Tn7 Transposition: Target DNA Recognition Is Mediated by Multiple Tn7-Encoded Proteins in a Purified In Vitro System

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Summary

We have reconstituted the transposition of the bacterial transposon Tn7 into its specific insertion site attTn7 with four purified Tn7-encoded proteins, TnsA+TnsB+TnsC+TnsD, and ATP. TnsA+TnsB+TnsC form a "core" recombination machine that recognizes the transposon ends and executes DNA breakage and joining; TnsD specifically recognizes attTn7. TnsA+TnsB+TnsC are specifically targeted to attTn7 through the TnsD-dependent interaction of TnsC, a nonspecific DNA-binding protein, with attTn7. Recombination appears to be activated by the assembly of a nucleoprotein complex containing the DNA substrates and Tns proteins. We suggest that TnsC plays a central role in communication between the transposon and the target DNA, particularly in directing insertion away from DNAs already containing a copy of Tn7.

Introduction

Transposition is a DNA rearrangement reaction that involves three distinct DNA segments: the two transposon ends and an insertion site in the target DNA (reviewed by Berg and Howe, 1989). Critical steps in transposition include the choice of an insertion site and the DNA strand breakage and joining reactions at the transposon ends, which separate the transposon from the donor DNA and join it to the target DNA.

We are interested in understanding the mechanism of transposition of the bacterial transposon Tn7, which plays unusual target site selectivity (Barth et al., 1976; reviewed by Craig, 1989, 1991). Tn7 is distinguished by its ability to insert at high frequency into a specific site called attTn7 in the chromosomes of Escherichia coli and many other bacteria (Barth et al., 1976; Lichtenstein and Brenner, 1982; Craig, 1989, 1991). As is true in all other transposition reactions, no DNA sequence homology between the Tn7 ends and attTn7 is involved in the choice of attTn7 as an insertion site (Lichtenstein and Brenner, 1982; Gay et al., 1990; McKown et al., 1988). When attTn7 is unavailable, Tn7 resembles most other transposable elements, inserting at low frequency into many different sites with little obvious target sequence selectivity (Rogers et al., 1986; Waddell and Craig, 1988; Kubo and Craig, 1990). The frequency of Tn7 insertion into a particular target DNA molecule is also highly influenced by whether the target replicon already contains a copy of Tn7. If the target already contains Tn7, the frequency of the insertion of a second Tn7 into that DNA is greatly reduced (Hauer and Shapiro, 1984; Arciszewska et al., 1989). Such target immunity dictated by a preexisting transposon in the target DNA is also displayed by the bacterial transposons bacteriophage Mu and the Tn3-like elements (Robinson et al., 1977; Lee et al., 1983; Adzuma and Mizuuchi, 1988).

Tn7 transposition to its different classes of target sites is mediated by two distinct but overlapping sets of Tn7-encoded transposition genes (Rogers et al., 1986; Waddell and Craig, 1988; Kubo and Craig, 1990). tnsABC+tnsD promote target site-specific insertions into sites that are related in nucleotide sequence, i.e., into attTn7 at high frequency and into pseudo-attTn7 sites at low frequency. In the random target site pathway of Tn7, tnsABC+tnsE promote low frequency insertion into many different sites that are distinct from attTn7 and are not apparently related to each other. Thus, tnsABC provide functions common to both types of Tn7 transposition, and tnsD and tnsE are alternative target site-determining functions. Tn7 transposition immunity is active in both the tnsD- and tnsE-dependent pathways (Arciszewska et al., 1989).

Molecular dissection of the mechanism and control of Tn7 transposition requires a biochemical approach. We have extended our earlier biochemical analysis of Tn7 insertion into attTn7 via the tnsABC+tnsD pathway (Bainton et al., 1991) by reconstituting this reaction in vitro with ATP and purified TnsA, TnsB, TnsC, and TnsD, establishing that the Tns proteins participate directly in recombination. What are the roles of these proteins? We demonstrate here that TnsD is a sequence-specific DNA-binding protein that binds to attTn7 and also show that the same sequences in the attTn7 region that are involved in specific interaction with TnsD provide attTn7 target activity, i.e., can promote high frequency, site-specific insertion of Tn7. These findings suggest that specific recognition of attTn7 by TnsD is the critical determinant in attTn7 target activity. We also establish that TnsA+TnsB+TnsC provide a core recombination machine that mediates the DNA strand breakage and joining reactions that underlie transposition and that this core machine does not display target site selectivity; these proteins alone can carry out insertion into many random target sites in the presence of the alternative cofactor AMP-PNP instead of the usual ATP cofactor. We propose that the critical role of TnsD in Tn7 insertion into
into its preferred target site, \textit{attTn7}, using four highly purified fractions containing TnsA, TnsB, TnsC, or TnsD (see below). We have previously described the purification of TnsB (Arciszewska et al., 1991) and TnsC (Gamas and Craig, 1992); we also report here the purification of TnsA and TnsD (Figure 1). TnsA was purified using a glutathione S-transferase (GST)-TnsA fusion protein; we have been unable to associate any partial transposition activity, such as DNA binding or cleavage, with TnsA (data not shown).

Purification of a \textit{tn.0} dependent \textit{attTn7} DNA binding activity selects for TnsD (Table 1); as described below, TnsD binds specifically to \textit{attTn7}.

\textit{Tn7} transposes efficiently in vitro when the substrate DNAs, a donor plasmid containing a mini-\textit{Tn7} element and a target plasmid containing \textit{attTn7}, are incubated at 30°C with highly purified Tns fractions, ATP, magnesium acetate (MgAc), and small molecules (Figure 2). Transposition is evaluated by extraction of DNA from the incubation mixtures, digestion with restriction enzymes, separation by gel electrophoresis, and hybridization with specific probes. Using a mini-\textit{Tn7} probe specific to sequences between the Tn7 ends, we observe that more than 80% of the donor substrate can be converted to simple insertion product (lane 1). The other product of transposition, the donor backbone from which the element is excised, is also present but is not detectable by this hybridization probe. More than 95% of the transposition products result from the site- and orientation-specific insertion of \textit{Tn7} into \textit{attTn7} (data not shown). Transposition proceeds via a non-replicative mechanism in which \textit{in n} is excised from the donor and specifically inserted into \textit{attTn7} (Figure 3).

