immunity in Mu transposi-
tion (Adzuma and Mizuuchi, 1989). Here we investigate
whether immunity from Tn7 transposition can be conferred
to a target plasmid that is catenated to (but not continuous
with) a plasmid containing a Tn7 end. These experiments
were performed with TnsABC^{+D}, to take advantage of
the target site-specificity of this reaction.

To generate a target substrate for this experiment, the
right end of Tn7 and an **attTn7** sequence were cloned into
a plasmid containing two recognition sites for f6 resolvase
(Figure 4A). Recombination of the resulting plasmid
generated two catenated circles, one containing the Tn7
end (which would bind TnsB) and the other containing the
**attTn7** site (to which TnsC will be bound in the
presence of TnsD). In our hands, ~50% of the DNA
molecules became catenated (data not shown). The mixture
of DNAs from the catenation reaction was used as targets
for Tn7 transposition, allowing us to evaluate the target
immunity of the unrecombined plasmid (in which **attTn7**
and the Tn7 end were present on the same DNA molecule)
and the catenated plasmid (in which **attTn7** and the Tn7
end were present on separate but tethered DNA circles).

As expected, the unrecombined **attTn7** + end’ plasmid
was found to be immune; insertions into the ‘**attTn7** + end’ target were reduced when immunity was switched
ON by including TnsB in the target preincubation (Figure
4B, compare lanes 2 and 3, upper arrow). Interestingly,
insertions into the **attTn7** circle catenated to the Tn7 end-
containing circle were also affected when immunity was
switched ON: 10-fold fewer insertions were seen into the
catenated **attTn7** circle when TnsB was present in the
target preincubation (Figure 4B, lanes 2 and 3, lower
arrow). This result suggests that a Tn7 end can impose
immunity on an **attTn7** plasmid that is tethered ‘close’ to
that end.

If the proximity of the Tn7 end (and thus TnsB) to the
**attTn7** target site is important in conferring immunity to
that target, then unlinking the **attTn7** plasmid from the
end-containing plasmid would be predicted to relieve the
immunity effect. The **attTn7** circle was released from the
Tn7 end-containing circle by treating the products of the
catenation reaction with the restriction enzyme NdeI
(Figure 4A). The unlinked **attTn7** target plasmid was no
longer found to be immune: insertions accumulated into
the unlinked **attTn7** target regardless of whether TnsB
was present or absent from the target preincubation (Figure
4B, compare lanes 4 and 5, lower arrow). This result is
Fig. 4. Immunity can be conferred on a target plasmid catenated to a Tn7 end-containing plasmid. (A) Generating the catenated target substrates. The ‘attTn7 + end’ plasmid PRCAT2 contains a Tn7 right end (▼) and an attTn7 target sequence (grey box) between two recognition sites (res) for NdeI resolvase. Resolvase-catalyzed recombination of PRCAT2 yields two catenated circles, one containing attTn7 and the other containing the Tn7 end. Treatment of the catenated product with NdeI (N) linearizes the attTn7 circle but leaves the Tn7 end-containing circle intact. (B) Transposition reactions. Reactions were staged as cartooned in Figure 2A. 50 ng of a target without ends (pRM2) were present in each reaction, along with ~100 ng of each of the ‘attTn7 + end’ and ‘recombined attTn7’ targets. Transposition was evaluated with supercoiled target DNAs (lanes 1–3) or NdeI-linearized target DNAs (lanes 4–5). However, all DNAs were digested with NdeI prior to electrophoresis. Therefore, insertions into the catenated attTn7 target and the unlinked attTn7 target migrate to the same position in the gel. Target preincubations contained 1× TnsC and 1× TnsD (lanes 2–5); 0.5× TnsB was added to the target preincubation (lanes 3 and 5) or added after the donor and target preincubations were combined (lanes 2 and 4). Reactions in lane 1 contained no Tns proteins. Donor preincubations were as described for Figure 3. The upper arrow indicates insertions into the ‘attTn7 + end’ target, the lower arrow indicates insertions into the catenated attTn7 target or the unlinked attTn7 targets. Cartoons of these insertion products are also provided.

consistent with the hypothesis that TnsB must be in close proximity to a target DNA to impose immunity on that target.

