Cell, Vol. 72, 931–943, March 26, 1993, Copyright © 1993 by Cell Press

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 displays 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., 1986; 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

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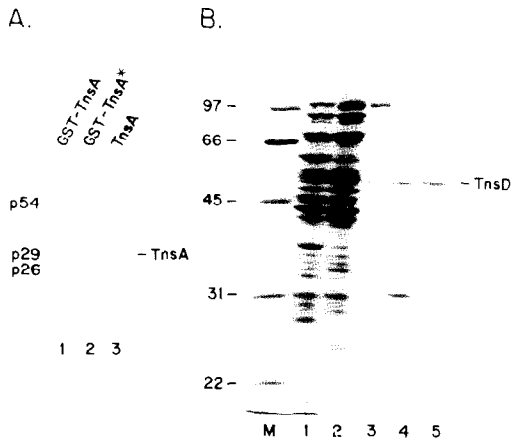

Figure 1. Purification of TnsA and TnsD

(A) Analysis of TnsA fractions by SDS-polyacrylamide gel electrophoresis and Coomassie blue R250 staining. Lanes 1 and 2, 1.0 μg of affinity-purified GST–TnsA fusion protein before and after (asterisk) thrombin cleavage, respectively (TnsA, 29 kd; GST domain, 26 kd); lane 3, 2.0 μg of cleaved TnsA protein after depletion of the released GST domain by affinity chromatography. The bar indicates TnsA.

(B) Analysis of TnsD fractions by SDS-polyacrylamide gel electrophoresis and Coomassie blue R250 staining. Lane 1, 95 μg of fraction I; lane 2, 95 μg of fraction II; lane 3, 10 μg of fraction III; lane 4, 5 μg of fraction IV; lane 5, 1.5 μg of fraction V. Numbers at the left indicate the positions of the molecular mass markers, and the bar indicates the position of TnsD.

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 *attTn7* target DNA, likely through binding to TnsD. Our finding that 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.

Results

Reconstitution of Tn7 Transposition In Vitro with Purified Tns Proteins and ATP

The development of a cell-free Tn7 transposition system using crude extracts has been previously reported (Bainton et al., 1991). We have now reconstituted Tn7 insertion into its preferred target site, *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 *tnsD*-dependent *attTn7* DNA binding activity selects for TnsD (Table 1); as described below, TnsD binds specifically to *attTn7*.

Tn7 transposes efficiently in vitro when the substrate DNAs, a donor plasmid containing a mini-Tn7 element and a target plasmid containing *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-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 Tn7 into *attTn7* (data not shown). Transposition proceeds via a nonreplicative mechanism in which Tn7 is excised from the donor and specifically inserted into *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 *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

Table 1. TnsD Purification

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
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. Affi-Gel–heparin	270	25.7	78,000	3,000	79	32
IV. Green 1	52	1.3	42,000	32,000	840	18
V. Hydroxylapatite	5.4	0.16	16,000	100,000	2,630	6.7

Specific activities of the pooled fractions were compared using the mobility shift assay to determine TnsD binding to *attTn7*. A unit of activity (U) was defined as the amount of protein required to shift 10% of the *attTn7* fragment in the presence of an E. coli lysate.

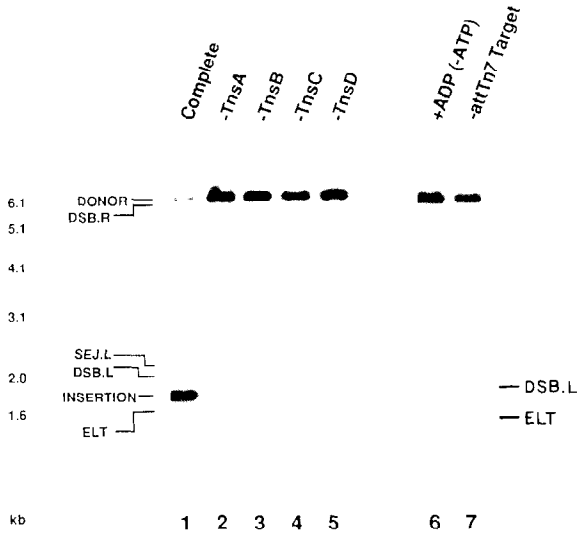

Figure 2. Reconstitution of Tn7 Transposition In Vitro with Purified Tns Proteins

An autoradiogram of a Southern blot is shown of transposition reactions digested with EcoRI, in which the indicated components were omitted. The DNA species, detected with a mini-Tn7 probe specific to sequences between the transposon ends, are labeled as in Figure 3. SEJ.L refers to the DSB-SEJ.L species; SEJ.Rs did exist but were not resolved with this digest. Reactions were performed as described in Experimental Procedures, with the following exceptions: lane 6, 2 mM ADP was substituted for exogenously added ATP; lane 7, a plasmid lacking *attTn7* sequences was used as a target, instead of the usual *attTn7*-containing plasmid.

end of a DSB.R) is observed in amounts equivalent to that of the SEJ.Ls. ELTs that have one transposon end joined to one strand of *attTn7* in the target DNA (ELT-SEJ.L and ELT-SEJ.R) can also be observed at very low levels (shown schematically in Figure 3; actual data not shown). These SEJ species appear late and accumulate in reactions (data not shown), which suggests that they are not transposition intermediates. Although these other species are present, simple insertions are by far the predominant transposition product.