Also detectable with the reconstituted system are several DNA species shown in the crude transposition system (Bainton et al., 1991) to be transposition intermediates, donor molecules broken by a single double-strand break between the backbone and the transposon (DSB.L; double-strand break, left end; or DSB.R, double-strand break, right end) and excised linear transposons (ELTs) cut away from the donor backbone by two double-strand breaks (Figure 3). DSBs in the purified system also display the kinetic properties of transposition intermediates (data not shown).

We have also observed in the reconstituted system DNA species in which the exposed left transposon ends of DSB.Ls are apparently joined to a single DNA strand of the \textit{attTn7} target; these species are called DSB-SEJ.L (single-end join of the left end of a DSB.L, as shown schematically in Figure 3; Figure 2, lane 1). Using different restriction analyses, a DSB-SEJ.R (single-end join of the right

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Fraction & Volume (ml) & Total Protein (mg) & Total Activity (U) & Specific Activity (U/mg) & Purification (fold) & Yield (%) \\
\hline
I. Cleared lysate & 130 & 6,240 & 240,000 & 38 & (1) & (100) \\
II. Polyethylenimine-ammonium sulfate & 360 & 3,600 & 180,000 & 50 & 1.3 & 75 \\
III. Affigel-heparin & 52 & 1.3 & 42,000 & 32,000 & 840 & 18 \\
IV. Green 1 & 5.4 & 0.16 & 16,000 & 100,000 & 2,630 & 6.7 \\
\hline
Specific activities of the pooled fractions were compared using the mobility shift assay to determine TnsD binding to \textit{attTn7}. A unit of activity (U) was defined as the amount of protein required to shift 10% of the \textit{attTn7} fragment in the presence of an E. coli lysate.
\end{tabular}
\caption{TnsD Purification}
\end{table}

\textit{Results}

\textbf{Reconstitution of \textit{Tn7} Transposition In Vitro with Purified Tns Proteins and ATP}

The development of a cell-free \textit{Tn7} transposition system using crude extracts has been previously reported (Bainton et al., 1991). We have now reconstituted \textit{Tn7} insertion

\textit{attTn7} is to recruit the core machine to this particular target site.

We also provide evidence here that TnsC plays a critical role in target DNA selection. We show that TnsC, an ATP-dependent non-sequence-specific DNA-binding protein (Gamas and Craig, 1992), can also specifically interact with \textit{attTn7} target DNA, likely through binding to TnsD. Our finding that \textit{Tn7} target immunity is abolished in the presence of the alternative AMP-PMP cofactor supports the proposal that TnsC plays an important role in target DNA selection.

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TnsD Binds Specifically to attTn7

TnsD is a sequence-specific DNA-binding protein that recognizes attTn7: TnsD forms a discrete, retarded complex in a gel mobility shift assay with attTn7 fragments (Figure S5A, lanes 5 and 11) not detectable with a non-attTn7 fragment (lane 8). Characterization of the TnsD–attTn7 interaction by footprint analysis revealed a protected region located entirely rightward of the specific Tn7 insertion point (data in Figure 6A; summary in Figure 7). The region of TnsD protection spans a ~30 bp region extending outward from a position about 25 bp rightward of the specific insertion point (+25 to +55).

The retarded complex observed with purified TnsD and attTn7 (Figure S5A, lane 5) is distinct in mobility from the complex observed with tnsD crude extract (lane 3); the addition of crude extract from a cell lacking TnsD to purified TnsD does reconstitute the crude extract tnsD-dependent complex (lane 4). Addition of host extract to purified TnsD also stimulates up to 10-fold the amount of complex observed (compare lanes 4 and 5). This host factor is not IHF or HU, as neither of these proteins obviously affects TnsD–attTn7 binding (K. K., O. Hughes, and N. L. C., unpublished data). This TnsD–host complex is not ob-
Figure 3. The Transposition Substrates, Intermediates, and Products

The 6.1 kb donor molecule contains a 1.6 kb mini-Tn7 element flanked by sequences unrelated to attTn7; the mini-Tn7 element contains segments from the left (Tn7L) and right (Tn7R) ends of Tn7 that provide the cis-acting transposition sequences. The 2.9 kb target molecule contains a 0.15 kb attTn7 segment flanked by EcoRI sites. Double-strand breaks at the junction of either Tn7L or Tn7R with the donor backbone (arrows) produce the DSB.L and DSB.R species, which are transposition intermediates. An ELT is generated by a second double-strand break in the DSBs. The excised mini-Tn7 element inserts site and orientation specifically into attTn7, producing a 4.5 kb simple insertion product and a 4.5 kb gapped donor backbone. EcoRI digestion of the simple insertion product releases a diagnostic 1.75 kb fragment. Other transposition species are SEJs made by a single Tn7 end from either a DSB or ELT intermediate joining to a target molecule. The identities of these species were determined by digestion with a variety of restriction enzymes and hybridization to specific probes. Restriction sites: E, EcoRI; B, BglII; S, ScaI.

served with Tns fractions highly active in transposition in vitro (data not shown) and thus does not appear to be essential to Tn7 recombination.

Nucleotide Sequences Required for attTn7 Target Activity and TnsD attTn7 Binding

How is TnsD binding to attTn7 related to attTn7 Tn7 target activity, i.e., to the ability to promote high frequency, site- and orientation-specific Tn7 insertion? A plasmid containing a short attTn7 segment (+23 to +58), in which attTn7 sequences leftward of +23, including the insertion point, have been replaced by heterologous sequences, has target activity comparable with a larger attTn7 segment (-52 to +64) that provides wild-type activity (Table 2). Tn7 insertion into attTn7 (+23 to +58) occurs with duplication of five target base pairs centered at either the 0 or +1 position (data not shown); such "wobble" is also observed with larger attTn7 segments (Gringauz et al., 1988; Bainton et al., 1991). Thus, the sequences that can promote high frequency and site-specific attTn7 target activity correspond to those involved in TnsD binding.