It should be noted that the unlinked attTn7 targets have undergone two changes at once: they are no longer tethered to the Tn7 end, but they are also no longer supercoiled. Although it could be argued that both changes contribute to the loss of immunity of the unlinked attTn7 circle, other experiments have established that DNA supercoiling is not strictly required for immunity to be imposed on Tn7 end-containing target DNAs (data not shown). However, supercoiling may influence the effectiveness with which TnsB and TnsC interact. We consistently observe that the 4.8 kb ‘attTn7 + end’ plasmid is less immune as a linear than as a supercoiled molecule (Figure 4B), although smaller (2–3 kb) plasmids are essentially equally immune when supercoiled or linearized (data not shown).

We interpret these results to suggest that the proteins responsible for target immunity (i.e. TnsB and TnsC) interact by random collision through space. Thus, the Tn7 end-containing target DNA does not appear to be involved in propagating a signal between TnsB and TnsC. Instead, the DNA likely serves to increase the concentration of TnsB in the vicinity of TnsC.

TnsB can inactivate preassembled complexes on Tn7 end-containing targets

In all of the experiments presented above, target immunity is most effectively imposed when TnsB is present in the target preincubation. Why is the timing of TnsB’s addition so critical? It is possible that the establishment of target immunity requires a specific order of events. For example, TnsB might need to bind to the target DNA before TnsC (particularly if TnsB’s mode of action were to block the binding of TnsC to Tn7 end-containing targets). On the other hand, the order of events might not be critical, but some time might be required for TnsB to exert its effects.

To investigate these issues, we asked whether TnsB could impose immunity on target DNAs that had been preassembled with TnsC, and what the time course of that inactivation would be. These experiments were performed with the TnsC<sub>A225V</sub> mutant, to focus on the interactions of TnsB and TnsC in the absence of TnsD; however, we have observed similar results in reactions with TnsC+D (see below). The staging of the transposition reaction was altered (Figure 5A): instead of adding TnsB and TnsC<sub>A225V</sub> simultaneously to the target preincubation, TnsC<sub>A225V</sub> was added first. After 20 min, TnsB was added and the target preincubations were continued for various times. Transposition was then initiated by adding the donor preincubation mix. The relative amount of insertion into the Tn7 end-containing target was determined by Southern blotting and phosphorimager analysis; this ‘target reactivity’ is presented graphically as a function of the time the preincubation was extended upon the addition of TnsB.

The reactivity of Tn7 end-containing targets fell sharply when TnsB was added to the target preincubation (Figure 5B, filled circles), suggesting that TnsB can functionally inactivate the preassembled TnsC<sub>A225V</sub>–target complexes. However, the effects of TnsB were not instantaneous: the degree of target inactivation increased with increasing time of exposure to TnsB. As a control for the stability of the target complexes, the preincubations of an identical set of reactions were extended without the addition of TnsB. Under these conditions, the Tn7 end-containing target DNAs maintained at least 80% of their activity (Figure 5B, open circles). This result suggests that the binding of TnsC<sub>A225V</sub> to target DNA is fairly stable in the absence of TnsB, a hypothesis that has been verified by pulse–chase analysis (A.Stellwagen and N.L.Craig, in preparation). However, the presence of TnsB affects the reactivity of the TnsC<sub>A225V</sub>–target DNA complexes over...
The ability of TnsB to inactivate the Tn7 end-containing target DNA complexes was also found to be affected by the type of ATP nucleotide present in the reaction. TnsC is an ATP-dependent DNA-binding protein with a modest ATPase activity (Gamas and Craig, 1992; A.Stellwagen and N.L.Craig, in preparation). Non-hydrolyzable ATP analogs are known to increase TnsC's affinity for DNA (Gamas and Craig, 1992) and to compromise immunity in TnsABC+D transposition reactions (Bainton et al., 1993). Here, we found that TnsB was much less successful at imposing immunity on Tn7 end-containing complexes that were assembled in the presence of the non-hydrolyzable analog AMP–PNP (Figure 5C). The reactivity of the Tn7 end-containing target dropped only 2-fold upon prolonged (30 min) exposure to TnsB (Figure 5B, filled squares), in contrast to the 11-fold loss of activity seen in reactions with ATP (Figure 5B, filled circles). These results suggest that the interactions between TnsB and TnsC that confer immunity to a target DNA involve TnsC’s ability to hydrolyze ATP.

