Gain-of-Function Mutations in TnsC, an ATP-Dependent Transposition Protein That Activates the Bacterial Transposon Tn*7*

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ABSTRACT

The bacterial transposon Tn*7* encodes five genes whose protein products are used in different combinations to direct transposition to different types of target sites. TnsABC+D directs transposition to a specific site in the *Escherichia coli* chromosome called *attTn7*, whereas TnsABC+E directs transposition to non-*attTn7* sites. These transposition reactions can also recognize and avoid "immune" targets that already contain a copy of Tn*7*. TnsD and TnsE are required to activate TnsABC as well as to select a target site; no transposition occurs with wild-type TnsABC alone. Here, we describe the isolation of TnsC gain-of-function mutants that activate the TnsA+B transposase in the absence of TnsD or TnsE. Some of these TnsC mutants enable the TnsABC machinery to execute transposition without sacrificing its ability to discriminate between different types of targets. Other TnsC mutants appear to constitutively activate the TnsABC machinery so that it bypasses target signals. We also present experiments that suggest that target selection occurs early in the Tn*7* transposition pathway *in vivo*: favorable *attTn7* targets appear to promote the excision of Tn*7* from the chromosome, whereas immune targets do not allow transposon excision to occur. This work supports the view that TnsC plays a central role in the evaluation and utilization of target DNAs.

TRANSPOSONS are DNA elements that can move from one position to another in the genome of their host organism. The selection of a new insertion site is usually not a random process; instead, many transposons show characteristic preferences for certain types of target sites. In some cases, target selection reflects the DNA sequence preferences of the transposase itself. The bacterial transposon Tn*10*, for example, preferentially inserts into "hotspots" with a common sequence motif *in vivo* (HALLING and KLECKNER 1982), and Tn*10* transposase binds to hotspot sequences *in vitro* (D. HANIFORD, personal communication). In other cases, the transposase is directed to particular targets through interactions with cellular proteins or transposon-encoded factors. The transcription factors TFIIIB and TFIIIC, for example, recruit the yeast retrotransposon Ty*3* to insert into the promoters of genes transcribed by RNA polymerase III (KIRCHNER *et al.* 1995), whereas bacteriophage Mu insertions are targeted to DNA molecules bound by the phage-encoded accessory factor MuB (CRAIGIE and MIZUUCHI 1987; SURETTE *et al.* 1987; ADZUMA and MIZUUCHI 1988). Understanding how these factors modulate transposase activity to impose target site preferences will lend insight into the spread of transposons and viruses and may suggest ways to manipulate those target preferences.

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The bacterial transposon Tn*7* is distinctive in that it uses several element-encoded accessory proteins to evaluate potential target DNAs for positive and negative features, and to select a target site (for a recent review, see CRAIG 1996). Tn*7* encodes five genes whose protein products mediate its transposition (ROGERS *et al.* 1986; WADDELL and CRAIG 1988). Two of these proteins, TnsA and TnsB, collaborate to execute the catalytic steps of Tn*7* transposition (MAY and CRAIG 1996; SARNOVSKY *et al.* 1996); thus TnsA+B constitutes the Tn*7* transposase. The activity of this transposase is modulated by the remaining proteins, TnsC, TnsD, and TnsE, and also by the nature of the target DNA. TnsABC+D directs transposition at high frequency to a specific site in the *E. coli* chromosome called *attTn7* (BARTH *et al.* 1976; LICHTENSTEIN and BRENNER 1982; ROGERS *et al.* 1986; WADDELL and CRAIG 1988; KUBO and CRAIG 1990). An alternative combination of proteins, TnsABC+E, directs transposition at lower frequency to many non-*attTn7* sites in the chromosome (ROGERS *et al.* 1986; WADDELL and CRAIG 1988; KUBO and CRAIG 1990) and, preferentially, to conjugable plasmids (WOLKOW *et al.* 1996). Thus, *attTn7* and conjugable plasmids contain positive signals that recruit the transposon to these target DNAs.

The Tn*7* transposition machinery can also recognize and avoid targets that are unfavorable for insertion. Tn*7* transposition occurs only once into a given target molecule; repeated transposition events into the same target are specifically inhibited (HAUER and SHAPIRO 1984; ARCISZEWSKA *et al.* 1989). Therefore, a pre-ex-

isting copy of Tn*7* in a potential target DNA generates a negative signal which renders that target "immune" to further insertion. The negative target signal affects both TnsD- and TnsE-activated transposition reactions and is dominant to any positive signals present on a potential target molecule (ARCISZEWSKA *et al.* 1989). Several other transposons, such as Mu and members of the Tn*3* family, also display this form of negative target regulation (LEE *et al.* 1983; REYES *et al.* 1987; ADZUMA and MIZUUCHI 1988).

Target selection could be an early or late event in the course of a transposition reaction. For example, a transposon could constitutively excise from its donor position, and the excised transposon could then be captured at different frequencies by different types of target molecules. Tn*10* appears to follow this course of events *in vitro*, excising from its donor position before any interactions with target DNA occur (SAKAI *et al.* 1995; KLECKNER *et al.* 1996). Alternatively, the process of transposon excision could itself be dependent on the identification of a favorable target site. Tn*7* transposition shows an early dependence on target DNA signals *in vitro*: neither transposition intermediates nor insertion products are seen in the absence of an *attTn7* target (BAINTON *et al.* 1991, 1993). Thus, the nature of the target DNA appears to regulate the initiation of Tn*7* transposition *in vitro*.

How are positive and negative target signals communicated to the Tn*7* transposase? Dissection of the TnsABC+D reaction *in vitro* has implicated TnsC as a pivotal connector between the transposase and the target DNA. TnsC is an ATP-dependent, nonspecific DNA binding protein (GAMAS and CRAIG 1992). However, TnsC can be specifically recruited to *attTn7* targets through interactions with TnsD, an *attTn7*-specific DNA binding protein (BAINTON *et al.* 1993). The TnsC-TnsD-*attTn7* complex has been visualized by DNA protection and mobility-shift assays, and the preassembled complex can attract insertions (BAINTON *et al.* 1993). Direct interactions between TnsC and the TnsA+B transposase have also been observed (A. STELLWAGEN and N. L. CRAIG, unpublished results). Therefore, TnsC may serve as a "connector" or "matchmaker" between the transposase and the TnsD + *attTn7* target complex (BAINTON *et al.* 1993; SANCAR and HEARST 1993). This connection is not constitutive, but instead appears to be regulated by the ATP state of TnsC. Only the ATP-bound form of TnsC is competent to interact with target DNAs and activate the TnsA+B transposase; the ADP-bound form of TnsC has neither of these activities and cannot participate in Tn*7* transposition (GAMAS and CRAIG 1992; BAINTON *et al.* 1993). TnsC hydrolyzes ATP at a modest rate (A. STELLWAGEN and N. L. CRAIG, unpublished results) and therefore can switch from an active to an inactive state. The modulation of TnsC's ATP state may be a central mechanism for regulating Tn*7* transposition.

The proposal that TnsC regulates the connection between the transposase and the target site predicts that TnsC mutants could be isolated which would constitutively activate Tn*7* transposition. In this paper, we report the identification of gain-of-function TnsC mutants that can activate the TnsA+B transposase in the absence of TnsD or TnsE. We have characterized the ability of these mutants to promote insertions into various targets and to respond to regulatory signals on those targets. Interestingly, one class of TnsC mutants activates transposition in a way that is still sensitive to target signals, whereas a second class of TnsC mutants activates transposition in a way that appears to bypass target signals. As had been observed *in vitro*, the critical communication between the transposon and the target DNA appears to be an early event in the Tn*7* reaction pathway *in vivo*, preceding the double-strand breaks at the transposon ends that initiate transposition.