All four Tns proteins are required for the generation of the simple insertion product (Figure 2, lanes 2–5). It is notable that no transposition intermediates are detectable when TnsA, TnsB, or TnsC is omitted from the incubation, indicating that these proteins are critical to the initiation of recombination, i.e., to the double-strand breaks that separate the transposon from the donor backbone. However, very low levels of the transposition intermediates, DSBs and ELTs, are observed when TnsD is omitted from the reaction, i.e., with only TnsA, TnsB, and TnsC present (lane 5), revealing that these proteins provide the active site(s) for transposition. (The activity of the "core " TnsA+ TnsB+TnsC machinery is considered in detail below.) Also notable is the fact that the presence of *attTn7* is required for the efficient production of transposition intermediates or products: although donor cleavage apparently precedes the joining of the transposon to the target DNA during Tn7 transposition, little donor cleavage occurs in the absence of *attTn7* (lane 7).

ATP is an essential cofactor for Tn7 transposition. No recombination intermediates or products are observed when ADP is substituted for ATP (lane 6). (ATP cannot be completely omitted, as it is present in the TnsC fraction to increase the solubility of TnsC.) TnsC is an ATP-dependent DNA-binding protein and thus is likely the site of ATP action (Gamas and Craig, 1992).

These experiments provide evidence that the four Tns proteins participate directly in transposition and also establish that ATP is an essential cofactor. As transposition does occur with highly purified Tns fractions, Tn7 transposition in vitro does not obviously require any E. coli proteins; however, it should be noted that the addition of crude extracts from cells lacking the *tns* genes can increase both the rate and extent of transposition, particularly under suboptimal conditions (data not shown). Thus, the possibility that host proteins, though not apparently essential, may be able to act as accessory proteins in Tn7 transposition should not be discounted.

TnsC and TnsD Can Interact with Target DNA: Efficient Tn7 Transposition Requires Preincubation of *attTn7*, TnsD, TnsC, and ATP

Our reaction mixtures require the presence of MgAc (Figure 4, lane 1). Recombination is most efficient, however, when all other components except MgAc are preincubated for several minutes prior to MgAc addition (lane 3), rather than when MgAc is present from the beginning (lane 2). The stimulatory preincubation step is effective only if TnsC, TnsD, *attTn7*, and ATP are all present (lanes 4–7); efficient recombination can still occur if TnsA, TnsB, and the donor DNA are later added with MgAc (lane 8). These observations suggest a special interaction between TnsC, TnsD, and the target DNA. As shown below, TnsD is a sequence-specific DNA-binding protein that recognizes *attTn7* and that directs TnsC to *attTn7* through formation of an ATP-dependent TnsC–TnsD–*attTn7* complex.

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 5A, 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 5A, 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-