TnsB can also impose immunity on target DNAs preassembled with TnsC+D (data not shown). The rates of target inactivation were similar in reactions with Tn7 end-containing TnsABC+D and non-hydrolyzable ATP analogs severely reduced the effects of TnsB in both reactions. In fact, with the exception of their TnsD-dependence (and thus their target site specificity), we have found TnsABC+D reactions to behave similarly throughout this work. Therefore, we speculate that similar interactions underlie target immunity in both the TnsABC+D and TnsABC+D reactions.

TnsB promotes the recycling of target binding proteins

How does TnsB inactivate Tn7 end-containing target DNAs? Some possible mechanisms would include: (i) dissociating TnsC from the target DNA; (ii) inactivating TnsC molecules bound to the target; or (iii) obstructing TnsC’s interactions with the Tn7 transposase. A mechanism that involves the dissociation of TnsC from the target DNA might be advantageous to Tn7: TnsC could then potentially recycle and reassociate with other, more favorable, target DNAs, rather than being trapped on the Tn7 end-containing target. On the other hand, TnsC’s interactions with TnsD might keep TnsC tethered to the Tn7 end-containing target DNA.

We looked for evidence of the release of proteins from the Tn7 end-containing target by staggering the addition of target DNAs during the target preincubation. Here we focus on TnsABC+D transposition reactions (Figure 6); reactions with TnsABC+D are discussed below. TnsC+D was preincubated with target #1 in the presence or absence of TnsB. After 20 min, target #2 was added and the preincubation was continued. This second target functioned as a sink to capture Tns proteins that had been released from target #1. Transposition was initiated by adding the donor preincubation mix, and the level of insertion into the two targets was compared. If TnsB promotes the removal and recycling of proteins (TnsC and/or others) from the Tn7 end-containing target, then we would predict that the use of the second target would increase when immunity was turned ON by the inclusion of TnsB in the target preincubation. However, if TnsB causes the permanent inactivation of proteins that associate with the Tn7 end-containing target, we would expect the use of the second target to be unchanged when immunity was applied. A limiting concentration of TnsC was used in these experiments, so that (when immunity was OFF) most of the protein would interact with the first target, therefore the use of the second target would be low. By contrast, TnsD was present in excess and preincubated with both target DNAs (see Figure 6A and legend for details), so that the use of the second target would depend on the recycling of TnsC but not the recycling of TnsD.

Figure 6B shows the distribution of insertions between a target with Tn7 ends (target #1) and a target without
Fig. 6. TnsB increases the use of target DNAs added late into the target preincubation. (A) The staging of the reaction. Two target DNAs were introduced into the reaction at different times. 1 × TnsC (5 ng) and 0.5 × TnsD (11 ng) were preincubated with target #1 in the presence of ATP or AMP–PNP. After 20 min, target #2 (which had been separately preincubated with 0.5 × TnsD) was added to each reaction, and the target preincubation was continued for another 20 min. 0.5 × TnsB was added to the target preincubation (even lanes) or added after the donor and target preincubations were combined (odd lanes). Donor preincubations were as described for Figure 3. After a further 20 min incubation, the reactions were stopped and the DNAs collected and analyzed. (B) Transposition reactions. Lanes 1–4: a Tn7 end-containing target (pRM2L) was added first (T#1), a target without ends (pKAO4-3) was added second (T#2). Lanes 5–8: a target without Tn7 ends (pRM2) was added first (T#1), a different target without ends (pKAO4-3) was added second (T#2). The nucleotide present in each reaction is indicated.

ends (target #2) (lanes 1–4). When the target preincubations were done in the absence of TnsB (lane 1), the majority of insertions (81%) occurred into target #1. This suggests that despite the prolonged target preincubation, most of the TnsC+D remained associated with the first target DNA. However, when TnsB was present in the target preincubation (lane 2), the use of target #2 increased (from 19% to 84% of the total insertions), coincident with immunity being imposed on target #1. The shift in target usage suggests that TnsB does not cause TnsC+D to become permanently inactivated and/or trapped on a Tn7 end-containing target. Instead, functional Tns proteins appear to become available to associate with the second target DNA.

We also examined the recycling of Tns proteins in the presence of the non-hydrolyzable ATP analog AMP–PNP. We have previously shown that target immunity is much less effective when AMP–PNP is substituted for ATP in the Tn7 transposition reaction (Bainton et al., 1993 and Figure 5). If the redistribution of insertions between the first and second targets is a consequence of target immunity, then AMP–PNP should also affect this redistribution. We observed that the commitment of insertions to the first target preincubated with TnsC+D was very strong in the presence of AMP–PNP: 98% of insertions occurred into target #1 (lane 3). This is consistent with previous observations that TnsC’s affinity for DNA is increased when AMP–PNP is substituted for ATP (Gamas and Craig, 1992). The commitment to the first target was maintained even when TnsB was present in the target preincubation; the use of target #2 did not increase (lane 4). Thus, when ATP hydrolysis was blocked, the Tns proteins appear to remain stably and functionally bound to Tn7 end-containing targets despite the presence of TnsB.