MATERIALS AND METHODS

Media, chemicals, and enzymes: LB broth and agar were prepared as described by MILLER (1972). Trimethoprim selection was on Isosensitest agar (Oxoid). Lac phenotypes were evaluated on MacConkey lactose agar (Difco). Antibiotic concentrations used were 100 μg/ml carbenicillin (Cb), 30 μg/ml chloramphenicol (Cm), 7.5 μg/ml gentamicin (Gn), 50 μg/ml kanamycin (Km), 10 μg/ml nalidixic acid (Nal), 20 μg/ml tetracycline (Tet) and 100 μg/ml trimethoprim (Tp). Hydroxylamine was purchased from Sigma. DNA modifying enzymes were purchased from commercial sources and used as recommended by the manufacturer.

Bacterial strains, phage and plasmids: BR293 is *E. coli* F^- Δ*(lac-pro) thi rpsL* Δ*(gal* − λ*G)* + *lacZ* pL cI^{+}_{434} pRS_7 (ELESPURU and YARMOLINSKY 1979; YARMOLINSKY and STEVENS 1983). BR293 is identical to NK8027 (HANIFORD *et al.* 1989), and was generously provided by NANCY KLECKNER. NLC51 is *E. coli* F^- *araD139* Δ*(argF-lac) U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* Val^R *recA56* (MCKOWN *et al.* 1987). CW51 is *E. coli* F^- *ara arg* Δ*lac-proXIII recA56* Nal^R Rif^R (WADDELL and CRAIG 1988). λKK1 is lambda 780 *hisG9424*::Tn*10 del*16 *del*17::*attTn7*::miniTn*7*-Km^R (MCKOWN *et al.* 1988). Tns transposition proteins were provided by pCW15 (*tnsABC*), pCW23 (*tnsD*), pCW30 (*tnsE*), or pCW4 (*tnsABCDE*) (WADDELL and CRAIG 1988). Target plasmids were derivatives of pOX-G, a conjugable derivative of the F plasmid that carries Gn^R (JOHNSON and REZNIKOFF 1984). pOX-*attTn7* carries a (−342 to +165) *attTn7* sequence (ARCISZEWSKA *et al.* 1989). The immune plasmid pOX-*attTn7* EP-1::miniTn*7*-Cm^R was made by transposing miniTn*7*-Cm^R (MCKOWN *et al.* 1988) onto pOX-*attTn7* using TnsABC+E to direct the insertion into a non-*attTn7* position. Construction of the immune target plasmid pOX-G::miniTn*7-dhfr* is described below. The transposon donor plasmid for the papillation assay was pOX-G::miniTn*7lac*, containing promoterless *lacZY* between the transposon ends (HUGHES 1993). The high copy transposon donor for mating-out assays was pEMΔ, containing miniTn*7*-Km^R (BAINTON *et al.* 1993).

Manipulation and characterization of DNA: Phage and plasmid isolation, transformation, and standard cloning techniques were performed as described by SAMBROOK *et al.* (1989). Conjugation and P1 transduction were performed as described by MILLER (1972). DNA sequencing was done on an automated ABI sequencer. Two plasmids were constructed

in this work: (1) pOX-G::miniTn *7-dhfr*. MiniTn *7-dhfr* was constructed by replacing the Km^R cassette in pLA1 (ARCISZEWSKA *et al.* 1989) with a *dhfr* cassette from pSD511 (DEVINE and BOEKE 1994), which had been amplified by PCR to add flanking *Sal*I sites. The PCR fragment was ligated into the TA vector (Invitrogen), the *dhfr* cassette was then removed by *Sal*I digestion and inserted into the *Sal*I site of pLA1, replacing the Km^R gene. The resulting plasmid was transformed into NLC51 + pCW4 + pOX-G, and grown for several days to allow transposition to occur. pOX-G plasmids which had received a miniTn *7-dhfr* insertion were identified by mating into CW51 and selecting for Tp^R. (2) TnsA($BC^{\Delta 1\text{-}293}$). The *tnsABC* plasmid pCW15 was briefly treated with *Bam*HI, and molecules that had lost the 0.9-kb fragment from the 5′ end of *tnsC* were identified by gel electrophoresis, using low melting temperature agarose (SeaPlaque, FMC Corp., Marine Colloids Div.). Gel slices containing these molecules were isolated and the DNA was religated to generate *tnsA*($BC^{\Delta 1\text{-}293}$).

SOS induction assays: SOS induction was evaluated in BR293 strains. Tn *7* and miniTn *7*-Km^R elements were introduced by P1 transduction; target plasmids were introduced by conjugation. *lacZ* expression served as a reporter for the SOS state of the cells, and was evaluated by patching cells on MacConkey lactose plates at 30° for 18–36 hr. β-galactosidase activity was quantitated in cultures that were grown at 30°, using the assay described by MILLER (1972).

Mutagenesis of *tnsC*: The *tnsABC* plasmid pCW15 was exposed to 1 M hydroxylamine hydrochloride in 0.45 M NaOH (final pH ~7.0) at 37° for 20 hr (ROSE *et al.* 1990). The DNA was recovered by multiple ethanol precipitations, and *Pvu*II-*Sph*I fragments containing mutagenized *tnsC* were subcloned into untreated pCW15, replacing the wild-type *tnsC*. These plasmids were then introduced into CW51 + pOX-G::miniTn *7lac* by electroporation, and transformants were selected on MacConkey lactose plates containing Cm. The plates were incubated at 30° for 3–4 days and screened for the emergence of Lac^+ papillae, indicating transposition of miniTn *7lac*.

λ hop transposition assay: Tn *7* transposition was evaluated in NLC51 strains into which *tns* functions were introduced by transformation, and pOX-G was introduced by conjugation (for Figure 5). The protocol of MCKOWN *et al.* (1988) was followed: Cells were grown in LB and 0.2% maltose at 37° to an OD_{600} of 0.4–0.6 and then concentrated to 1.6×10^9 cells/ml by centrifugation and resuspension in 10 mM $MgSO_4$; 0.1 ml cells were combined with 0.1 ml λKK1 containing miniTn *7*-Km^R at a multiplicity of infection of 0.1 phage per cell. The infection proceeded for 15 min at 37° and was terminated by the addition of 10 mM sodium citrate in 0.8 ml LB. Cells were allowed to recover with aeration for 60 min at 37° and then spread on plates containing Km and citrate. Transposition frequency is expressed as the number of Km^R colonies/pfu of λKK1.

Mating-out transposition assay: Tn *7* transposition was evaluated in the derivatives of BR293 used to monitor SOS induction (Table 3) or in NLC51 strains into which *tns* functions were introduced by transformation, and pOX-G or pOX-G::miniTn *7-dhfr* were introduced by conjugation. MiniTn *7*-Km^R was present in the NLC51 strains either in the chromosomal *attTn7* site (Figure 4 and Table 1) or the high copy plasmid pEMΔ (Table 2). The protocol was adapted from WADDELL and CRAIG (1988): The donor strains described above and the recipient strain CW51 were grown at 37° to an OD_{600} of 0.4–0.6 with gentle aeration. Donors and recipients were mixed at a ratio of 1:5, and growth was continued for another hour. Mating was disrupted by vigorous vortexing, and the cells were diluted and plated. The total number of exconjugants was determined by selection on GnNal plates. Tn *7*-containing exconjugants were selected on TpNal plates, and miniTn *7*-Km^R exconjugants were selected on KmNal plates. Transposition frequencies are expressed as the number of Tp^R- or Km^R-exconjugants/total number of exconjugants.

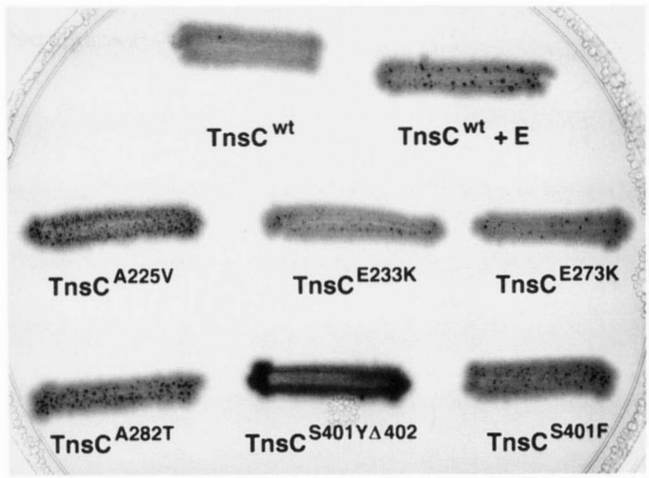

FIGURE 1.—Papillation phenotypes of the TnsC gain-of-function mutants. Cells were patched on MacConkey lactose plates and photographed after three days' incubation at 30°. TnsA+B was present in each strain; the TnsC species present is indicated below each patch.