It has previously been reported that an even smaller attTn7 (+28 to +55) fragment has much diminished attTn7 target activity (Waddell and Craig, 1989). Although TnsD–host binding to attTn7 (+28 to +55) is observed (Waddell and Craig, 1989; Figure 5A, lane 13), we have now established that attTn7 (+28 to +55) does not bind TnsD as effectively (lane 14) as do larger attTn7 fragments that have full attTn7 target activity in vivo (lanes 5 and 11). The correlation of impaired attTn7 target activity and TnsD binding activity supports the hypothesis that TnsD binding is the critical determinant of attTn7 target activity.
Figure 4. Tn7 Transposition In Vitro Is Stimulated by a Preincubation Step That Lacks MgAc

Shown is an autoradiogram of transposition reactions, performed as described in Experimental Procedures, that displays Bglll-digested species detected with a mini-Tn7-specific probe. Lanes 1-3, MgAc was omitted entirely from the reaction (lane 1), added at the beginning of the reaction (lane 2), or added after a 30 min preincubation step performed in the absence of MgAc (lane 3). Lanes 4-8, indicated reaction components were initially omitted and added to reactions after the 30 min preincubation step lacking MgAc.

Formation of a TnsC-TnsD-attTn7 Complex

In the presence of ATP, TnsC and TnsD can form a distinct complex on attTn7. Although TnsC can bind to DNA, no binding of TnsC to a labeled attTn7 fragment is observed in a gel mobility shift assay because of the presence of a large excess of unlabeled competitor DNA (Figure 5B, lane 2). However, when TnsC and ATP are mixed with TnsD and the attTn7 fragment, a novel protein-DNA complex is observed (lane 4) that is distinct from the TnsD-attTn7 complex (lane 3). Formation of this novel complex requires ATP (lanes 6 and 7). Formation of the ATP-dependent TnsC-TnsD-attTn7 complex is specific to TnsC; no such complex is observed when TnsA or TnsB is substituted for TnsC (data not shown). Formation of the TnsC-TnsD-attTn7 complex is dependent on attTn7 as well as TnsD, as no complexes are observed with a non-attTn7 fragment (lane 9).

The TnsC-TnsD complexes detected by mobility shift assay appears to correspond to attTn7 complexes formed during the preincubation step of the recombination reaction, since formation of the shifted complex is also blocked by the presence of MgAc (lane 5). Furthermore, although formed under somewhat different conditions, the TnsC-TnsD-attTn7 complexes detected by gel shift are competent transposition substrates (data not shown).

Characterization of the TnsC-TnsD-attTn7 complex by footprint analysis revealed a protection pattern distinct from that observed with TnsD-attTn7: the TnsC+TnsD pattern is larger and the degree of protection much greater. In the presence of TnsC+TnsD, the protected region spans about 55 bp (about +7 to +60) and is principally extended toward the point of Tn7 insertion, unlike the pro-

Figure 5. Analysis of TnsD and TnsC-TnsD Binding to attTn7 by Mobility Shift Assays

(A) TnsD interacts specifically with attTn7. The DNA fragments include: lanes 1-5, attTn7 (-25 to +64); lanes 6-8, non-attTn7 sequences; lanes 9-11, attTn7 (+23 to +58); lanes 12-14, attTn7 (+28 to +55). As indicated by plus signs, protein additions were crude host lysate (5 ug), crude TnsD lysate (5 ug), and TnsD (15 ng of fraction V).

(B) Formation of ATP-dependent TnsC-TnsD complexes on attTn7. The DNA fragment in lanes 1-7 includes attTn7 (-25 to +64); the DNA fragment in lanes 8 and 9 lacks attTn7 sequences. Binding reactions indicated by plus signs include TnsC (20 ng), TnsD (20 ng), and exogenous ATP. In lane 5, MgAc was added to 15 mM at the beginning of the binding reaction, and in lane 7, ADP (2.0 mM) is substituted for ATP.
Figure 6. Footprinting Analysis of TnsD and TnsC–TnsD Complexes with attTn7

Protein complexes with attTn7 were formed and treated with micrococcal nuclease (A) or DNAase I (B and C), and the DNA was recovered from isolated complexes following electrophoresis on a polyacrylamide gel. Top and bottom strands of attTn7 and the numbering of the nucleotide positions are as shown in Figure 7. The vertical lines indicate the extent of protection, and asterisks indicate individual protected positions observed outside the contiguous protection region. Lanes marked AG contain a Maxam–Gilbert A+G reaction of the same fragment.

(A) Micrococcal nuclease footprint of the TnsD–attTn7 complex. Left (lanes 1 and 2) and right (lanes 3 and 4) sections show top and bottom strands, respectively. Proteins added were: lanes 1 and 3, TnsD (2 μg/ml); lanes 2 and 4, no addition.

(B) DNAase I footprint of the top strand in TnsD–attTn7 and TnsC–TnsD–attTn7 complexes. The left (lanes 1 and 2) section shows TnsD–attTn7, and the right shows TnsC–TnsD–attTn7 complexes. Proteins added were: lane 1, TnsD (2 μg/ml); lane 2, no addition; lane 3, TnsC (5 μg/ml) and TnsD (2 μg/ml); lane 4, no addition.

(C) DNAase I footprint of the bottom strand in TnsD–attTn7 and TnsC–TnsD–attTn7 complexes. The left (lanes 1 and 2) section shows TnsD–attTn7, and the right (lanes 3 and 4) shows TnsC–TnsD–attTn7 complexes. Proteins added were: lane 1, no addition; lane 2, TnsD (2 μg/ml); lane 3, no addition; lane 4, TnsC (5 μg/ml) and TnsD (2 μg/ml).