A similar redistribution of insertions has also been observed in reactions activated by TnsC(A225V) in the absence of TnsD (data not shown). This result, together with the ATP-dependence of the process, strongly suggests that TnsC is the key protein whose distribution among DNAs is being influenced by TnsB. Therefore, we interpret the insertion patterns seen in these experiments to reflect which target DNAs are bound by TnsC and are therefore capable of interacting with the Tn7 transposase. The fate of TnsD—whether it remains bound to the Tn7 end-containing target DNA or whether it is dislodged along with TnsC—has not yet been resolved.

We also investigated whether TnsB could affect TnsC’s distribution among target DNAs without Tn7 ends (Figure 6B, lanes 5–8). In this set of staggered target experiments, TnsC+D was preincubated first with a 3.2 kb target plasmid and a slightly smaller target plasmid was added second; neither of these target DNAs contained a Tn7 end. In the absence of TnsB (lane 5), the majority of insertions (95%) occurred into target #1. However, in the presence of TnsB (lane 6), a modest increase in insertions into target #2 was seen (from 5% to 27%), accompanied by a drop in the use of target #1. This ‘evening out’ of insertions between the first and second targets was not seen when AMP–PNP was substituted for ATP in these reactions (lanes 7 and 8).

These experiments suggest that TnsB promotes the dissociation of TnsC from target DNAs in general, but that the process is more extensive when a potential target DNA contains Tn7 ends, and thus has a higher local concentration of TnsB. Blocking TnsC’s ability to hydrolyze ATP appears to immobilize it on a target DNA, reducing TnsB’s effectiveness both at imposing immunity and redistributing insertions to alternative target DNAs.

**Discussion**

This work has investigated the mechanism by which target DNAs containing Tn7 ends are rendered ‘immune’ to Tn7 transposition. The transposon end-binding protein TnsB and the ATP-dependent DNA-binding protein TnsC were found to be central to the recognition and avoidance of Tn7 end-containing target DNAs. TnsC interacts with both the target DNA and the TnsA+B transposase to assemble an active transposition complex; only after this complex is built can Tn7 transposition be initiated (Bainton et al., 1993). An attractive model (illustrated in Figure 7) that emerges from this work is that TnsB dissociates TnsC from target DNAs containing Tn7 ends, leaving those target DNAs unable to interact successfully with the TnsA+B transposase and thus immune to Tn7 transposition. TnsC molecules that have dissociated from a Tn7 end-containing target can recycle and reassociate with other target DNAs. Therefore, target immunity is an
Target immunity in Tn7 transposition

**Tn7 target immunity: the TnsB signal**

We have demonstrated that the association of TnsB with Tn7 end-containing target plasmids discourages transposition from occurring into those target plasmids *in vitro*. Importantly, we have also shown that TnsB can confer immunity to a target plasmid that is catenated to (but not continuous with) a Tn7 end-containing plasmid. This result argues strongly that TnsB and the proteins present at the insertion site (TnsC and/or others) communicate by protein–protein interactions rather than by tracking or signaling along the DNA. Therefore, ‘immune’ targets are simply those DNAs which have a high local concentration of TnsB, and consequently a high frequency of interaction between TnsB and the proteins at the insertion site.

In the bacterial chromosome, immunity can operate over impressive DNA distances: a Tn7 end can reduce the frequency of transposition into sites up to 190 kb away (DeBoy and Craig, 1996). The effects of TnsB do not appear to be uniform over this distance; an inverse correlation was found between the magnitude of the immunity effect and the distance between the Tn7 ends and the *attTn7* target site in the chromosome (DeBoy and Craig, 1996). Variations in relative TnsB concentration may contribute to the effects of distance on target immunity *in vivo*. Chromosomal regions in close linear and/or physical proximity to Tn7 ends would be in a zone of comparatively high TnsB concentration and thus would undergo a strong immune response. By contrast, chromosomal regions separated from the Tn7 ends by large DNA distances or chromosomal condensation would have very few interactions with TnsB and would not be immune to insertions.