RESULTS

Isolation of TnsC gain-of-function mutants: To focus on the contributions of TnsC to the evaluation and utilization of target DNAs, we sought to isolate gain-of-function TnsC mutants that could activate the TnsA+B transposase in the absence of TnsD or TnsE. Since overexpression of wild-type TnsC does not relieve the requirement for TnsD or TnsE (WADDELL and CRAIG 1988), these gain-of-function mutations are predicted to affect the biochemical properties of TnsC, rather than its expression or stability.

A visual assay for Tn *7* transposition has been developed to facilitate the identification of transposition mutants (HUGHES 1993; HUISMAN and KLECKNER 1987). This assay uses a miniTn *7lac* element that carries promoterless *lacZY* genes between the *cis*-acting sequences at the transposon ends. The miniTn *7lac* element is located in a transcriptionally silent position on a donor plasmid; cells containing this plasmid are phenotypically Lac^-. When Tns functions are provided in *trans*, miniTn *7lac* can transpose to new sites in the *E. coli* chromosome. Some of those transposition events place the element downstream from active promoters, resulting in increased *lacZ* expression. This is observed on MacConkey lactose color indicator plates as the emergence of red (Lac^+) papillae in an otherwise white (Lac^-) colony. Therefore, the number of papillae reflects the amount of transposition that occurred during the growth of that colony.

Cells containing miniTn *7lac* and various Tns functions were patched on color indicator plates (Figure 1).

Virtually no Lac$^+$ papillae were seen in cells containing only TnsABC^{wt}, whereas cells containing TnsABC^{wt} + E produced many Lac$^+$ papillae. Southern blotting has demonstrated that TnsABC^{wt} + E papillae result from translocations of miniTn *7lac* to a variety of chromosomal locations rather than from intramolecular rearrangements of the donor plasmid (HUGHES 1993). It should be noted that most TnsABC^{wt} + D events are silent in this assay, because there is no appropriately oriented promoter adjacent to *attTn7* which would fire into miniTn *7lac* insertions in that target site (HUGHES 1993; R. DEBOY and N. L. CRAIG, unpublished results).

We used this visual assay to screen for TnsC mutants that had acquired the ability to activate Tn *7* transposition in the absence of TnsD or TnsE. Randomly mutagenized *tnsC* was cloned into a plasmid containing *tnsAB*; these *tns* genes were introduced into cells containing miniTn *7lac* and evaluated for their ability to promote papillation. Six gain-of-function TnsC mutants were identified, from ~3000 transformants screened (Figure 1). Transposition activated by these TnsC mutants still required the TnsA+B transposase and intact transposon ends, and comparable levels of wild-type and mutant TnsC proteins were observed by Western blotting (data not shown). The papillation phenotypes of the TnsC mutants varied considerably, suggesting that different mutants were activating different amounts of miniTn *7lac* transposition. Several TnsC mutants promoted more transposition than TnsABC^{wt} + E, with TnsABC$^{S401Y\Delta402}$ achieving the highest level of transposition. Indeed, cells containing TnsC$^{S401Y\Delta402}$ produced so many papillae that, after several days' incubation, the entire patch of cells appeared red. At earlier time points, however, individual papillae could be seen in the TnsABC$^{S401Y\Delta402}$ patches as well (data not shown).

The amino acid changes responsible for the mutant phenotypes were determined by DNA subcloning and sequencing. *tnsC* encodes a protein of 555 amino acids, with Walker A and B motifs in the amino-terminal half of the protein (FLORES *et al.* 1990). Walker A and B motifs have been implicated by structural and mutational analyses to be directly involved in nucleotide binding and/or hydrolysis in a variety of ATPases and GTPases (WALKER *et al.* 1982; see SARASTE *et al.* 1990 for review). The *tnsC* mutations primarily result in single amino acid substitutions whose locations are scattered across the TnsC protein sequence (Figure 2). As will be described below, the TnsC mutants segregate into two phenotypic classes, which are indicated above and below the cartoon. Transposition reactions activated by the Class I mutants are sensitive to immune targets and the target selection factors TnsD and TnsE, whereas transposition reactions activated by the Class II mutants are compromised in their responses to these signals. Interestingly, the residues affected in two of the mutants (TnsC^{A225V} and TnsC^{E233K}) lie in or very close to TnsC's Walker B motif.

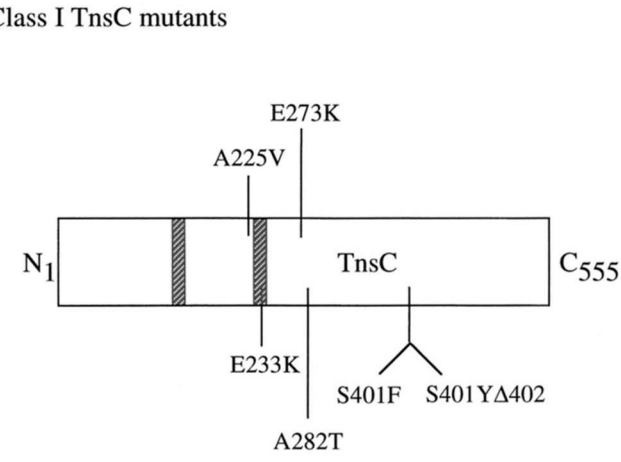

FIGURE 2.—Amino acid changes in the TnsC mutants. The TnsC protein sequence is cartooned, with the residues altered in the Class I mutants indicated above the protein and the Class II mutants below the protein. Hatched boxes represent Walker A and Walker B motifs.

TnsC mutants promote intermolecular transposition: The papillation assay is a powerful screen for transposition activity, but it does not necessarily report only intermolecular transposition events. Internal rearrangements of the miniTn *7lac* donor plasmid, which fortuitously place the miniTn *7lac* element downstream from a promoter, would also produce Lac$^+$ papillae. Therefore, we investigated whether the TnsC mutants facilitate the TnsA+B transposase to do intramolecular recombination or whether the mutants promote intermolecular transposition.

The λ hop assay measures the translocation of a miniTn *7*-Km^R element from a replication- and integration-defective λ phage to the bacterial chromosome during a transient infection. The miniTn *7*-Km^R element carries a kanamycin resistance cassette with a constitutive promoter. Therefore, the λ hop assay reports the total number of transposition events occurring into the chromosome, unlike the papillation assay, which only scores those events which occur downstream from active promoters. TnsABC^{wt} had no detectable transposition activity in the λ hop assay, whereas TnsABC^{wt} + E generated 2.2×10^{-7} Km^R colonies/pfu, a transposition frequency ≥20-fold above the threshold of sensitivity (Figure 3). Transposition promoted by TnsABC^{wt} + D occurred at a much greater frequency, generating 1.8×10^{-4} Km^R colonies/pfu. All of the TnsC mutants could promote the translocation of miniTn *7*-Km^R. TnsABC^{A225V} and TnsABC$^{S401Y\Delta402}$ had striking activities, promoting eight- and 50-fold more transposition than TnsABC^{wt} + E. Other TnsC mutants promoted more modest levels of transposition. Although all of the TnsABC^{mutant} reactions show a gain-of-function over TnsABC^{wt}, none of the mutant reactions reach the level of transposition seen with TnsABC^{wt} + TnsD.