Figure 7. Summary of attTn7 Nuclease Protection Experiments

The attTn7 sequence is shown, with boxed nucleotides indicating the 5 bp sequence duplicated upon Tn7 insertion (Lichtenstein and Brenner, 1982; Gay et al., 1990; McKown et al., 1988). The central base pair of the target duplication is designated 0; sequences extending to the left are given negative values and those to the right positive values. Thick bars above and below the sequence show the extent of protection on the top and bottom strands, respectively, in the presence of the indicated Tns proteins. The bars extend to the outermost positions that are inaccessible to DNAase I and micrococcal nuclease (MN), and arrows designate accessible positions within protected regions (arrows marked X indicate enhanced cleavage positions). The hatched portion designates a region of partial protection. Open circles mark the positions nearest the protected regions that are accessible. The dashed line indicates the modest protection observed for TnsD on the top strand.
Recognition of a Specific Tn7 Insertion Site

Table 2. Evaluation of attTn7 Target Activity

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>attTn7 Sequence</th>
<th>Transposition/pfu (mean ± SEM [n])</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSK⁺</td>
<td>None</td>
<td>(2.5 ± 2.5) x 10⁻¹ [2]</td>
</tr>
<tr>
<td>pkk26</td>
<td>-52 to +64</td>
<td>(2.5 ± 2.1) x 10⁻² [3]</td>
</tr>
<tr>
<td>pkk28</td>
<td>+23 to +58</td>
<td>(3.7 ± 2.7) x 10⁻³ [3]</td>
</tr>
</tbody>
</table>

λ hop assays were performed in LA3, an attTn7::Tn7 strain, containing the indicated plasmids. Translocation of a kanamycin-resistant mini-Tn7 element from a replication- and integration-defective phage X derivative after phage infection was evaluated by the appearance of kanamycin-resistant colonies. pfu, plaque-forming units; SEM, standard error of the mean.

* LA3 cells lacking any plasmids show a similar transposition frequency (Kubo and Craig, 1990).

The ability of Tn7s to insert specifically and selectively into attTn7 is not entirely abolished when AMP-PMP is used as a cofactor instead of ATP. In TnsA+TnsB+TnsC+TnsD reactions performed in the presence of AMP-PMP, specific insertions into attTn7 and random insertions into the target backbone are both observed (Figure 8A, lane 6). AMP-PMP is also an effective cofactor in promoting the formation of TnsC–TnsD–attTn7 complexes, as evaluated in mobility shift assays (data not shown). These data suggest that although AMP-PMP can dramatically change the insertion specificity, it does not abolish the ability of TnsD to direct TnsC to attTn7.

![Figure 8. Tn7 Transposition In Vitro in the Presence of AMP-PMP](image)

(A) TnsA+TnsB+TnsC can promote efficient transposition in the presence of AMP-PMP. EcoRI-digested species detected with the mini-Tn7-specific probe are shown from transposition reactions performed in the presence of ATP (lanes 1–3) or AMP-PMP (lanes 4–6). In lanes 3 and 5, a target plasmid (pBluescript KS(+) that lacked attTn7 sequences was used instead of the standard attTn7 plasmid (pKAO4-3) and an attTn7 plasmid (pLA11) that also contained a Tn7R segment located about 1 kb from the attTn7 target site. The concentration of the target plasmids is 3-fold less than that of the standard conditions, as described in Experimental Procedures. Reactions were performed in the presence (lanes 1–4) or absence (lanes 5–8) of TnsD.

(B) Tn7 transposition immunity is altered by the presence of AMP-PMP. EcoRI-digested species detected with a mini-Tn7-specific probe are shown from transposition reactions using the standard attTn7 target plasmid (pKAO4-3) and an attTn7 plasmid (pLA11) that also contained a Tn7R segment located about 1 kb from the attTn7 target site. The concentration of the target plasmids is 3-fold less than that of the standard conditions, as described in Experimental Procedures. Reactions were performed in the presence (lanes 1–4) or absence (lanes 5–8) of TnsD.
A prominent feature of the standard Tn7 transposition reactions performed with ATP is that recombination is most efficient when TnsC, TnsD, and attTn7 and A1p are preincubated in the absence of MgAc to facilitate the formation of TnsC-TnsD complex on the target DNA. In TnsA+TnsB+TnsC reactions with AMP-PMP, recombination also proceeds more efficiently when TnsC and the target DNA and nucleotide are preincubated prior to the addition of MgAc and the other reaction components (data not shown).

Lack of Target Immunity in Tn7 Transposition In Vitro with AMP–PMP

Tn7 displays transposition immunity in vivo; in other words, DNA molecules that already contain Tn7 are poor targets for transposition. The presence in the target DNA of the sequences from the right end of Tn7 that participate directly in recombination is sufficient to provide immunity (Arciszewska et al., 1989). We previously established that Tn7 immunity is active in vitro in the crude extract system (Bainton et al., 1991). Tn7 transposition immunity is also active in the reconstituted TnsA+TnsB+TnsC+TnsD system when ATP is used as a cofactor (lane 2). In these reactions with ATP, which include both a standard attTn7 target plasmid and an immune attTn7 plasmid (i.e., one containing both attTn7 and a segment that includes the right end of Tn7 cis-acting transposition sequences located more than 1 kb from the attTn7 target site), no insertions into the immune target (5.3 kb) are observed, although the standard attTn7 plasmid remains an effective target (1.75 kb); this is consistent with target immunity working only in cis on the target plasmid, which already contains a Tn7 element. Transposition immunity is virtually abolished, however, when AMP–PMP is used as a cofactor instead of ATP (lane 4). In the presence of AMP–PMP, insertions occur into the plasmid target (5.3 kb) and, as expected, into attTn7 (1.75 kb) and the target backbone (4.2 kb) of the nonimmune target plasmid. A similar result is also seen in the absence of TnsD (lane 8).