Target immunity *in vitro* was found to depend on the number of TnsB binding sites present on the target DNA; the stability with which TnsB binds to those sites is likely also involved. Furthermore, we found that time is also required to establish immunity: several minutes were needed for TnsB to fully inactivate the Tn7 end-containing targets (Figure 5). This is the same time frame needed for a mTn7 element, TnsA and TnsB to assemble with a target complex and initiate transposition (Bainton et al., 1993; A.Stellwagen and N.L.Craig, unpublished data). Thus, TnsB is involved in two processes—target inactivation and target use—which appear to occur at similar rates. In the test tube, immunity is efficiently established when these processes are temporally separated, through the careful staging of the transposition reactions. It will be interesting and challenging to discover whether the timing of TnsB’s interactions with target DNA complexes is also important for target immunity in the cell.

**Tn7 target immunity: the response to TnsB**

Our experiments suggest that TnsB imposes target immunity through its interactions with TnsC. We observed that target immunity was severely compromised in the presence of non-hydrolyzable ATP analogs, yet TnsB itself has no known interactions with ATP (Gamas and Craig, 1992; A.Stellwagen and N.L.Craig, unpublished data). Therefore, ATP hydrolysis appears to be important not in the TnsB signal but in the response to TnsB. TnsC, the only Tn7-encoded protein known to interact with ATP, is strongly implicated to be involved in that response. Support for the critical role of TnsC also comes from the observations of target immunity in reactions with TnsABC<sup>A223V</sup> in the absence of TnsD. Although several mechanisms can be imagined by which TnsB could interact with TnsC to inactivate a potential target DNA, perhaps the most attractive model to explain the data is that TnsB triggers the dissociation of TnsC from Tn7 end-containing target DNAs.

How might TnsB affect TnsC’s association with DNA? The ATP state of TnsC has been demonstrated to regulate its DNA-binding activity: TnsC+ATP is competent to bind DNA, whereas TnsC+ADP is not (Gamas and Craig, 1992). TnsC also has a modest ATPase activity (A.Stellwagen and N.L.Craig, in preparation), enabling TnsC to switch between the ATP- and ADP-bound states. TnsB could promote the removal of TnsC from a potential target DNA molecule by influencing TnsC’s ATP interactions—for example, by stimulating TnsC’s rate of ATP hydrolysis—or by facilitating TnsC’s dissociation from DNA following ATP hydrolysis. Either possibility would explain why non-hydrolyzable ATP analogs reduce TnsC’s ability to respond to TnsB.

The effects of ATP binding and hydrolysis on TnsC’s activities are reminiscent of the actions of molecular switch proteins, whose ability to participate in various biological processes is controlled by hydrolysis of ATP.
or GTP. A classic example of a molecular switch is p21-Ras, a GTPase that regulates many signal transduction cascades (Bourne et al., 1990, 1991). GTP hydrolysis enables Ras to switch from an active to an inactive conformation; the rate at which Ras switches between conformations is modulated by GTPase-activating proteins (GAPs) and GTP–GDP exchange factors (GEFs). We propose that TnsC acts as a molecular switch to regulate Tn7 transposition, and we are very interested in determining whether TnsC’s ATP interactions are modulated by other Tns proteins. For example, TnsB could be the functional equivalent of a GAP, promoting ATP hydrolysis and thus TnsC inactivation, while other factors (perhaps TnsD) might stabilize the ATP-bound form of TnsC.

**Comparison of target immunity in Tn7 and Mu**

The proposed mechanism for Tn7 target immunity—the TnsB-dependent removal of TnsC from Tn7 end-containing target DNAs—has many parallels to the mechanism of target immunity described by Adzuma and Mizuuchi (1988, 1989) for the bacteriophage Mu. Mu transposition is executed by the MuA transposase, which directs insertions into target DNAs bound by MuB, an ATP-dependent DNA-binding protein (Chaconas et al., 1985; Maxwell et al., 1987). However, MuB is actively removed from target DNAs containing Mu ends: MuA, bound to the Mu end, stimulates MuB’s ATPase activity and promotes MuB’s dissociation from those target DNAs (Maxwell et al., 1987; Adzuma and Mizuuchi, 1988). Thus, there are strong similarities between the interactions of MuA and MuB and the interactions of TnsB and TnsC in establishing target immunity. In both cases, immunity can be conferred to a target DNA that is catenated to a transposon end (Adzuma and Mizuuchi, 1989 and this work), suggesting that high local concentrations of the end-binding protein (MuA or TnsB) are responsible for immunity in each system.