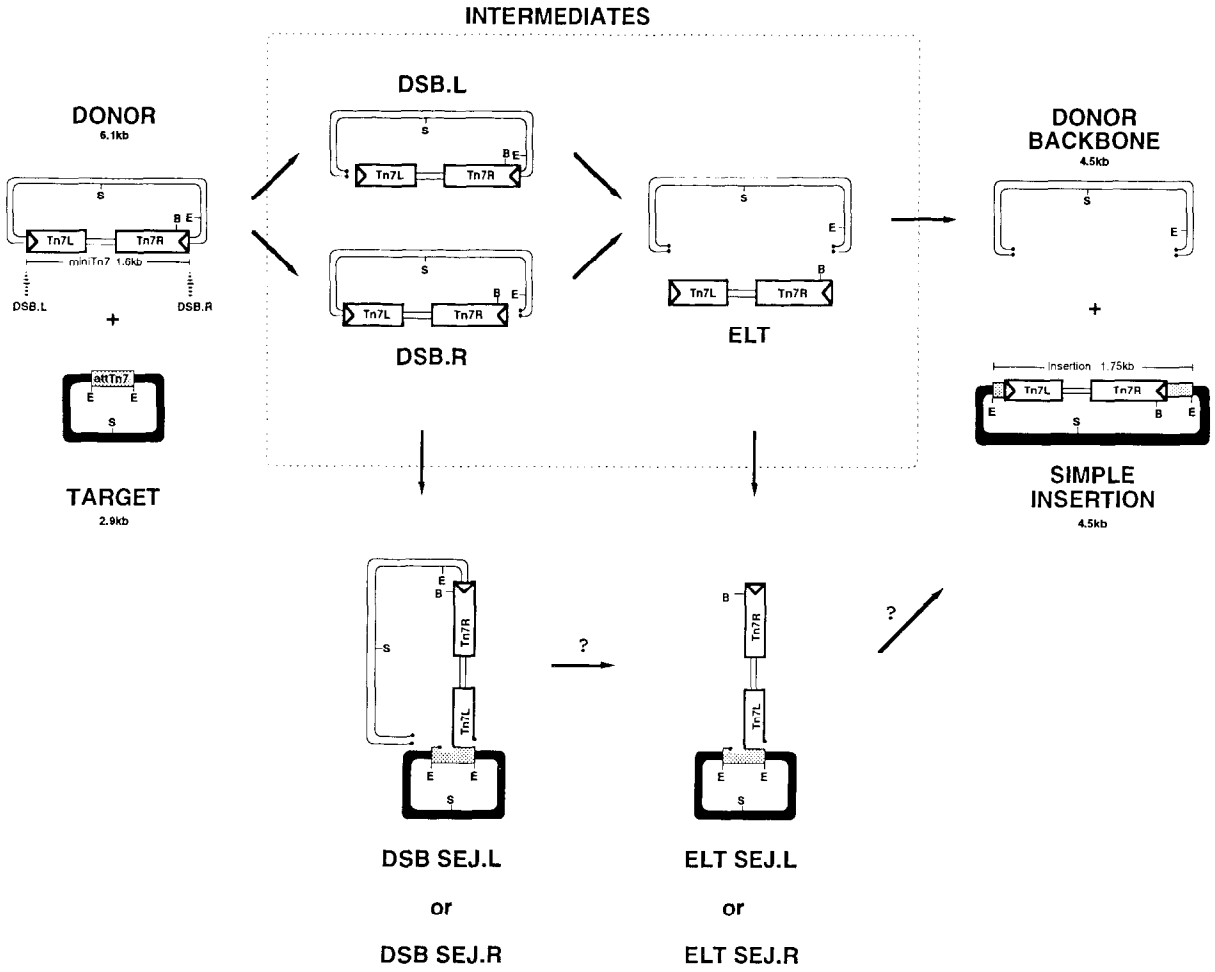

Figure 3. The Transposition Substrates, Intermediates, and Products

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.

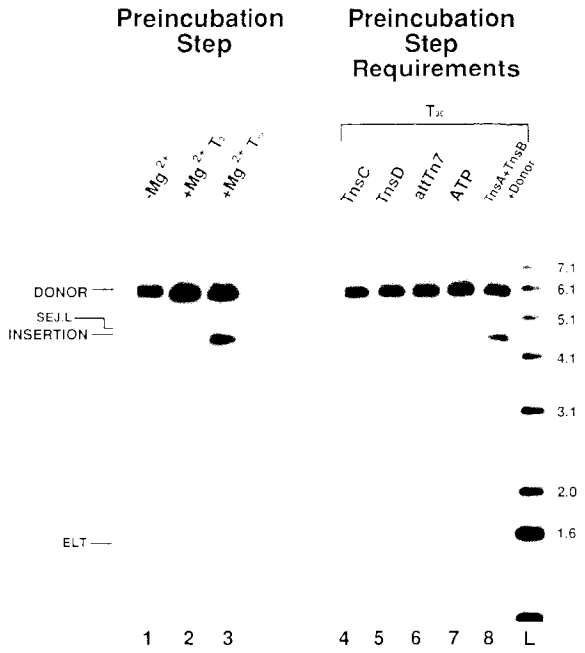

Figure 4. Tn7 Transposition In Vitro Is Stimulated by a Preincubation Step That Lacks MgAc

Shown is an autoradiogram of transposition reactions, performed as described in Experimental Procedures, that displays BglII-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-