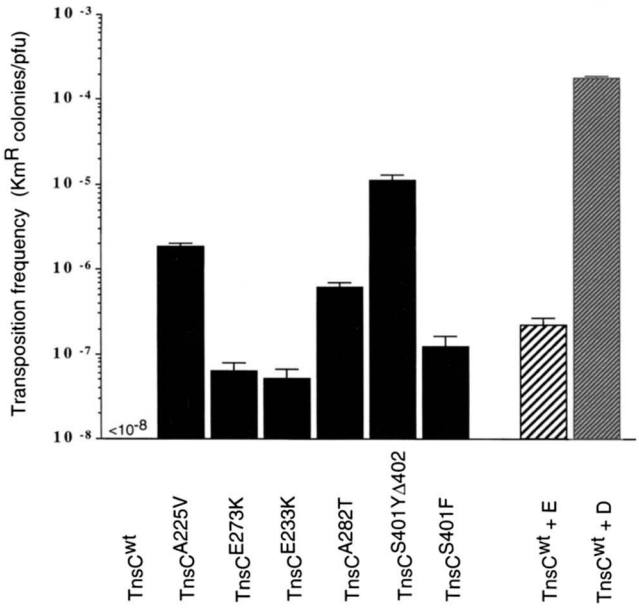

FIGURE 3.—TnsC mutants promote transposition to the chromosome. Frequencies of transposition of miniTn *7*-Km^R from a λ phage to the chromosome were measured by the λ hop assay. TnsA+B was present in each strain; the TnsC species present is indicated below each column.

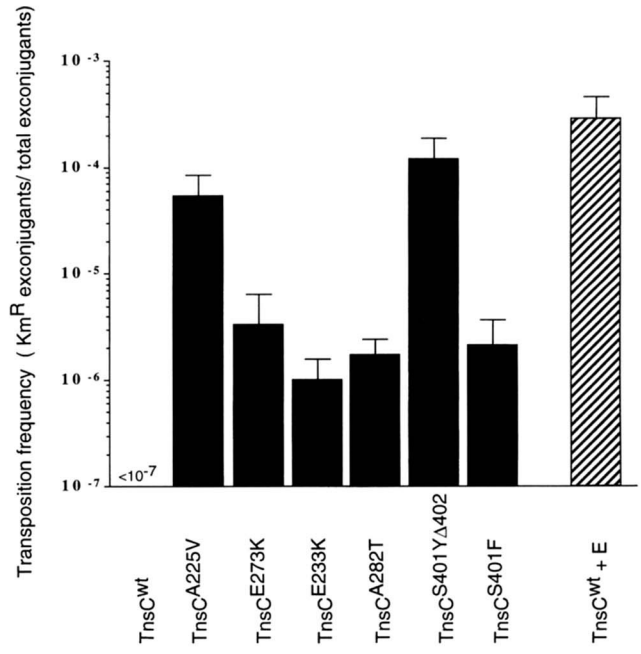

FIGURE 4.—TnsC mutants promote transposition to conjugable plasmids. Frequencies of transposition of miniTn *7*-Km^R from the chromosome to the conjugable target plasmid pOX-G were measured by the mating-out assay. TnsA+B was present in each strain; the TnsC species present is indicated below each column.

The mating-out assay was used to explore the ability of the TnsC mutants to promote translocations into a different type of target molecule. This assay measures the frequency of transposition of miniTn *7*-Km^R from the chromosome to pOX-G, a conjugable derivative of the *E. coli* F factor. The TnsABC^{wt} + E machinery preferentially selects conjugable plasmids as targets for transposition, whereas the TnsABC^{wt} + D machinery does not recognize pOX-G unless it contains *attTn7* sequences (ROGERS *et al.* 1986; WADDELL and CRAIG 1988; WOLKOW *et al.* 1996). The TnsC mutants could promote transposition to pOX-G (Figure 4), with the frequencies of transposition ranging over two orders of magnitude, from the modestly active TnsABC^{E233K} to the strongly active TnsABC$^{S401Y\Delta402}$. However, none of the mutant reactions promoted more transposition to pOX-G than TnsABC^{wt} + E. This is in contrast to results from the papillation and λ hop assays, in which several of the TnsC mutant reactions promoted more transposition than TnsABC^{wt} + E. Since the λ hop assay reports the total amount of transposition in a cell, rather than just transposition to pOX-G, we hypothesize that the majority of the transposition events activated by the TnsC mutants are directed to the chromosome. The distribution of TnsABC^{mutant} transposition events between the chromosome and pOX-G is addressed below.

Thus, we have demonstrated that the gain-of-function TnsC mutants can promote intermolecular transposition, using two independent assays. We have also looked directly for intramolecular rearrangements of the miniTn *7lac* donor plasmid in the papillation strains; no Lac$^+$ rearrangements were seen out of 24 papillae examined (data not shown). This suggests that the TnsC mutants primarily activate inter- rather than intramolecular transposition events.

Response to the target selectors TnsD and TnsE: TnsD and TnsE are required to activate the TnsABC^{wt} machinery and to direct transposition into particular target DNAs (ROGERS *et al.* 1986; WADDELL and CRAIG 1988; KUBO and CRAIG 1990; WOLKOW *et al.* 1996). The TnsABC^{mutant} machineries, by definition, do not require the inputs of TnsD or TnsE. However, we investigated whether TnsD or TnsE could influence the frequencies or distribution of transposition events promoted by the TnsC mutants.

The λ hop assay was used to evaluate the effects of TnsD and an available *attTn7* site on transposition promoted by the TnsC mutants (Figure 5). All of the mutant reactions were responsive to TnsD + *attTn7*, but those responses varied widely. Reactions activated by TnsABC^{A225V} and TnsABC^{E273K} were strongly stimulated by TnsD + *attTn7*, promoting 500- and 5000-fold more transposition, respectively, in the presence of TnsD + *attTn7* than with TnsABC^{A225V} or TnsABC^{E273K} alone. The remaining mutant reactions were less profoundly influenced by TnsD: TnsABC^{S401F} reactions showed a moderate (50-fold) stimulation, whereas reactions activated by TnsC^{E233K}, TnsC$^{S401Y\Delta402}$ and TnsC^{A282T} were somewhat inhibited in the presence of TnsD.

We also examined the effects of TnsE and the conjugable plasmid pOX-G on the transposition events activated by the TnsC mutants. Transposition frequencies were

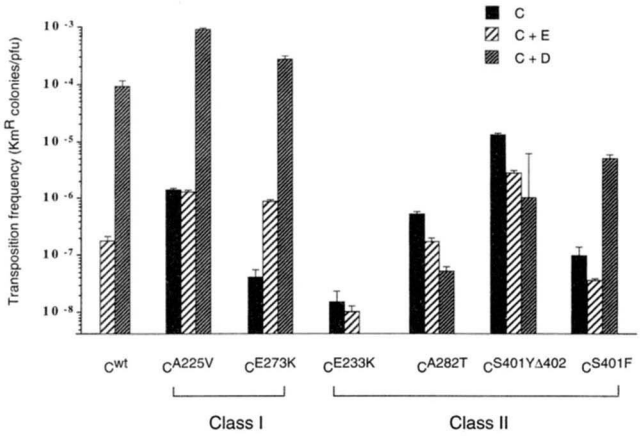

FIGURE 5.—Effects of the target selection factors TnsD and TnsE. Frequencies of transposition of miniTn *7*-Km^R from a λ phage to the chromosome and/or pOX-G were measured in strains containing TnsA+B and the TnsC mutants, either alone or in combination with TnsD or TnsE. The preferred target for TnsD reactions, *attTn7*, was present in the chromosomes of these strains. The preferred target for TnsE reactions, pOX-G, was introduced by conjugation into strains containing the TnsC mutants or the TnsC mutants + TnsE. The distribution of miniTn7-Km^R insertions between the chromosome and pOX-G was determined by mating the pOX-G plasmids from the Km^R products of a λ hop assay into the Km^S strain CW51, and testing whether Km resistance was plasmid linked. No transposition was detected in strains containing TnsABC^{wt} alone or TnsABC^{E233K} + TnsD.

measured by the λ hop assay (Figure 5), and the distribution of insertions between the chromosome and pOX-G was also determined (data not shown). In the absence of TnsE, the vast majority of the TnsABC^{mutant} transposition events were targeted to the chromosome rather than pOX-G. However, in the presence of TnsE, preferential insertion into pOX-G was observed with some of the TnsC mutants. TnsC^{E273K}-activated reactions were most clearly affected by TnsE: 20-fold more transposition was seen with TnsABC^{E273K} + TnsE than with TnsABC^{E273K} alone, and the majority of the TnsABC^{E273K} + TnsE transposition events were targeted to pOX-G. The distribution of insertions in TnsC^{A225V}-activated reactions was also affected by TnsE: targeting to pOX-G was detected in the majority of the TnsABC^{A225V} + TnsE reactions examined, although the frequency of transposition was unchanged. By contrast, transposition activated by the remaining TnsC mutants was predominantly targeted to the chromosome despite the presence of TnsE, and the frequencies of those reactions were unchanged or modestly inhibited by TnsE.