These mechanistic similarities are striking since Mu and Tn7 proteins generally have very little primary sequence homology, although specific proteins sometimes share functional motifs. For example, MuB and TnsC both contain motifs commonly found in nucleotide binding proteins (Walker et al., 1982, Chaconas et al., 1985; Flores et al., 1990), but otherwise the amino acid sequences of MuB and TnsC are widely divergent.

MuB and TnsC also have an important functional difference: whereas MuB is sufficient to activate its transposase, TnsC is not. Instead, Tn7 transposition is dependent on multiple target selection factors, which direct Tn7 transposition to different types of target sites (Rogers et al., 1986; Waddell and Craig, 1988; Wolkow et al., 1996). Interestingly, these extra complexities do not appear to override or interfere with the basic immunity mechanism shared by Mu and Tn7. In fact, we will argue below that Tn7 actually exploits the interactions that underlie target immunity to facilitate its selection of preferred target sites.

**The target immunity paradox**

Target immunity poses a molecular paradox: the same proteins that discourage transposition from occurring into target DNAs containing transposon ends are also responsible for promoting transposition into target DNAs without ends. For example, MuA bound to Mu ends in a donor molecule readily captures and utilizes MuB–target DNA complexes, but MuA bound to a Mu end on a target DNA destroys the MuB–target DNA complex (Adzuma and Mizuuchi, 1988). The situation is even more perplexing for Tn7, in which transposition and immunity are accomplished by a single transposon-encoded protein (Gill et al., 1979; Lee et al., 1983; Maekawa et al., 1996).

The study of Tn7 target immunity may have given us some insight into this paradox. Tn7’s transposition activities are distributed among many proteins, and different combinations of these proteins appear to mediate target use versus target immunity. Promoting transposition requires both TnsA and TnsB: the active sites responsible for DNA breakage and joining appear to be divided between TnsA and TnsB and the catalytic activities of TnsA and TnsB are interdependent (May and Craig, 1996; Sarnovsky et al., 1996). By contrast, the requirements for target immunity appear to be more minimal: TnsB bound to a single transposon end can interact with TnsC to prevent target use; TnsA does not appear to be involved. Future work is needed to determine whether the interaction(s) between TnsA+B and TnsC that result in transposition are the same or different from the interaction(s) between TnsB and TnsC that result in immunity. Toward that end, it would be very interesting to screen for TnsB mutants that retain the ability to promote transposition but fail to promote target immunity.

**Comparison with other mechanisms for silencing DNA**

Tn7 and Mu achieve target immunity by preventing a region of DNA from being able to engage the transposition machinery. This phenomenon is conceptually similar to transcriptional silencing, in which regions of the chromosome—for example, the silent mating type loci and telomeres in yeast—become unable to engage the transcriptional machinery. Silent chromosomal regions are packaged in specialized heterochromatin-like structures that render the DNA inaccessible to many proteins, including the basal transcriptional machinery (reviewed in Loo and Rine, 1995; Zakian, 1996). Thus, genes within silent chromatin are unable to interact with RNA polymerase, just as target sites on immune plasmids are unable to interact with transposase. Although the outcomes of these two processes are similar, the strategies used to achieve the ‘silent’ state are significantly different: transcriptional silencing involves the assembly of an inactive protein–DNA structure, whereas target immunity involves the disassembly of an active structure.

**Tn7 transposition: extending the immunity mechanism to multiple forms of target site selection**

Tn7 and Mu both evaluate potential target DNAs for negative signals, namely the presence of transposon ends. However, Tn7 is uniquely able to evaluate potential target DNAs for positive signals. Tn7 transposition is preferentially directed into attTn7 sequences or into plasmids undergoing conjugation (Barth et al., 1976; Hauer and Shapiro, 1984; Wolkow et al., 1996). We propose that the interactions between TnsB and TnsC described in this work may facilitate all of these types of Tn7 target site selection. By promoting TnsC’s dissociation...
from DNA in general and from immune targets in particular (Figure 6B). TnsB increases TnsC’s ability to ‘sample’ the various target molecules present in the cell or the test tube. Therefore, TnsC would be more likely to find favorable target sites (such as attTn7) and less likely to be trapped on unfavorable targets. Non-hydrolyzable ATP analogs, by contrast, appear to reduce TnsC’s ability to sample different target DNAs. In the presence of AMP–PNP, TnsC appears to be locked to an active conformation; its affinity for DNA is increased (Gamas and Craig, 1992) but its ability to respond to the positive and negative features of a potential target DNA is decreased. Therefore, Tn7 is less successful at finding attTn7 target sites in the presence of AMP–PNP (Bainton et al., 1993), as well as less successful at avoiding Tn7 end-containing targets.