Discussion

Tn7 Transposition Is Mediated by Four Tn7-Encoded Proteins

We have extended our biochemical analysis of Tn7 transposition, establishing here that Tn7 insertion into its preferred target site, attTn7, occurs efficiently with and requires four purified Tn7-encoded proteins, TnsA, TnsB, TnsC, and TnsD, and ATP. The direct participation of the Tns proteins in recombination accounts for the fact that Tn7 transposition in vivo requires these tns genes (Rogers et al., 1988, Waddell and Craig, 1989). TnsA+TnsB+TnsC form the core transposition machinery that specifically recognizes Tn7 and executes DNA strand breakage and joining. We have also presented evidence that TnsC plays a central role in mediating communication between the target DNA and the transposon ends. Application of this core machinery to attTn7 results from the positioning of TnsC, an ATP-dependent, nonspecific DNA-binding protein (Gammas and Craig, 1992), on attTn7 through collaboration of TnsC with TnsD, an attTn7-specific DNA-binding protein. Other experiments have shown that TnsB binds specifically to the cis-acting recombination sequences at the Tn7 ends (Arciszewska et al., 1991; Tang et al., 1991). The specific role of TnsA has not yet been identified. The highly coupled nature of Tn7 transposition, in which the presence of all the recombination proteins and attTn7 is required for the production of recombination intermediates and products, indicates that recombination actually occurs within a nucleoprotein complex containing the Tns proteins and three DNA substrates: the Tn7 ends and attTn7. Assembly of this complex is apparently required for the initiation of recombination; the DNA cleavages that separate the transposon from flanking donor DNA.

attTn7 Target Activity Is Mediated by the Specific DNA Binding Activity of TnsD

It has previously been proposed that TnsD is a target DNA-binding protein that specifically recognizes attTn7 (Waddell and Craig, 1989; Kubo and Craig, 1990). By characterization of purified TnsD, we have now shown directly that TnsD recognizes attTn7 and have determined that the sequences required for TnsD binding closely correlate with the sequences required for attTn7 target activity; i.e., they result in the high frequency, site- and orientation-specific insertion of Tn7. Notably, the sequences required for attTn7 target activity do not include the actual point of Tn7 insertion; the TnsD interaction sequences span a region of 30 nt positioned about 25 nt from the point of insertion. Interestingly, these TnsD sequences lie within the carboxy-terminal coding sequence of the bacterial glmS gene, which is involved in cell wall biosynthesis. Tn7 insertion occurs downstream of glmS, thereby avoiding inactivation of this essential gene. The likely presence of glmS-like genes in a number of different bacteria may also explain the ability of Tn7 to insert in a site-specific manner in a wide range of bacteria (reviewed by Craig, 1989; Gadri et al., 1989). TnsD-dependent insertion also occurs at low frequency into pseudo-attTn7 sites, which are related in sequence to attTn7 (Kubo and Craig, 1990). The region of homology between pseudo-attTn7 sites and attTn7 coincides with the attTn7 TnsD recognition sequences, suggesting that TnsD directs insertion into pseudo-attTn7 sites by binding to these sites. An attractive hypothesis is that the low target activity of the pseudo-attTn7 sites reflects reduced TnsD binding.

TnsC Is Directed to attTn7 by TnsD

Several observations provide evidence that TnsC, an ATP-dependent, nonspecific DNA-binding protein, is directed to attTn7 by TnsD. We have observed the formation of specific TnsC–TnsD–attTn7 complexes in the presence of ATP by gel mobility shift and DNA footprint assays and have also found that recombination in vitro is stimulated by conditions that favor the formation of such complexes. We propose that TnsC is directed to attTn7 by interaction with TnsD. No specific interaction of TnsC with attTn7 is observed in the absence of TnsD; furthermore, no specific
Recognition of a Specific Tn7 Insertion Site

The thin horizontal line represents a BTn7 sequences, with 0 indicating the center base pair of the 5 bp (hatched box) usually duplicated upon insertion (arrowheads); the thick horizontal line indicates the sequences sufficient to provide attTn7 target activity. The boundaries of DNAase I protection proved by TnsD alone (top line) and TnsC+TnsD (bottom line) on attTn7 are indicated by ovals. We propose that the rest of the transposition machinery, the ends of Tn7, TnsB, and TnsA, are directed to the point of insertion by interaction with TnsD on the target DNA.

Figure 9. A Model for Tn7 Transposition

The picture that has emerged from our Tn7 experiments provides the critical specificity determinant by directing the nonspecific TnsA+TnsB+TnsC machine to attTn7. Since TnsC is a DNA-binding protein whose apparent affinity for DNA is increased in the presence of nonhydrolyzable ATP analogs such as AMP-PMP (Gamas and Craig, 1992), we suspect that AMP-PMP promotes recombination under conditions in which ATP does not (i.e., in the absence of TnsD) by promoting a more stable interaction of TnsC with DNA.

The hypothesis that the ATP-dependent interaction of TnsC with the target DNA plays a central role in Tn7 transposition is also supported by the differential effects of ATP and AMP-PMP on target immunity in vitro. The frequency of Tn7 insertion in vivo into a target DNA is much reduced by the presence of Tn7 or even just the right end of Tn7 in that DNA; that is, a Tn7-containing target is immune (Arciszewska et al., 1989). In the presence of ATP, Tn7 target immunity is active in the crude extract (Bainton et al., 1991) and reconstituted transposition systems. By contrast, target immunity is abolished when AMP-PMP is present: insertion into a target containing a Tn7 end occurs efficiently in the presence of this ATP analog. Thus, the nature of the adenine cofactor has a profound impact on target immunity, in other words, on the ability of the Tn7 transposition machinery to evaluate the target DNA for the presence of a Tn7 end. An attractive hypothesis is that TnsC mediates the recognition of immune and nonimmune target DNAs through its ATP-dependent binding to DNA. We postulate that in the presence of AMP-PMP, TnsC binds stably to all target DNAs, including those containing Tn7 ends, and that when ATP is used as a cofactor, the binding of TnsC to immune targets is discouraged. In this view, immune DNA molecules (those that contain a Tn7 end) are poor targets for transposition, because TnsC cannot effectively interact with these DNAs.