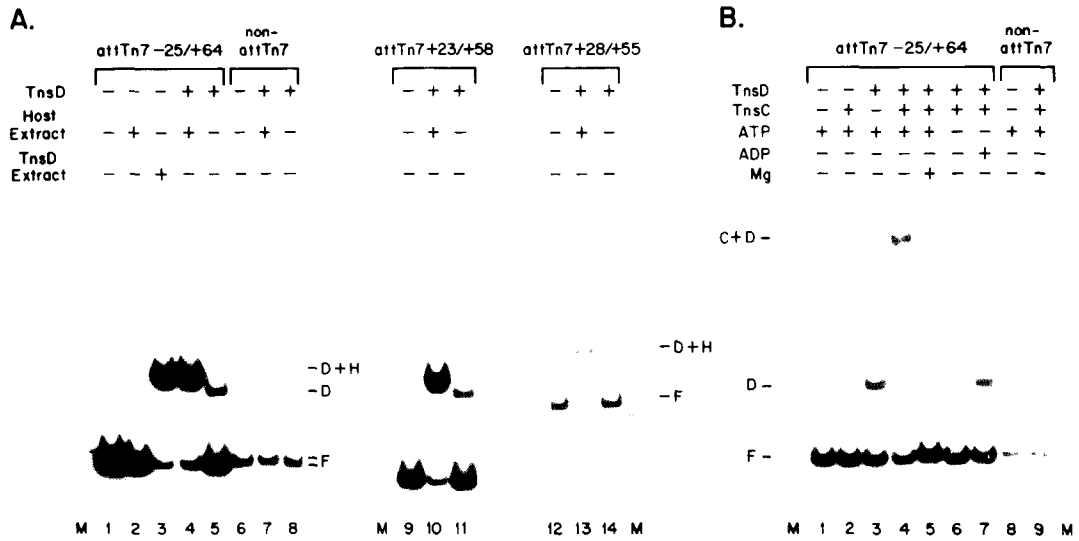

Figure 5. Analysis of TnsD and TnsC–TnsD Binding to *attTn7* by Mobility Shift Assays

(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 μg), crude TnsD lysate (5 μg), 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.

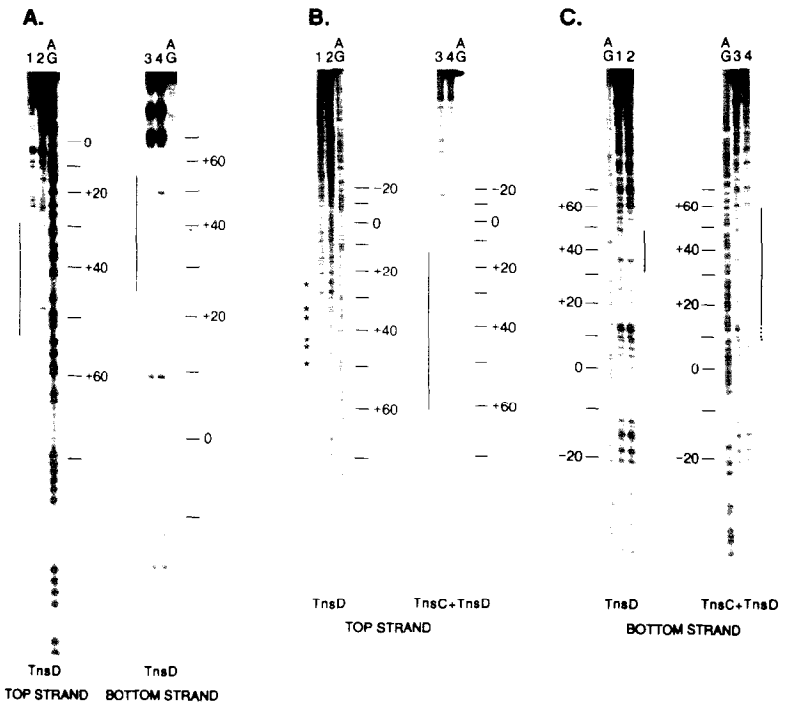

Figure 6. Footprinting Analysis of TnsD and TnsC–TnsD Complexes with *attTn7*

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).