In conclusion, our investigations into the mechanism of target immunity may have revealed a theme that is common to all forms of Tn7 target site selection: the importance of controlling the distribution of TnsC. Although TnsC binds to DNA with no particular sequence specificity (Gamas and Craig, 1992), its localization among target DNAs is hardly random. TnsC is recruited to attTn7 sites by TnsD; the formation of TnsC–TnsD–attTn7 complexes has been documented by DNA protection and mobility shift assays (Bainton et al., 1993). TnsC may be similarly recruited to conjugating plasmids by TnsE. Finally, experiments in this work suggest that TnsC is removed from Tn7 end-containing targets by TnsB. Therefore, we speculate that TnsC’s distribution is a molecular reflection of the positive and negative features of the available target DNAs, and provides a means of attracting the Tn7 transposase to the most favorable targets.

Materials and methods

DNA substrates

The donor plasmid pEMA (5.9 kb) contains a 1.6 kb mini-Tn7 element (mTn7), in which 166 bp from the left end of Tn7 and 199 bp from the right end of Tn7 flank a kanamycin resistance marker (Bainton et al., 1993). All of the target plasmids used in this work are derivatives of pUC (except the catenation plasmids, which are derivatives of pBR322). The target plasmids pKA04-3 (2.8 kb) and pMR2 (3.2 kb) contain the sequences necessary for attTn7 sites. The target plasmid pLA11 (3.6 kb) was derived from pRM2, and contains a Tn7 right end (including the RI–199 sequences) located ~1 kb from the attTn7 site (Bainton et al., 1991). The target plasmid pRM2L (3.7 kb) was generated in this study by cloning the HindIII–ScaI fragment from pLA19 (Arciszewska et al., 1989), containing L1–166 sequences, into pMR2. The left end sequences in the resulting plasmid were ~0.8 kb from the attTn7 site. The plasmids pLA62 (2.8 kb) and pLA77 (3.1 kb) contain the sequences R38–199 from the Tn7 right end and L109–166 from the Tn7 left end, respectively (Arciszewska et al., 1989, 1991); these plasmids do not contain attTn7 sites.

The catenation-competent plasmid pRCAT2 (4.8 kb) was constructed in two steps. First, an EcoRI–ScaI fragment from pEMA, containing R1–199 sequences, was cloned into pNG210 (Boocock et al., 1995) to generate pRCAT1. An attTn7 site was then introduced by replacing the ApII–NdeI fragment of pRCAT1 with the ApII–NdeI fragment from pKA04-3. The right end sequences and the attTn7 site were ~2.2 kb apart in the resulting plasmid pRCAT2.

Tns proteins

TnsA was purified as a fusion with glutathione S-transferase (GST) and then released from the GST moiety by thrombin cleavage (May and Craig, 1996). TnsA was stored at ~9°C in 25 mM HEPES, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 5% glycerol and 0.25 mM PMSF. TnsB was TnsB-His6 (Gary et al., 1996) and was stored in 25 mM HEPES, pH 7.5, 500 mM KCl, 2 mM DTT, 1 mg/ml BSA and 25% glycerol. TnsC was fraction III (Gamas and Craig, 1992) and was stored in 15 mM HEPES, pH 7.5, 1.5 mM NaCl, 2.5 mM DTT, 1 mM ATP, 10 mM MgCl2, 0.1 mM EDTA, 10 mM CHAPS and 10% glycerol. Before use, TnsC was diluted 1:5 in a buffer identical to the storage buffer except without ATP. TnsD was TnsD-His (Sarnovsky et al., 1996; P.L.Sharpe and N.L.Craig, in preparation) and was stored in 50 mM Tris, pH 7.5, 2 mM DTT, 500 mM KCl, 1 mM EDTA and 25% glycerol.