Our proposal for the role of TnsC in Tn7 transposition is reminiscent of the proposed roles of MuB in bacteriophage Mu transposition. MuA protein, a Mu end-binding protein (Craigie et al., 1984), ATP, and MuB collaborate to promote recombination (Surette et al., 1987; Craigie and Mizuuchi, 1987). Like TnsC, MuB is a nonspecific DNA-binding protein (Chaconas et al., 1985) that requires ATP for stable association with DNA; upon ATP hydrolysis, MuB dissociates from DNA (Adzuma and Mizuuchi, 1988, 1989). MuB can stimulate Mu transposition by interacting with both the target DNA and MuA bound to the transposon ends (Baker et al., 1991; Surette et al., 1991; Surette and Chaconas, 1991). MuB plays a key role in Mu target immunity (Adzuma and Mizuuchi, 1988, 1989). MuB selectively dissociates from immune target DNAs through stimulation of its ATPase activity, which is provoked by an interaction with MuA bound to the transposon ends on the immune target; transposition immunity is abolished by the presence of the nonhydrolyzable ATP analog, which promotes the stable interaction of MuB with an immune target DNA.

The picture that has emerged from our Tn7 experiments is that TnsC can interact with the target DNA during transposition, insertion being directed to DNAs on which TnsC is stably associated. An attractive view is that TnsC both
contacts the target DNA and communicates with TnsA, TnsB, or both, perhaps thereby mediating interactions with the transposon ends.

A Proposal for the Mechanism of TnsE-Dependent Tn7 Transposition
We have suggested that during TnsD-dependent transposition the target site is specifically recognized by the binding of TnsD and that the interaction of TnsD with TnsC then stably positions TnsC (and consequently TnsA, TnsB, and the Tn7 ends) on the target DNA, thereby provoking transposition. What relationship might the mechanism of TnsE-mediated transposition to random target sites have to site-specific TnsD-dependent transposition? We presume (but have yet to show directly) that TnsE directs the basic TnsA+TnsD+TnsC machine to promote Tn7 insertion. It seems likely that the overall mechanisms of TnsD- and TnsE-dependent transposition reactions are similar. TnsC interaction with the target DNA being central to both pathways, but that the reactions are distinguished most by the way particular target sites are chosen, i.e., the way TnsC is stably directed to a target site. Perhaps TnsE interacts with TnsC and is, like TnsD, a DNA-binding protein, but one with a different sequence preference; TnsE may therefore fix TnsC to target DNA at many different sites and provoke transposition. Alternatively, TnsE itself may not interact directly with DNA, but may function exclusively as an effector to modulate the ability of TnsC to interact stably with DNA, for example, by influencing the interactions of TnsC with ATP.

These models suggest that two alternative types of nucleoprotein complexes, one with TnsD and one with TnsE, may mediate Tn7 transposition. The two complexes could share the same basic machinery, TnsA+TnsB+TnsC, but a nucleoprotein complex with TnsD directs insertions to attTn7 and a TnsE-dependent complex promotes insertions to random sites. This combinatorial assembly of nucleoprotein complexes provides regulated and differential activities analogous to other processes involved in recombination, replication, transcription, and translation (Echols, 1990; Hershey, 1991). The study of Tn7 transposition in vitro provides a defined and readily manipulable system that can be used to dissect the mammalian recombination mechanisms that underlie such elaborate protein--nucleic acid transactions.

Experimental Procedures
tns Plasmids and Bacterial Strains
TnsA was expressed as a GST fusion protein in E. coli strain DH5a (Bethesda Research Laboratories). The gene construct encoding the GST--TnsA fusion was generated by inserting a Ncol-Hindlll tnsA fragment into pKK181 (Orle and Craig, 1991), a derivatives digested with HindIII, between the Ncol and Hindlll sites of pGEX2 (Smith and Johnson, 1988).

The pKaO18 expression plasmid was constructed by inserting a Ncol-Hindlll tnsA fragment from pKAO41 (Craig and Craig, 1991), after partial digestion with HindIII, between the Ncol and Hindlll sites of pGEX2. pGDI08 is a derivative of pln-III--OmpA-1 (Ghrayeb et al., 1984; G. Dalbadie-McFarland and J. Abelson, personal communication), which contains a synthetic linker inserted into OmpA-1. In pKAO41, the proposed tnsD initiation ATG was modified to produce a Ncol site, which produces a change in the second amino acid from the N-terminus of TnsD (Gly to Ser). TnsD protein was prepared from NC5233 (E. coli K-12 [lac-]) and Tnarc (Shand et al., 1991) carrying the pKKA181 tnsD plasmid.

Host lysate for analysis of TnsD binding and transposition activity was prepared from MC4100 (F- araD139 ;argF-lacU169 ;proA1 recA1 relA1 thi-1 F' metI argE1 proA1 ) and pGDI08 (pPS2G105). For nuc assays, LAB3 (MC4100 vA" recA56 attTn6::Tn7; McKown et al. 1987) was used.

Purification of TnsA
GST--TnsA fusion expression and purification and GST release by thrombin cleavage were adapted from Smith and Johnson (1988). Cells were grown at 30°C in 1 liter of Luria broth with 100 µg/ml ampicillin. At an ODo of 0.7, fusion protein expression was induced by 40 µM isopropyl-β-D-thiogalactoside, and growth was continued to an ODo of 1.2-1.4. Cells were harvested by centrifugation and washed in buffer A (25 mM HEPES [pH 7.5], 1 mM EDTA). Subsequent steps, unless otherwise noted, were performed at 4°C. Cells were resuspended with 2 ml of buffer A per 1 g of cells with 100 µM KCI, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication, debris was removed by centrifugation for 30 min at 16,800 x g, and supernatant was collected. NaCl (5 M) and 250 mM CHAPS were added to final concentrations of 600 mM and 10 mM, respectively, and the lysate was incubated with glutathione--agarose beads (Sigma) for 1 hr, followed by extensive washing in buffer A with 500 mM NaCl, 10 mM CHAPS, and 2 mM DTT. GST--TnsA was eluted with 10 mM reduced glutathione, and the beads were removed by centrifugation. The supernatant containing the fusion protein was dialyzed against thrombin lysis buffer, and TnsA was released from the GST domain by incubation for 30 min at 25°C with 30 U of human thrombin (Sigma). Cells used for purification were prepared in a TnsA pool and three additional amino acids (Gly-Ser-Pro) at its N-terminus. Purification procedures were incubated for 6 hr with glutathione--agarose beads, and the supernatant, selectively enriched in TnsA, was collected. These fractions were stored at -80°C and were stable for at least several months. The transposition activity of purified TnsA that is added to crude extracts lacking TnsA is comparable to authentic TnsA in crude extract (data not shown).