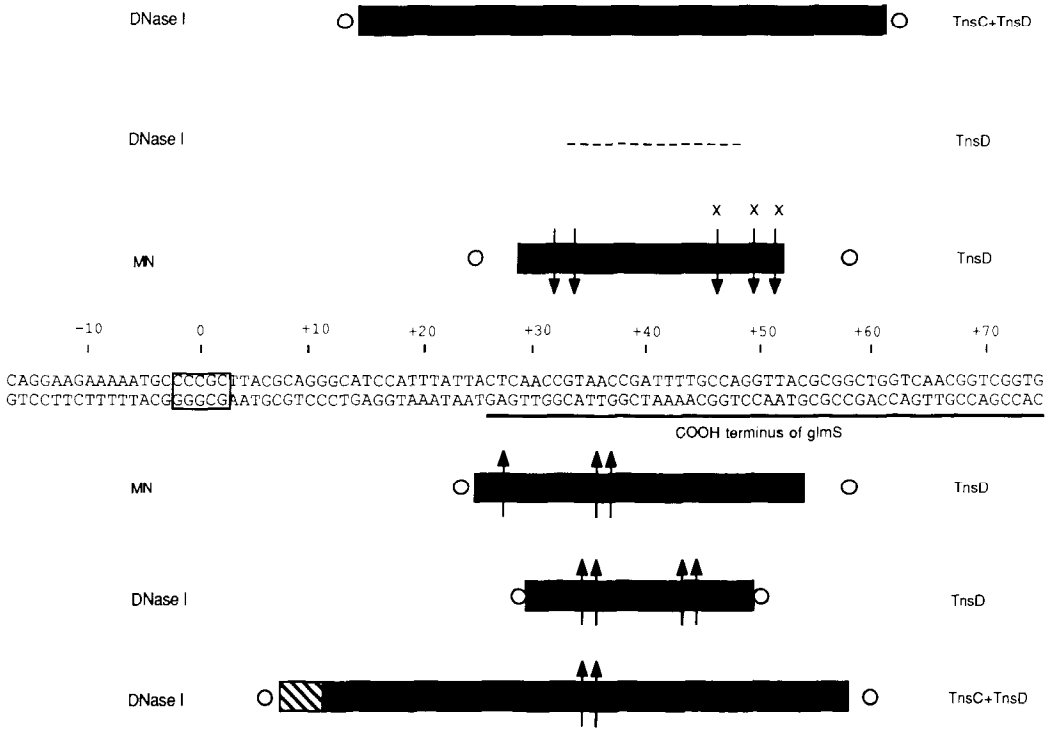

Figure 7. Summary of *attTn7* Nuclease Protection Experiments

The *attTn7* sequence is shown, with boxed nucleotides indicating the 5 bp sequence duplicated upon Tn7 insertion (Lichtenstein and Brenner, 1982; Gay et al., 1986; 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.

Table 2. Evaluation of *attTn7* Target Activity

Plasmid	*attTn7* Sequence	Transposition/pfu (mean ± SEM [n])
pSK[a]	None	$(2.5 \pm 2.5) \times 10^{-7}$ [2]
pkk26	−52 to +64	$(2.5 \pm 2.1) \times 10^{-3}$ [3]
pkk28	+23 to +58	$(3.7 \pm 2.7) \times 10^{-3}$ [3]

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 λ derivative after phage infection was evaluated by the appearance of kanamycin-resistant colonies. pfu, plaque-forming units; SEM, standard error of the mean.

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

tection by TnsD alone. However, an important feature of the TnsC+TnsD protection is that the specific point of Tn7 insertion remains exposed and thus does not appear to be directly contacted by either TnsC or TnsD. The actual dispositions of TnsC and TnsD in the complex remain to be established, but a simple view is that TnsC occupies the region of *attTn7* DNA that extends from the observed edge of the binding region for TnsD alone (about +25) to the border of the TnsC+TnsD protected region closest to the point of insertion (about +7).

TnsA+TnsB+TnsC Can Promote Efficient Transposition in the Presence of AMP-PMP

The substitution of AMP-PMP for ATP in the reconstituted system has profound effects on both transposition frequency and target site selection. In the presence of AMP-PMP, considerable TnsA+TnsB+TnsC transposition is observed (Figure 8A, lane 5), in contrast with the very low level of ATP-dependent recombination in the absence of TnsD (lane 1) or *attTn7* (lane 3). The TnsA+TnsB+TnsC AMP-PMP-dependent insertions, like the ATP-dependent insertions in the standard reaction, generate 5 bp target duplications (data not shown). In marked contrast with the stringent target site selectivity of TnsD-dependent insertion, in which virtually all insertions occur into *attTn7*, efficient insertion into target molecules lacking *attTn7* is observed with TnsA+TnsB+TnsC in the presence of AMP-PMP (Figure 8A, lane 5); in addition there is no apparent preference for *attTn7* insertion, even when this site is present in the target molecule (lane 4). Analysis of the distribution of insertions mediated by TnsA+TnsB+TnsC in the presence of AMP-PMP reveals that many different insertion sites are used at comparable frequencies with little observed target site preference; i.e., target site selection is apparently random (data not shown).

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*.

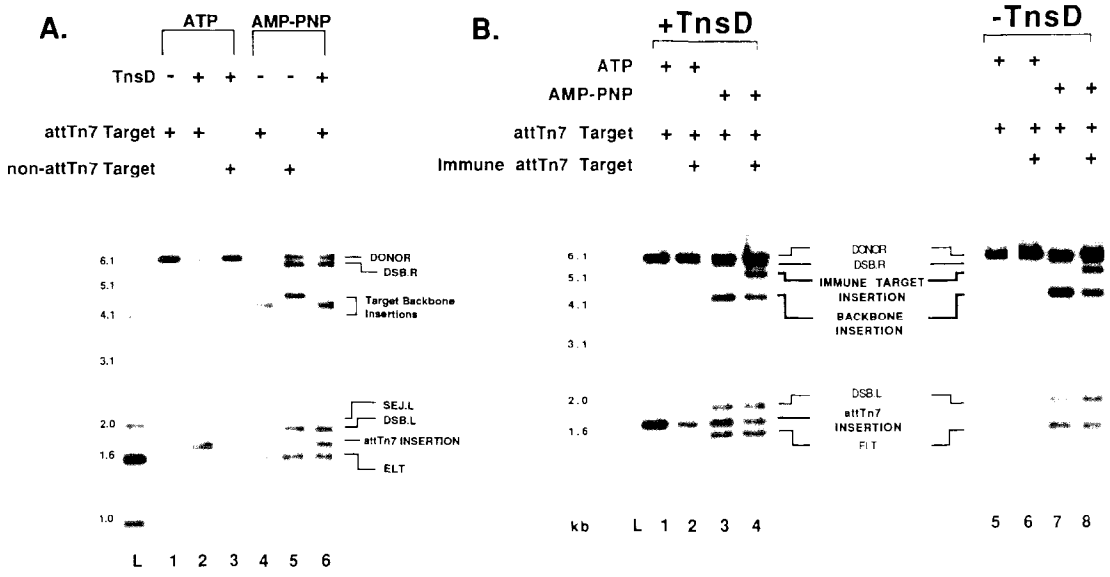

Figure 8. Tn7 Transposition In Vitro in the Presence of AMP-PMP

(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).