These differential responses suggest that the six TnsC mutants are not activating Tn7 transposition through a single mechanism. Instead, the mutants can be segregated into two classes, based on their ability to respond to TnsD and TnsE. Transposition activated by the Class I mutants, TnsC^{A225V} and TnsC^{E273K}, can be stimulated by TnsD and targeted to pOX-G by TnsE. Transposition activated by the Class II mutants — TnsC^{E233K}, TnsC^{A282T}, TnsC$^{S401Y\Delta402}$ and TnsC^{S401F} — is not responsive to the positive effects of TnsD or TnsE or both. By these criteria, TnsC^{S401F} is proposed to be a member of Class II: although TnsC^{S401F}-activated reactions are somewhat stimulated by TnsD, the distribution of insertions in TnsC^{S401F}-activated reactions is not affected by TnsE. The grouping of the TnsC mutants into these two classes is supported by the differential responses of the TnsABC^{mutant} reactions to immune targets, as described below.

TABLE 1

Sensitivity of TnsC mutant reactions to transposition immunity

	TnsC	Fold inhibition of insertion into immune target
	C^{wt} + E	85 (11)
Class I mutants	TnsC^{A225V}	104 (6)
	TnsC^{E273K}	50 (6)
Class II mutants	TnsC^{E233K}	3.6 (6)
	TnsC^{A282T}	0.6 (6)
	TnsC$^{S401Y\Delta402}$	2.0 (6)
	TnsC^{S401F}	0.9 (6)

The frequencies of miniTn *7*-Km^R transposition from the chromosome into the nonimmune target pOX-G and the immune target pOX-G::miniTn *7-dhfr* were determined for each TnsC species, and the fold reduction of insertions into the immune target is indicated. TnsA+B was present in each strain. The number of trials is given in parentheses; the variability of a given experiment was less than twofold.

Recognition of negative target signals: The Tn*7* transposition machinery evaluates potential target DNAs not only for positive signals, such as *attTn7*, but also for negative signals, such as the presence of a preexisting copy of Tn*7*. While TnsD and TnsE are clearly involved in the recognition of positive target signals, the mechanism by which negative target signals are recognized is unknown. Is this information also communicated by TnsD or TnsE, or is TnsC more directly involved? We examined whether transposition activated by the TnsC mutants was responsive to negative target regulation by comparing frequencies of insertion into immune and nonimmune pOX-G targets in the mating-out assay (Table 1). Consistent with previous observations (ARCISZEWSKA *et al.* 1989), TnsABC^{wt} + E transposition showed a strong immune response: insertions into the immune target were reduced 85-fold compared with insertions into the nonimmune target. Transposition activated by two of the TnsC mutants, TnsC^{A225V} and TnsC^{E273K}, was also sensitive to the immune target, showing 100- and 50-fold inhibition, respectively. Thus, a minimal transposition machinery involving only the TnsA+B transposase and TnsC can still identify and avoid immune targets. TnsD and TnsE are not required for the recognition of this negative target signal.

Transposition activated by the four remaining TnsC

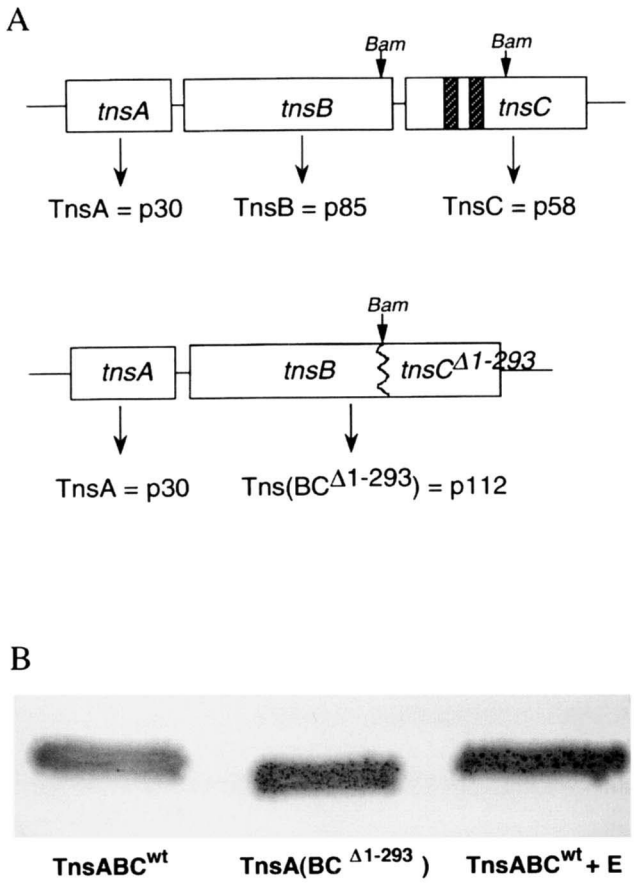

FIGURE 6.—The Tns(BC$^{\Delta 1\text{-}293}$) deletion-fusion mutant. (A) Construction of *tnsA(BC$^{\Delta 1\text{-}293}$)* from a *tnsABC* expression plasmid. Short arrows labeled "*Bam*" indicate *Bam*HI sites present in *tnsB* and *tnsC*; deletion of the intervening fragment resulted in the fusion of the 3′ half of *tnsC* to the end of *tnsB*. The molecular weights of the protein products are indicated below their respective genes; *tns(BC$^{\Delta 1\text{-}293}$)* encodes a protein of ~112 kD. Hatched boxes represent TnsC's ATP motifs, which are deleted from the Tns(BC$^{\Delta 1\text{-}293}$) mutant. (B) Papillation phenotypes of cells containing TnsA(BC$^{\Delta 1\text{-}293}$), as observed on MacConkey lactose plates after a 3-day incubation at 30°.

mutants was not dramatically inhibited by the immune target. Therefore, the ability to respond to immune targets segregates the TnsC mutants into the same two classes that were proposed based on the responses of the TnsC mutant-activated reactions to TnsD and TnsE. Transposition activated by the Class I mutants, TnsC^{A225V} and TnsC^{E273K}, maintains the ability to respond to both positive and negative target signals characteristic of wild-type Tn7 transposition. By contrast, the Class II TnsC mutants appear to promote transposition into any target DNA they encounter.