TnsC A225V mutation was introduced into Tn7 by treating the BluntI fragment of pPA101 (Gamas and Craig, 1992) by replacing the HindIII fragment of pPA101 with the 1.9 kb HindIII fragment from a pCW15 plasmid containing the TnsC A225V mutation (Stellwagen and Craig, 1997). TnsC A225V was expressed and purified using the protocol developed for TnsC and TnsD (Gamas and Craig, 1992). One modification was introduced: the ammonium sulfate pellet was extracted twice with Buffer A containing 0.1 M NaCl, to resuspend in 0.1 M NaCl at 4°C, rather than the ammonium sulfate pellet being resuspended in Buffer B + 1.0 M NaCl, precipitated by dialysis into Buffer B + 0.1 M NaCl, and then resuspended in the storage buffer. This modification has also been used when purifying wild-type TnsC; no change in protein activity has been observed but the protein yields are increased (A.Stellwagen and N.L.Craig, unpublished data).

Tn7 transposition in vitro

Reactions were adapted from the procedure of Bainton et al. (1993). The final reaction mixture (100 μl) contained 0.25 nM pEMA donor DNA, 0.83 nM each of various target DNAs, 26 mM HEPES, pH 7.5, 3.3 mM Tris, pH 7.5, 2.1 mM DTT, 70 μg/ml BSA, 0.06 mM EDTA, 0.1 mM MgCl2, 0.1 mM CHAPS, 12 mM NaCl, 15 mM KCl, 0.9% glycerol, 4 μM PMSF, 2 mM nucleotide (ATP or AMP–PNP) and 15 mM MgAc unless otherwise indicated. Tns proteins (1–2 μl each) were added to the following final amounts, unless otherwise indicated: 60 ng TnsA, 25 ng TnsB, 10 ng TnsC (or 20 ng TnsC A225V) and 20 ng TnsD.

The order of addition of the DNA substrates and the Tns proteins was varied in different experiments; details are provided in the figure legends. The general strategy was to preincubate the donor and target DNAs separately with different subsets of Tns proteins, and then to combine the preincubated DNAs to initiate transposition. Target precubinations (50 μl) usually contained TnsC and TnsD (or TnsC A225V and an equivalent volume of TnsD buffer) in the presence or absence of TnsB (at 0.5× its final concentration, i.e. 12.5 ng). ATP (or AMP–PNP, when indicated) was added to the target precubinations; MgAc was not. The donor precubinations (50 μl) always contained TnsA, 0.5× TnsB and 15 mM MgAc. Donor and target precubinations were carried out for 20 min at 30°C unless otherwise indicated. The precubinations were then combined, the MgAc concentration was adjusted back to 15 mM, and any proteins which had been omitted from the target precubination (i.e. 0.5× TnsB) were added. Therefore, the final composition of all reactions in a given experiment was always the same. Reactions were incubated for another 20 min and then stopped with the addition of 100 μl phenol:chloroform.

The DNAs from a transposition reaction were collected by ethanol precipitation, subjected to NdeI digestion (unless otherwise indicated) and loaded on a 0.6% TBE agarose gel. 0.05 μCi of a 35S-labeled DNA ladder (Amersham) was included on each gel. Electrophoresis proceeded at 2 V/cm for 15 h, to obtain good separation in the relevant size range. The DNAs were transferred to Gene Screen Plus (NEN Research Products) by capillary transfer. Blots were hybridized with a mTn7-specific probe, namely the kanamycin resistance cassette which is contained within mTn7. Probes were labeled by random priming with [α-32P]dCTP and the Klenow fragment of DNA polymerase I (MBI). Blots were analyzed by autoradiography using Kodak BioMax MR film or by a Molecular Dynamics Phosphorimager.

γ Resolution reactions

The 76 resolution reactions were performed essentially as described by Reed (1981). Reaction mixtures (100 μl) contained 3 μg pRCAT2, 22 mM Tris, pH 8.0, 10 mM MgCl2, 100 mM NaCl, 5% glycerol and 200 ng γδ resolvase protein (a gift from Nigel Grindley). Reactions were incubated at 37°C for 60 min. The reaction mixture was then split, and half of the material was digested with NdeI. All reactions were stopped by incubation with 0.4 mg/ml proteinase K in 0.25% SDS and 20 mM EDTA, followed by heat-inactivation. DNAs were recovered by two rounds of phenol:chloroform extraction, ethanol precipitation and resuspension in TE. The efficiency of the resolution reaction and the concentration of the resolved and unresolved products were evaluated by agarose gel electrophoresis.
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