Purification of TnsD
We previously identified a tnsD-dependent attTn7 DNA binding activity in crude extracts (Waddell and Craig, 1989). Purification of this activity selects for TnsD, as evaluated both by a mobility shift assay (Waddell and Craig, 1989), in which TnsD fractions were supplemented with 100 mM KCI (assuming the presence of propanol) to 0.25 mM. Cleavage of GST--TnsA with thrombin results in a TnsA polypeptide with three additional amino acids (Gly-Ser-Pro) at its N-terminus. Cleavage products were incubated for 6 hr with glutathione--agarose beads, and the supernatant, selectively enriched in TnsA, was collected. These fractions were stored at -80°C and were stable for at least several months. The transposition activity of purified TnsA that is added to crude extracts lacking TnsA is comparable to authentic TnsA in crude extract (data not shown).

Unless otherwise indicated, all steps were performed at 4°C. Two milliliters per gram of cells of buffer. D20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 2 mM DTT, 10% (v/v) glycerol with 1 M KCl was added to 57 g of cells. Cells were subjected to sonication, cell debris was removed by centrifugation for 30 min at 16,800 x g, and supernatant was collected (fraction I). Polysaccharide (pH 7.5) was added to 0.9% (v/v), the mixture was incubated for 10 min and centrifuged for 30 min at 16,800 x g, and supernatant was collected. Solid ammonium sulfate was added (423 mg/ml) with stirring for 40 min, the mixture was incubated an additional 20 min, and the resulting pellet was collected by centrifugation for 30 min at 16,800 x g. The pellet was resuspended in buffer B with 0.3 M KCl using volume to that used for sonication, followed by dialysis against buffer B with 0.3 M KCl with two additional hours. After centrifugation for 30 min at 16,800 x g, the supernatant was collected and diluted to 0.1 M KCl by the addition of buffer C with 0.1 M KCl. The diluted sample was centrifuged for 30 min at 16,800 x g, and supernatant was collected (fraction II). Fraction II was applied to an Affi-Gel heparin (Bio-Rad Laboratories) column (2.5 x 20 cm) equipilibrated in buffer B with 0.18 M KCl. The column was washed with the same buffer and eluted with a 500 ml linear
gradient of 0.18–0.3 M KCl in buffer B, followed by a 250 ml elution with 0.3 M KCl in buffer B. TnsD binding activity eluted beginning at 0.37 M KCl, and active fractions were pooled (fraction III). Fraction III was applied to a Protekt Green-1 (ICN Biomedicals) dye column (1.0 x 7.0 cm) equilibrated in buffer B with 0.4 M KCl. The column was washed with the same buffer and eluted with a 0.1 M linear gradient of 0.4–1.0 M KCl in buffer B. TnsD binding activity eluted in a broad peak beginning at 0.65 M, and active fractions were pooled (fraction IV). Fraction IV was applied to a Bio-Gel HT dextran/agarose (Bio-Rad Laboratories) column (0.8 x 2.0 cm) equilibrated in buffer B with 1.0 M KCl. The column was successively eluted with 0.5 M KCl, 0.5 M KCl and 25 mM KPO4, (pH 8.0), and 0.5 M KCl and 50 mM KPO4, (pH 8.0) in buffer B. TnsD binding activity eluted at 0.5 M KCl and 50 mM KPO4, (pH 8.0); active fractions were dialyzed against 0.5 M KCl and 50 mM KPO4, (pH 8.0) with 25% (v/v) glycerol instead of 10% glycerol. Dialyzed fractions (fraction V) were frozen in liquid nitrogen and stored at −80°C. TnsD fractions were stable for at least several months.

Preparation of TnsB and TnsC

TnsB was fraction IV (Ariczewska et al., 1991) and TnsC was fraction III (Gamas and Craig, 1993).