A TnsC deletion-fusion mutant that can activate transposition: TnsC is a multifunctional protein that can interact with both the target DNA and the transposase (GAMAS and CRAIG 1992; BAINTON *et al.* 1993; A. STELLWAGEN and N. L. CRAIG, unpublished results). An early report in the Tn7 literature, which predates the identification of the *tns* genes, implicates a domain of TnsC

TABLE 2

Tns(BC$^{\Delta 1\text{-}293}$) promotes intermolecular transposition

Tns functions	Transposition frequency
TnsABC^{wt}	$<10^{-7}$
TnsA(BC$^{\Delta 1\text{-}293}$)	$2.5 \pm 3.3 \times 10^{-6}$
TnsABC^{A225V}	$8.8 \pm 8.1 \times 10^{-6}$
TnsABC^{wt}DE	$5.5 \pm 1.1 \times 10^{-4}$

Frequencies of transposition of miniTn7-Km^R from a high copy plasmid to pOX-G were determined using the mating-out assay and are expressed as the number of Km^R exconjugants/total exconjugants. Each value is the average of three independent measurements.

in the process of activating the TnsA+B transposase. SMITH and JONES (1984) created a Tn7 element that produced TnsA and a deletion-fusion protein, in which the amino-terminal half of TnsC (including the Walker A and B motifs) was deleted and the remainder fused in frame to TnsB; this deletion-fusion is hereafter referred to as Tns(BC$^{\Delta 1\text{-}293}$). TnsA(BC$^{\Delta 1\text{-}293}$) was capable of promoting transposition, although at a frequency 1000-fold less than native Tn7. Unlike TnsABC^{wt}, TnsA(BC$^{\Delta 1\text{-}293}$) transposition did not require TnsE or intact TnsD. Thus, the Tns(BC$^{\Delta 1\text{-}293}$) deletion-fusion protein appears to be a gain-of-function mutant, with the same phenotype as the TnsC point mutants isolated in this work. However, interpretation of SMITH and JONES' results is complicated by ambiguities in their experimental design: it is unclear whether these transposition events were targeted to *attTn7* or not, or whether TnsA(BC$^{\Delta 1\text{-}293}$) activity required the nearly full-length TnsD polypeptide.

We reconstructed the Tns(BC$^{\Delta 1\text{-}293}$) deletion-fusion mutant using our *tnsABC* plasmid (Figure 6A), to confirm and extend the observations of SMITH and JONES. We found that TnsA(BC$^{\Delta 1\text{-}293}$) promoted as many Lac$^+$ rearrangements as did TnsABC^{wt} + E in the papillation assay (Figure 6B). Therefore, TnsA(BC$^{\Delta 1\text{-}293}$) can, in fact, execute transposition in the absence of TnsD or TnsE. The amount of transposition activated by the Tns(BC$^{\Delta 1\text{-}293}$) mutant was highly dependent on the transposition assay used. TnsA(BC$^{\Delta 1\text{-}293}$) could promote intermolecular translocation of miniTn7-Km^R from a high copy plasmid to pOX-G, at a frequency ~200-fold less than TnsABC^{wt}DE (Table 2). However, TnsA(BC$^{\Delta 1\text{-}293}$)-activated transposition of miniTn7-Km^R from the chromosome or from a λ phage was undetectable (data not shown). These variations suggest that transposition activated by Tns(BC$^{\Delta 1\text{-}293}$) may have a greater dependence on the donor site or the donor copy number than transposition activated by the TnsC point mutants.

We also investigated whether TnsA(BC$^{\Delta 1\text{-}293}$) transposition reactions were responsive to positive or negative target signals. The frequency of TnsA(BC$^{\Delta 1\text{-}293}$) transposition was not affected by the presence of TnsD + *attTn7* or TnsE + pOX-G in either the papillation or mating-

out assays (data not shown). Thus, Tns(BC$^{\Delta 1\text{-}293}$) mutant reactions do not appear to be influenced by these target selectors. We were unable to determine whether the Tns(BC$^{\Delta 1\text{-}293}$) reactions could recognize and avoid immune targets, due to high levels of recombination between the miniTn *7-dhfr* on the immune target plasmid and miniTn 7-Km^R on the high copy donor plasmid that confounded the detection of transposition events.

How has Tns(BC$^{\Delta 1\text{-}293}$) acquired the ability to activate transposition? Other experiments have revealed that it is the removal of the amino-terminal domain of TnsC, not the fusion to TnsB, which is responsible for this gain-of-activity (A. STELLWAGEN, F. LU, and N. L. CRAIG, unpublished results). An attractive hypothesis is that the carboxy-terminal half of the protein interacts with the transposase to activate transposition, whereas the amino-terminal half is required for the recognition of target DNA signals and/or the coupling of transposition activation to those signals.

Target DNA regulates the initiation of transposition *in vivo*: The order of events in the Tn *7* transposition reaction has been studied in detail *in vitro*. Tn *7* transposition uses a cut-and-paste mechanism, in which the element is first excised from the donor DNA by double-strand breaks, and then inserted into *attTn7* (BAINTON *et al.* 1991, 1993). The *attTn7* target is required for both steps in the reaction pathway: neither excision nor insertion is observed unless an *attTn7* target is present. This suggests that the nature of the target DNA regulates the initiation of Tn *7* transposition *in vitro*. Here we have investigated whether target DNA plays a similar role in controlling transposition initiation *in vivo*, by evaluating the effects of different target DNAs on the process of Tn *7* excision.

Chromosomal damage caused by Tn *7* excision could potentially induce the SOS response, the cellular response to DNA damage (WALKER 1996), as has been observed for other transposable elements (ROBERTS and KLECKNER 1988; LANE *et al.* 1994). Tn *7* transposition was evaluated in a strain in which *lacZ* expression served as a reporter of the SOS state of the cell. This strain contains a transcriptional fusion of *lacZ* to the pL promoter of a lysis-defective λ prophage (ELESPURU and YARMOLINSKY 1979); thus, *lacZ* expression is inhibited by λ repressor. In cells that have experienced DNA damage, λ repressor is cleaved as part of the SOS response (ROBERTS and ROBERTS 1975), and the subsequent increase in *lacZ* expression can be detected on MacConkey lactose color indicator plates or in bacterial extracts.

We observed that cells that were undergoing high frequency Tn *7* transposition into *attTn7* became Lac$^+$ (Table 3). Mobilization of a single copy of Tn *7* resulted in a small but reproducible increase in *lacZ* expression, detectable as a change in color of these cells on MacConkey lactose plates. However, cells containing two transposable elements, Tn *7* in the chromosomal *attTn7* site and miniTn 7-Km^R elsewhere in the chromosome, experienced more transposition events and had more *lacZ* expression. The chromosomal location of the miniTn 7-Km^R element was important: SOS induction was assayed in strains with miniTn 7-Km^R elements in 12 different locations, and the amount of *lacZ* expression varied considerably (data not shown). This could reflect different accessibilities of these DNA damage sites to the SOS-inducing machinery or the DNA repair apparatus, or the variations could reflect different transposition frequencies from these different donor sites. The observed Lac$^+$ phenotypes were dependent on RecA, the master regulator of the SOS response (WALKER 1996), and RecBCD, a DNA unwinding enzyme which is required to convert the double-strand breaks caused by some DNA damaging agents into SOS inducing signals (KARU and BELK 1982; CHAUDHURY and SMITH 1985) (data not shown). Cells undergoing Tn *7* transposition also had a fivefold higher frequency of spontaneous induction of a wild-type λ lysogen, another standard measure of SOS induction (data not shown).

All of the components necessary for high frequency Tn7 transposition — a transposon (line 2), the Tns proteins (line 3), and an *attTn7* target (line 7) — were required to trigger the SOS response. TnsE-mediated transposition to pOX-G did not induce a detectable SOS response, probably because these transposition events occurred at 1000-fold lower frequency than TnsD-mediated transposition to *attTn7*. Taken together, these results suggest that the process of Tn *7* transposition induces the cellular SOS response, and the strength of that SOS induction correlates with the amount of ongoing transposition.

What step in the transposition pathway is responsible for SOS induction? An attractive model is that the double-strand breaks left in the chromosome after transposon excision trigger the SOS response. SOS induction is also observed as a consequence of Tn *10* transposition (ROBERTS and KLECKNER 1988), and there is strong evidence that the excision of Tn *10* from the chromosome is the event that induces the SOS response: Tn *10* transposase mutants that can promote excision but not insertion maintain the ability to induce SOS (HANIFORD *et al.* 1989). If the breaks in the chromosome which result from Tn *7* excision are similarly responsible for SOS induction, then the observation that *attTn7* targets are required for SOS induction suggests that Tn *7* target sites are being evaluated before transposon excision *in vivo*. This would be consistent with results *in vitro*, in which breaks at the transposon ends are not observed unless *attTn7* is present (BAINTON *et al.* 1991). Therefore we propose that target DNA regulates Tn *7* excision *in vivo*, and that this feature of the reaction mechanism has been reproduced *in vitro*.