(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 ATP 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 immune 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 dissection 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., 1986; Waddell and Craig, 1988). 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. Apposition of this core machinery to *attTn7* results from the positioning of TnsC, an ATP-dependent, nonspecific DNA-binding protein (Gamas 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; Qadri 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

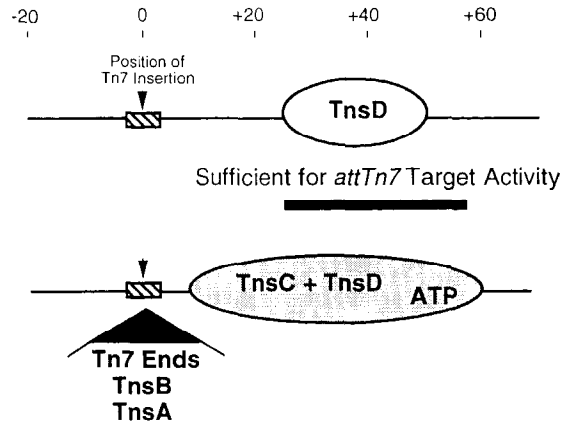

Figure 9. A Model for Tn7 Transposition

The thin horizontal line represents *attTn7* sequences, with 0 indicating the center base pair of the 5 bp (hatched box) usually duplicated upon Tn7 insertion (arrowhead); 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 TnsC on the target DNA.

sequences in *attTn7* outside the region of *attTn7* that can interact with TnsD alone appear to be required for *attTn7* target activity. A reasonable hypothesis is that TnsD binds specifically to *attTn7* and TnsC also interacts with *attTn7* through specific protein–protein contacts with TnsD and nonspecific contacts with DNA; however, other interactions cannot be excluded, for example, that TnsD induces a particular conformational change in *attTn7* DNA that is recognized by TnsC, or that additional specific TnsC contacts exist within this TnsD–binding region. The nonspecific DNA binding activity of TnsC is modulated by ATP; it remains to be determined how this ATP-dependent interaction of TnsC with DNA may be modulated by TnsD.

The region of target DNA immediately surrounding the actual point of Tn7 insertion in the ATP-dependent TnsC–TnsD–*attTn7* complexes is not obviously contacted by either TnsC or TnsD. We are intrigued by the hypothesis that the insertion region is actually contacted by the transposon ends, TnsA and TnsB (Figure 9); we speculate that TnsC may also interact with TnsB, TnsA, or both, thereby juxtaposing the transposon ends with the target DNA within a Tns protein complex and provoking the initiation of recombination.

A Core Recombination Machine That Is Not Target Site-Specific: TnsA+TnsB+TnsC Perform DNA Strand Breakage and Joining

We have found that transposition mediated by only TnsA+TnsB+TnsC can occur efficiently when AMP–PMP is used in place of the usual ATP cofactor. Thus, the active site(s) for donor DNA cleavage and strand transfer is contained within these polypeptides. The finding that TnsA+TnsB+TnsC-mediated insertion is not target site specific, i.e., uses random target sites, supports the view that TnsD 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 Mu 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 in 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+TnsB+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 little sequence preference; TnsE may thereby 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 macromolecular interactions 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 DH5α (Bethesda Research Laboratories). The gene construct encoding the GST–TnsA fusion was generated by inserting a NcoI–HindIII *tnsA* fragment from pKAO46 (Orle and Craig, 1991), after partial digestion with HindIII, between the NcoI and HindIII sites of pGEX2 (Smith and Johnson, 1988).

The *tnsD* expression plasmid pKK18 was constructed by inserting a NcoI–HindIII *tnsD* fragment from pKAO41 (Orle and Craig, 1991), after partial digestion with HindIII, between the NcoI and HindIII sites of pGD108. pGD108 is a derivative of pIN-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 NcoI site, which produces a change in the second amino acid from the N-terminus of TnsD (Gly to Ser). TnsD protein was prepared from NCM533 (E. coli K-12[λ^+] *lacZ*::Tn5 *lacI*^{q1}; Shand et al., 1991) carrying the pKK18 *tnsD* plasmid.