Protein Analysis

Protein concentration was determined using Bio-Rad Protein Assay with bovine serum albumin (BSA) as standard. SDS-polyacrylamide gel electrophoresis using 12.5% gels (for TnsA) and 10% gels (TnsD) was carried out by the method of Laemmli (1970). Prior to loading, TnsD fractions were concentrated by 10% trichloroacetic acid precipitation of a 1.6 kb mini-Tn7 element flanked by sequences unrelated to attTn7 (Ariczewska et al., 1991). The mini-Tn7 element contains tns end segments that include the cis-acting transposition sequences (a 166 bp fragment from the left end [Tn7L] and a 199 bp fragment from the right end [Tn7R]) flanking a kanamycin resistance gene cassette; the kanamycin cassette was used as the mini-Tn7-specific probe in the hybridization experiments. The donor plasmid pEMA contains the mini-Tn7-flanking sequence and nearby flanking sequences (61 nt and 83 nt adjacent to the Tn7L and Tn7R ends, respectively) of pEM-1. The mini-Tn7 element and flanking donor DNA sequences were generated by PCR amplification using pEM-1 as a template with oligonucleotides containing pBluescript KS(-) (Stratagene) was used as a target DNA lacking active attTn7 sequences. The 115 bp EcoRI-EcoRI attTn7 (-25 to +64) fragment from pEM-1 was isolated by agarose gel electrophoresis and used as a probe in Southern hybridizations. The donor plasmid (pEM-1 or pEMA), 0.6 ag (2.5 nM) of pKAO4-3 attTn7 target plasmid, 2 mM ATP, 26 mM HEPES (pH 8.0), 0.02 mM EDTA, 2.1 mM DTT, 15 mM MgAc, 0.1 mM MgCl2, 0.01 mM CaCl2, 100 μg/ml tRNA, 50 μg/ml BSA, 0.1 mM CHAPS, 50 ng of TnsA, 50 ng of TnsB, 50 ng of TnsC, and 40 ng of TnsD. Reactions shown in Figures 2, 8, and 9 also contained an additional 1.0 mM Tris–HCl (pH 8.0), 5.8 mM NaCl, 15 mM KCI, and 0.25% glycerol. In Figure 9, the preincubation step was performed in which the reaction components were mixed and incubated for 30 min at 30°C prior to the addition of MgAc. MgAc (20 μM; 4 μl) was then added, and incubation was continued for an additional 30 min at 30°C. In Figure 4, the preincubation step was performed in reaction mixtures (96 μl) that also contained 1.0 mM Tris–HCl (pH 8.0), 5.8 mM NaCl, 15 mM KCI, and 0.25% glycerol but omitted the indicated Tns proteins; after preincubation, the omitted components and MgAc were added, reresulting in final reaction mixtures (104 μl) that also contained 2.0 mM Tris–HCl (pH 8.0), 11.5 mM NaCl, 30 mM KCI, and 0.5% glycerol. As described by Bainton et al. (1991), after incubation was completed, reaction DNAs were isolated by urea–spermine precipitation and ethanol precipitation, digested with restriction enzyme, and electrophoresed through 0.8% agarose. The resolved DNAs were transferred to Nytran (Schleicher & Schuell) and analyzed by Southern hybridization with a radioactive DNA probe specific for the mini-Tn7 element, i.e., the kanamycin segment that lies between the Tn7 end sequences. Blots were examined by autoradiography, and reaction products were quantified with a PhosphorImager (Molecular Dynamics).

Mobility Shift Assays

Protein–DNA complexes were examined using mobility shift assays (Fried and Crothers, 1981; Garner and Revzin, 1981). In Figure 5A, reaction mixtures (20 μl) contained 50 mM Tris–HCl (pH 8.0), 140 mM KCI, 1 mM EDTA, 1.8 mM DTT, 9.8% (v/v) glycerol, 340 μg/ml BSA, 17 μg/ml sheared salmon sperm DNA, and approximately 0.01 pmol of end-labeled DNA fragment and, as indicated in Figure 5, TnsA and host extract prepared by boiling MCI4100 crude cell lysate for 2 min and collecting the supernatant after centrifugation for 2 min at room temperature (Bainton et al., 1991). In Figure 5B, DNA binding reactions (20 μl) contained 50 mM Tris–HCl (pH 8.0), 0.25 mM HEPES (pH 7.5), 120 mM KCI, 10 mM NaCl, 0.1 mM EDTA, 1.8 mM DTT, 0.1 mM MgCl2, 0.1 mM CHAPS, 0.8 mM ATP, 8% (v/v) glycerol, 300 μg/ml BSA, 15 μg/ml sheared sperm DNA, and approximately 0.01 pmol of end-labeled DNA. After incubation for 20 min at room temperature, reactions were electrophoresed through 5% polyacrylamide gels (29:1 acrylamide:N,N'-methylene-bisacrylamide) in Tris–borate-EDTA buffer at 11.5 V/cm for 1.75 hr. Gels were vacuum dried and exposed to X-ray film.
Nucleosome Protection Experiments
Micrococcal nuclease protection assays were done according to Zhang and Gralla (1989). The reactions (100 μl) contained 45 mM Tris-HCl (pH 8.0), 0.5 mM KCl, 0.1 mM EDTA, 1.8 mM DTT, 320 μg/ml BSA, 20 μg/ml sheared salmon sperm DNA, 2 μg/ml Tn5 (fraction V), and ∼0.15 pmol of 3'-end-labeled DNA fragments. After incubation for 20 min at 30°C, 5 μl of 20 mM CaCl₂ and 5 μl of 1.4 mg/ml micrococcal nuclease (Boehringer Mannheim Biochemicals) were added. Incubation was continued for 2 min, and reaction was stopped by addition of EDTA to 10 mM.

DNAase I protection assays were performed using the method of Andrews et al. (1987). Reaction mixtures (100 μl) contained 40 mM Tris-HCl (pH 8.0), 1.3 mM HEPEs (pH 7.5), 85 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 1.7 mM DTT, 0.5 mM MgCl₂, 0.6 mM CHAPS, 1 mM ATP, 8.5% (v/v) glycerol, 350 μg/ml BSA, 20 μg/ml sheared salmon sperm DNA, with approximately 0.15 pmol of 3'-end-labeled DNA and TnsD, TnsC, or both. Reaction mixtures were incorobulated for 15 min at 30°C and then treated with DNAase I by addition of 5 μl of 20 mM MgCl₂ + 2 mM CaCl₂ solution and 5 μl of 0.4 mg/ml DNAase I (Boehringer Mannheim Biochemicals). Incubation was continued for 1 min, and the reaction was stopped by addition of EDTA to 10 mM. Protein-DNA complexes were resolved by electrophoresis, as for mobility shift assays; the half-lives of the TnsD-affTns7 and TnsC-TnsD-affTns7 complexes were longer than the attack periods, so these assays do reflect protection, not binding interference. Gel slices containing DNA of interest were isolated. DNA was electroeluted, recovered by isopropanol precipitation, and then analyzed on a sequencing gel. DNA sequencing was performed using the method of Maxam and Gilbert (1980).

Evaluation of Tn7 Transposition In Vivo
affTns7 target activity was evaluated by a Tn7-hap assay (McKown et al., 1988) in which the affTns7-containing plasmids are being introduced into host strains by transformation. To evaluate transposition proteins, the transcriptional and protective-defective JXK1 (Kubo and Craig, 1990) contains mini-Tn7 Km, which translocates to the affTns7 target plasmid from JXK1 upon infection, giving kanamycin-resistant colonies.

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Recognition of a Specific Tn7 Insertion Site