We also used the SOS induction assay to examine when negative target signals are evaluated. An immune target that contained an *attTn7* sequence and a miniTn 7-

TABLE 3

Tn7 transposition induces the cellular SOS response

Transposon[a]	Target[b]	Transposition frequency[c]		*lacZ* expression[d]	
		Tn7	miniTn7-Km^R	MacLac phenotype	Miller units
—	—	—	—	White	64 ± 4
—	pOX-*attTn7*	—	—	White	ND
miniTn7-Km^R	pOX-*attTn7*	—	—	White	ND
Tn7	—	—	—	White	ND
Tn7	pOX-*attTn7*	$8.9 \pm 3.7 \times 10^{-3}$	—	Pink	79 ± 11
Tn7 + miniTn7-Km^R	pOX-*attTn7*	$6.8 \pm 7.1 \times 10^{-2}$	$1.7 \pm 1.4 \times 10^{-2}$	Red	218 ± 69
Tn7 + miniTn7-Km^R	pOX-G	$1.5 \pm 1.8 \times 10^{-6}$	$1.8 \pm 1.9 \times 10^{-5}$	White	64 ± 9
Tn7 + miniTn7-Km^R	pOX-*attTn7*; EP-1::miniTn7-Cm^R	$2.3 \pm 0.8 \times 10^{-4}$	$8.4 \pm 2.0 \times 10^{-6}$	White	ND

[a] Tn7 was located in the chromosomal *attTn7* site and miniTn7-Km^R (which does not encode the Tns proteins) was located in the chromosomal position EC-2 (KUBO and CRAIG 1990) of the pL-*lacZ* transcriptional fusion strain BR293.

[b] Nonimmune targets were pOX-*attTn7* and pOX-G (which does not contain *attTn7*); the immune target was pOX-*attTn7* containing a miniTn7-Cm^R located in a position designated EP-1.

[c] Transposition frequency was measured by mating-out assays, with each value representing the average of at least five independent measurements.

[d] *lacZ* expression was evaluated on MacConkey lactose (MacLac) color indicator plates, or quantitated using the assay described by MILLER (1972), with each value representing the average of at least six independent measurements.

Cm^R element elsewhere in the plasmid was introduced into the SOS reporter strain. The frequency of insertion into this target was reduced more than 200-fold in comparison to a nonimmune *attTn7* target (Table 3). Transposon excision also appeared to be inhibited: no SOS response was observed in strains containing the immune target. Thus, negative features as well as positive features of a potential target molecule appear to be evaluated prior to the initiation of Tn7 transposition *in vivo*.

Finally, we examined whether the TnsC mutant reactions triggered the SOS response. In theory, TnsC mutants might exist that primarily stimulate transposon excision, perhaps by facilitating the assembly of the TnsA+B transposase with the transposon ends or by uncoupling the initiation of transposition from target regulation. The activity of such excision-promoting mutants might be underrepresented by insertion-based assays such as λ hops or mating-outs. Tn*10* transposase mutants that specifically activate excision have been described (HANIFORD *et al.* 1989). However, no SOS induction was detected in transposition reactions activated by any of the TnsC mutants (data not shown). This underscores the limited sensitivity of the SOS induction assay for Tn7 transposition: DNA damage apparently needs to occur at TnsD-activated frequencies to be detected. None of the TnsABC^{mutant} reactions generate as much insertion as TnsABC^{wt} + D reactions (Figure 3); the mutant reactions also appear to be promoting less excision than TnsABC^{wt} + D. Therefore, the six gain-of-function TnsC mutants identified in this work do not appear to be hyper-excision mutants, but instead promote both the excision and insertion of Tn7. Future studies may be able to exploit the SOS induction assay as a screen to identify hyper-excision mutants among the Tns proteins.

DISCUSSION

This work has investigated when and how target DNA regulates Tn7 transposition *in vivo*. We have presented experiments that suggest that positive and negative target signals are evaluated before Tn7 excision, providing inputs that control the initiation of transposition. Multiple transposition proteins are involved in target evaluation; here we have focused on the contributions of TnsC. We have isolated TnsC gain-of-function mutants that can activate the TnsA+B transposase without the target selection factors TnsD and TnsE. The TnsC mutant-activated transposition machinery can promote insertions into a variety of targets and, in some cases, still avoid unfavorable targets. This work supports the view that TnsC plays a key role in target evaluation and utilization.

Target evaluation occurs early in Tn7 transposition: Tn7 transposition is accomplished by two chemical steps, the excision of the element from its donor site and the insertion of the element into a target DNA (BAINTON *et al.* 1991). The nature of the target DNA could, in theory, regulate either or both of these steps: target DNA could provide a signal that allows the transposition reaction to initiate, and/or target DNA could be involved in late events such as the capture of the excised transposon. We have demonstrated here that Tn7 transposition induces the cellular SOS response, and we speculate that the primary event responsible for that induction is the excision of the transposon from the chromosome. If so, then the nature of the target DNA influences whether transposon excision will occur: favorable *attTn7* targets appear to provoke the double-strand breaks at the transposon ends that initiate Tn7 transposition, whereas unfavorable targets, such as im-

mune plasmids, do not promote these breaks. Thus, we propose that target DNAs provide both positive and negative signals that control the initiation of Tn*7* transposition *in vivo*. Similar conclusions have been reached from studies of the Tn*7* transposition reaction *in vitro*, in which the initiating double-strand breaks at the transposon ends can be examined directly. No breaks occur unless the transposition proteins have had a chance to interact with a nonimmune *attTn7* target (BAINTON *et al.* 1991, 1993). Thus the reaction pathway *in vitro* appears to accurately reflect the role of target DNA *in vivo* in regulating transposition initiation.

Coupling the initiation of transposition to the identification of a favorable target DNA could be advantageous for the transposon and its host. If transposition only initiated when it could be successfully completed, fewer copies of the transposon would be unproductively excised and lost, and less damage to the host chromosome would occur. However, this form of target regulation is not universally observed. The bacterial transposon Tn*10*, for example, follows a dramatically different reaction pathway *in vitro*. Tn*10* transposase can synapse the transposon ends and initiate cleavage at those ends before interactions with target DNA occur (SAKAI *et al.* 1995). In fact, the Tn*10* transposase cannot capture a target DNA unless the transposon end cleavages have occurred (KLECKNER *et al.* 1996). Bacteriophage Mu transposase (MuA) can also synapse and nick the Mu transposon ends before the introduction of target DNA into the reaction *in vitro* (CRAIGIE and MIZUUCHI 1987; SURETTE *et al.* 1987). It will be interesting to determine whether these assembly and cleavage steps remain uncoupled from target interactions *in vivo*. Recently, MuA transposase mutants have been identified that impose a dependence upon target DNA interactions on Mu transposition *in vitro* (MIZUUCHI *et al.* 1995). These mutants cannot interact with the Mu enhancer sequence, which normally promotes the assembly of the synaptic complex. Instead, the transposase mutants require an appropriate target DNA and the target-binding protein MuB to initiate transposition. The MuB-target complex has been proposed to serve as a scaffold that directs the assembly of the synaptic complex. Thus, Mu transposition as executed by these transposase mutants has become mechanistically more similar to Tn*7* transposition, in which transposition initiation is dependent on inputs from target DNA both *in vivo* and *in vitro*.

Proteins involved in target evaluation: How is an appropriate target for Tn*7* transposition identified? We have hypothesized that TnsC may serve as a "connector" or "matchmaker", linking the transposase and the target DNA in a manner regulated by the ATP state of TnsC (BAINTON *et al.* 1993; SANCAR and HEARST 1993). TnsC has the biochemical properties necessary for that connection: it can directly interact with target DNA (GAMAS and CRAIG 1992) and with the TnsA+B transposase (A. STELLWAGEN and N. L. CRAIG, unpublished results). However, wild-type TnsC is not sufficient to activate transposition. Instead, Tn*7* transposition is dependent on TnsD or TnsE to activate the TnsABC^{wt} machinery and select a target site. TnsD is an *attTn7* binding protein (BAINTON *et al.* 1993) that recruits TnsC to this target. The resulting TnsC-TnsD-*attTn7* complex can then attract the transposase *in vitro* (BAINTON *et al.* 1993). The mechanism by which TnsE activates transposition is not yet known. TnsE might be preferentially localized to conjugating plasmids and subsequently recruit TnsC to those molecules, or TnsE might modify TnsC so that TnsC's binding activity is now directed to those targets. Alternatively, TnsE might modify the transposase directly, without proceeding through TnsC. However, we are attracted to the hypothesis that TnsD and TnsE provide alternative inputs into TnsC, which in turn recruits the TnsA+B transposase to the target DNA.