Host lysate for analysis of TnsD binding and transposition activity was prepared from MC4100 (F$^-$ *ara*D139A Δ[*argF-lac*]U169 *rps*L150 *rel*A1 *flb*B5301 *deo*C1 *pts*F25 *rbs*R). For λ hop assays, LA3 (MC4100 *val*^{R} *rec*A56 *attTn7*$_{84}$::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 carbenicillin. At an OD_{600} of 0.7, fusion protein expression was induced by 40 μM isopropyl β-D-thiogalactopyranoside, and growth was continued to an OD_{600} 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 mM KCl, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication, debris was removed by centrifugation for 30 min at 16,800 × g, and supernatant was collected. NaCl (5 M) and 250 mM CHAPS were added to final concentrations of 500 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) per milligram of TnsA fusion protein. Reaction was stopped by addition of 100 mM phenylmethylsulfonyl fluoride (in isopropanol) 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 with 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 crude extract from cells lacking TnsD (see Table 1 and Figure 5), and by monitoring the TnsD polypeptide by imumunoblotting with anti-TnsD antiserum (Orle and Craig, 1991). Cells used for purification were grown at 37°C in a 200 liter fermentor in Luria broth (Miller, 1972) supplemented with 100 μg/ml carbenicillin. At an OD_{600} of 1.0, isopropyl β-D-thiogalactopyranoside was added to 100 μM. Growth was continued for an additional 2 hr, and cells were harvested by centrifugation. Cell paste was frozen and stored at −80°C.

Unless otherwise indicated, all steps were performed at 4°C. Two milliliters per gram of cells of buffer B (50 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 × g, and supernatant was collected (fraction I). Polyethylenimine (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 × g, and supernatant was collected. Solid ammonium sulfate was added (423 mg/ml) with stirring over 40 min, the mixture was incubated an additional 20 min, and the resulting pellet was collected by centrifugation for 30 min at 16,800 × g. The pellet was resuspended in buffer B with 0.3 M KCl using volume equal to that used for sonication, followed by dialysis against buffer B with 0.3 M KCl with two 10 hr changes. After centrifugation for 30 min at 16,800 × g, the supernatant was collected and diluted to 0.18 M KCl by the addition of buffer B with 0.1 M KCl. The diluted sample was centrifuged for 30 min at 16,800 × g, and supernatant was collected (fraction II). Fraction II was applied to an Affi-Gel–heparin (Bio-Rad Laboratories) column (2.5 × 20 cm) equilibrated 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 0.27 M KCl, and active fractions were pooled (fraction III). Fraction III was applied to a Protrans Green-1 (ICN Biomedicals) dye column (1.0 × 7.0 cm) equilibrated in buffer B in 0.4 M KCl. The column was washed with the same buffer and eluted with a 120 ml 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 hydroxylapatite (Bio-Rad Laboratories) column (0.8 × 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 KPO_4 (pH 8.0), and 0.5 M KCl and 50 mM KPO_4 (pH 8.0) in buffer B. TnsD binding activity eluted at 0.5 M KCl and 50 mM KPO_4 (pH 8.0); active fractions were dialyzed against 0.5 M KCl in buffer B 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 (Arciszewska et al., 1991) and TnsC was fraction III (Gamas and Craig, 1992).

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 with insulin (Sigma) as carrier.

DNA Substrates

The donor plasmid pEM-1 contains an E. coli chromosomal insertion of a 1.6 kb mini-Tn7 element flanked by sequences unrelated to *attTn7* (Arciszewska et al., 1989). The mini-Tn7 element contains Tn7 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 pEMΔ contains the mini-Tn7 element and nearby flanking sequences (51 nt and 83 nt adjacent to the Tn7L and Tn7R ends, respectively) of pEM-1. The mini-Tn7 element and flanking donor DNAs were generated by PCR amplification using pEM-1 as a template with oligonucleotides complementary to the flanking donor sequences positioned 51–30 nt from the Tn7L end and 83–64 nt from the Tn7R end. The amplified DNA was treated with Klenow enzyme and introduced into the SmaI site of pTrc99 (Pharmacia).

The *attTn7* target plasmid pKAO4-3 (McKown et al., 1988) contains a 150 bp segment that includes the sequences necessary for *attTn7* target activity (*attTn7* −25 to +64). pLA11 is an *attTn7* plasmid that also contains a segment that includes the Tn7R transposition sequences located about 1 kb from the *attTn7* target site (Bainton et al., 1991). pKK20 contains the BamHI–EcoRI *attTn7* (−25 to +64) fragment from pKAO4-3 inserted between the BamHI and EcoRI sites of pUC18. pKK24 contains the BamHI–EcoRI *attTn7* (−25 to +64) from pKAO4-3 inserted between the BamHI and EcoRI sites of pUC19. pKK25 contains the BamHI–EcoRI *attTn7* (−52 to +64) fragment from pEG31 (Gringauz et al., 1988) inserted between the BamHI and EcoRI sites of pUC19. pKK26 contains the BamHI–EcoRI *attTn7* (−52 to +64) fragment from pEG31 inserted between the BamHI and EcoRI sites of pBluescript SK(+) (Stratagene). pKK28, which contains *attTn7* (+23 to +58), an EcoRI linker, and HindIII and SalI staggered ends, was obtained by annealing two 46 nt synthetic oligonucleotides, 5′-AGCTTTACTCAACCGTAACCGATTTTGCCAGGTTACGCGGAAT TCG and AATGAGTTGGCATTGGCTAAAACGGTCCAATGCGCCTTAAGCAGCT-5′, followed by insertion between the HindIII and SalI sites of pBluescript SK(−). pKK27 contains the HindIII–EcoRI *attTn7* (+28 to +55) fragment from pCW80 (Waddell and Craig, 1989) inserted between the HindIII and EcoRI sites of pBluescript SK(−) (Stratagene). pBluescript KS(+) (Stratagene) was used as a target DNA lacking *attTn7* sequences.