The successful isolation of TnsC gain-of-function mutants reveals that the TnsABC machinery is capable of engaging target DNA and promoting insertions without TnsD or TnsE. However, the mutant reactions have not mimicked the abilities of TnsD or TnsE to direct transposition into particular targets: transposition activated by the TnsC mutants does not show the preferential insertion into conjugable plasmids seen with TnsE-activated reactions, nor the *attTn7* specificity of TnsD-activated reactions. Therefore, TnsD and TnsE are essential to recognize these positive target signals. However, we have demonstrated that TnsD and TnsE are not required to recognize the negative signal contained on immune targets. Transposition activated by a subset of the gain-of-function TnsC mutants maintains the ability of wild-type Tn*7* transposition to recognize and avoid targets that already contain a copy of Tn*7*. This strongly suggests that the ability to evaluate immune targets lies in TnsC and the transposase, not in the positive target selectors TnsD and TnsE.

TnsC is therefore hypothesized to receive a variety of inputs, from TnsD, TnsE and from immune targets, that control its activity. The activity of TnsC can also be influenced by mutation. Six gain-of-function TnsC point mutants have been described in this work, which segregate into two classes. The fact that we recovered different classes of TnsC mutants with different transposition activities is consistent with the hypothesis that there are multiple routes to activating TnsC. The Class I mutants, TnsC^{A225V} and TnsC^{E273K}, enable the TnsABC machinery to execute transposition without sacrificing its ability to respond to both positive and negative target signals. Both are substantial gain-of-function mutants, with TnsABC^{A225V} promoting eight-fold more transposition to the chromosome than TnsABC^{wt} + E (Figure 3). Transposition activated by these Class I mutants can be profoundly stimulated by TnsD + *attTn7* or directed to conjugable plasmids by TnsE, as well as being able to discriminate between immune and nonimmune targets.

Thus, the gain-of-function phenotypes seen with the Class I mutants have been achieved while preserving the ability of these TnsCs to transduce information between the target DNA and the transposase.

The TnsC mutants that fall into the second class behave much more like constitutively activated versions of TnsC. Some of these mutants also promote considerable amounts of transposition: TnsABC$^{S401Y\Delta402}$ results in 50-fold more transposition to the chromosome than TnsABC^{wt} + E (Figure 3). However, the nature of the transposition reactions promoted by the Class II TnsC mutants is quite different than those seen with the Class I mutants. Immune and nonimmune targets are used essentially equivalently in reactions with the Class II mutants, and TnsD and TnsE are not able to profoundly influence the frequency or distribution of these transposition events. Intriguingly, a similar loss of responsiveness to target signals is seen when Tn*7* transposition is activated by nonhydrolyzable ATP analogs *in vitro*. Transposition can still occur when TnsC's ATPase activity is blocked with AMP-PNP, but those transposition events no longer require TnsD and are no longer targeted to *attTn7* (Bainton *et al.* 1993). Instead, any DNA molecule, including immune targets, can serve as a target for Tn*7* insertion. Thus, TnsABC transposition can be constitutively activated by AMP-PNP or by the Class II TnsC mutants. It will be interesting to explore whether the ATP interactions of the TnsC mutants have been altered. In that regard, it is provocative that the amino acid affected in TnsC^{E233K} lies in one of TnsC's ATP motifs.

To be an effective communicator of multiple target DNA signals, TnsC is probably a multidomain protein. Some parts of TnsC may be involved in contacting target DNAs, other parts of TnsC may contact the transposase, and still other domains may bind and hydrolyze ATP. The TnsC point mutants are scattered across the TnsC protein sequence and could affect any or all of these activities. We have also analyzed a TnsC deletion mutant that may identify a domain in TnsC that contacts the TnsA+B transposase. Based on the observations of Smith and Jones (1984), we created a Tns(BC$^{\Delta1\text{-}293}$) deletion-fusion mutant in which the amino-terminal half of TnsC (including the Walker A and B motifs) is removed and the remainder of TnsC is fused to TnsB. Unlike TnsABC^{wt}, TnsA(BC$^{\Delta1\text{-}293}$) can activate transposition in the absence of TnsD or TnsE and without the ability to respond to these factors. Thus, Tns(BC$^{\Delta1\text{-}293}$) behaves like a Class II TnsC mutant. The deletion may have exposed a domain that interacts with (and is now covalently linked to) the transposase, whereas the activity of that domain may normally be regulated by ATP and/or inputs from target DNAs or target selection factors.

Comparison to other elements: The use of an ATP-dependent protein such as TnsC to regulate target site selection is not unique to Tn*7*. Bacteriophage Mu transposition is also profoundly influenced by its ATP-utilizing protein MuB. MuB is an ATP-dependent DNA binding protein (Chaconas 1985; Maxwell *et al.* 1987), which is required for efficient transposition *in vivo* (Faelen *et al.* 1978; O'Day *et al.* 1978). *In vitro*, the MuA transposase preferentially directs insertions into targets that are bound by MuB (Craigie and Mizuuchi 1987; Surette *et al.* 1987; Adzuma and Mizuuchi 1988). Although there is no particular sequence specificity to MuB binding, its distribution on DNA is not random: MuB binding to target molecules that already contain Mu sequences is specifically destabilized through an ATP-dependent mechanism (Adzuma and Mizuuchi 1988). Therefore Mu, like Tn*7*, recognizes and avoids immune targets; moreover, MuB and TnsC^{A225V} appear to play functionally similar roles in regulating transposition.

Mu and Tn*7* belong to a family of transposons that encodes proteins with ATP binding/hydrolysis motifs; other members include IS*21* (Reimmann *et al.* 1989; Koonin 1992), Tn*552* (Rowland and Dyke 1990), Tn*5053* (Kholodii *et al.* 1995), and Tn*5090* (Radstrom *et al.* 1994). Therefore, the strategy of using an ATP binding protein to regulate target site selection may extend to the entire family. Tn*5053* is particularly interesting, since it encodes three proteins that are required for its transposition: a presumptive transposase containing a D, D(35)E motif characteristic of transposases and integrases, a potential regulatory protein containing Walker A and B motifs, and a third protein of unknown function (Kholodii *et al.* 1995). Tn*5053* shows some degree of target site specificity, inserting predominantly into the *par* locus of the conjugable plasmid RP4. It is tantalizing to speculate that the third protein of Tn*5053* is a target selector, like TnsD or TnsE, directing insertions into the *par* locus.

This work has illustrated the role of target DNA in controlling Tn*7* transposition *in vivo* and has strongly implicated TnsC as a central player in this regulation. Single amino acid changes in TnsC can disrupt the communication between the transposon and the target site, reducing the stringency of Tn*7*'s target site selectivity. However, close relatives of Tn*7* containing functional equivalents of these TnsC gain-of-function mutants have yet to be identified. It will be interesting to see if such elements exist or whether the precise targeting conferred by TnsD and TnsE is more advantageous for Tn*7*'s survival and dissemination. TnsD promotes Tn*7* insertion at high frequency into *attTn7*, a safe haven in the bacterial chromosome, whereas TnsE allows Tn*7* access to conjugable plasmids, and thus a means to spread through bacterial populations. Avoidance of immune targets also promotes the spread of the element, rather than local hopping, and prevents one Tn*7* element from inserting into another. We hypothesize that TnsC integrates all of these target signals and communicates that information to the transposase.

Further genetic and biochemical analysis of the TnsC mutants will reveal the mechanism of that communication.

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