Preparation and Labeling of DNA Fragments

Plasmid DNA was digested with appropriate restriction enzymes and end labeled at 3′ ends with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. End-labeled fragments were purified by separation in a nondenaturing polyacrylamide gel and visualized by autoradiography. DNA fragments were recovered by electroelution followed by isopropanol precipitation.

For mobility shift assays, fragments containing the following sequences were used: the 122 bp PvuI–HindIII pUC18 fragment for non-*attTn7* sequences, the 115 bp EcoRI–BamHI pKK20 fragment for *attTn7* (−25 to +64), the 92 bp BamHI–ApaI pKK28 fragment for *attTn7* (+23 to +58), and the 256 bp PvuII–HindIII pCW80 fragment for *attTn7* (+28 to +55).

For micrococcal nuclease protection studies, the pKK20 EcoRI–HindIII fragment (151 bp), labeled at either the HindIII or EcoRI end for analysis of the top or bottom strand, respectively (as shown in Figure 5), was used. For DNAase I protection studies, the pKK24 HindIII–PvuI fragment (235 bp), labeled at the HindIII end and containing *attTn7* (−25 to +64), was used to examine the top strand of *attTn7*. The EcoRI–PvuI pKK25 fragment (260 bp), labeled at the EcoRI end, contained *attTn7* (−52 to +64) and was used to analyze the bottom strand.

Tn7 Transposition In Vitro

Reactions were performed essentially as described by Bainton et al. (1991). Reaction mixtures (100 μl) contained 0.1 μg (0.25 nM) of the donor plasmid (pEM-1 or pEMΔ), 0.6 μg (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 $MgCl_2$, 0.01 mM $CaCl_2$, 100 μg/ml tRNA, 50 mg/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 KCl, and 0.25% glycerol. The reactions in Figure 9 contained a lower concentration (1 nM each) of target plasmids pKAO4-3 and pLA11. A 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 (375 mM, 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 KCl, and 0.25% glycerol but omitted the indicated Tns proteins; after preincubation, the omitted components and MgAc were added, resulting in final reaction mixtures (104 μl) that also contained 2.0 mM Tris–HCl (pH 8.0), 11.5 mM NaCl, 30 mM KCl, 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 0.6% 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 KCl, 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, TnsD and host extract prepared by boiling MC4100 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 KCl, 10 mM NaCl, 0.1 mM EDTA, 1.8 mM DTT, 0.1 mM $MgCl_2$, 0.1 mM CHAPS, 0.8 mM ATP, 9% (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.

Nuclease 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), 90 mM KCl, 0.1 mM EDTA, 1.8 mM DTT, 320 μg/ml BSA, 20 μg/ml sheared salmon sperm DNA, 2 μg/ml TnsD (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 $CaCl_2$ and 5 μl of 1.4 μg/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 $MgCl_2$, 0.5 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 incubated for 15 min at 30°C and then treated with DNAase I by addition of 5 μl of 20 mM $MgCl_2$ + 2 mM $CaCl_2$ solution and 5 μl of 4 μg/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–*attTn7* and TnsC–TnsD–*attTn7* 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

attTn7 target activity was evaluated by a Tn7 λ hop assay (McKown et al., 1988) in which the *attTn7*-containing plasmids to be assayed were introduced into LA3, a strain containing a chromosomal insertion of Tn7, to provide transposition proteins. The replication- and integration-defective λKK1 (Kubo and Craig, 1990) contains mini-Tn7Km, which translocates into the *attTn7* target plasmid from λKK1 upon infection, giving kanamycin-resistant colonies.

Acknowledgments

The first two authors contributed equally to this work. We are pleased to thank Margie Lopata for her excellent technical assistance. We thank Bruce Alberts, Sandy Johnson, Rick Myers, and members of the laboratory, in particular Anne Stellwagen, for advice and comments on the manuscript. R. J. B. was supported by funds from the Medical Scientist Training Program, Merck Sharp and Doehme Research Laboratories, the University of California at San Francisco (UCSF) Department of Biochemistry Tompkins Memorial Fund, and the UCSF Program in Biological Sciences. K. M. K. was supported by funds from the UCSF Genetics Training Grant. The work was supported by a grant from the National Institute of Allergy and Infectious Diseases to N. L. C.

Received December 4, 1992; revised January 22, 1993.

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