Identification of the Structural and Functional Domains of the Large Serine Recombinase TnpX from Clostridium perfringens*

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Members of the large serine resolvase family of site-specific recombinases are responsible for the movement of several mobile genetic elements; however, little is known regarding the structure or function of these proteins. TnpX is a serine recombinase that is responsible for the movement of the chloramphenicol resistance elements of the Tn4451/3 family. We have shown that TnpX binds differentially to its transposon and target sites, suggesting that resolvase-like excision and insertion were two distinct processes. To analyze the structural and functional domains of TnpX and, more specifically, to define the domains involved in protein-DNA and protein-protein interactions, we conducted limited proteolysis studies on the wild-type dimeric TnpX1–707 protein and its functional truncation mutant, TnpX1–597. The results showed that TnpX was organized into three major domains: domain I (amino acids (aa) 1–170), which included the resolvase catalytic domain; domain II (aa 170–266); and domain III (aa 267–707), which contained the dimerization region and two separate regions involved in binding to the DNA target. A small polypeptide (aa 533–587) was shown to bind specifically to the TnpX binding sites providing further evidence that it was the primary DNA binding region. In addition, a previously unidentified DNA binding site was shown to be located between residues 583 and 707. Finally, the DNA binding and multimerization but not the catalytic functions of TnpX could be reconstituted by recombining separate polypeptides that contain the N- and C-terminal regions of the protein. These data provide evidence that TnpX has separate catalytic, DNA binding, and multimerization domains.

Mobile genetic elements, such as conjugative plasmids, transposons, integrons, and genomic islands are important vehicles for the transmission of virulence and antibiotic resistance genes in many microorganisms including Gram-positive bacteria such as Clostridium sp. (1, 2). Antibiotic resistance transposons identified in the clostridia include the integrative mobilizable elements Tn4451 and Tn4453a, which confer chloramphenicol resistance. Integration and excision of these elements is mediated by the large serine recombinase TnpX (3–5).

Members of the serine recombinase family of site-specific recombinases catalyze strand exchange by a non-replicative DNA breakage and repair mechanism that involves a 2-bp staggered break across all four DNA strands and the formation of covalent phosphoserine linkages between the DNA strands and recombinase subunits (6). The large resolvases represent a subgroup within the serine recombinase family (7). The members of this subgroup are significantly larger (50–82 kDa) than most other serine recombinase proteins (20 kDa) and catalyze a wider range of reactions (7). Little is known regarding the domain organization of large resolvases, and to date, the function and the mechanism by which the recombination process occurs is unclear.

Unlike Tn3-like resolvase proteins that only catalyze excision reactions, large serine recombinases have the ability to catalyze both the excision and integration of specific mobile DNA elements such as bacteriophage genomes, genomic islands, and mobile integrative elements (7). Several phage-encoded serine integrases related to TnpX have been shown to catalyze the insertion of their respective genomes into a specific target site (attP/attB recombination). The efficient excision of these integrated phage genomes has been demonstrated or postulated to require a recombination directionality factor in addition to the phage integrase (8–12). By contrast, TnpX does not require any other proteins for excise recombination (13).

Typical small resolvase proteins consist of two domains: an N-terminal catalytic domain (residues 1–140) that also mediates dimerization and a C-terminal helix-turn-helix DNA binding domain (7, 14, 15). The large serine recombinases identified to date show a high level of N-terminal sequence similarity to the catalytic domain of small resolvases, especially over the first 100 amino acids (aa).1 The critical serine residue within the catalytic domain is conserved across all of the serine recombinases (7), and mutation of the proposed catalytic serine in TnpX abolishes recombinase activity (16). The catalytic resolvase domain is followed by a region of sequence conservation found only between members of the large serine recombinase group (7). None of the large recombinases appear to have a helix-turn-helix motif (7).

Although the biological properties of the truncated TnpX1–597

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1 The abbreviations used are: aa, amino acid; His$_6$, hexahistidine; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; Tricine, N-$\alpha$-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; FCS, Phe-Cys-Ser.

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Fig. 1. Schematic organization of TnpX. The boxes show the location of defined TnpX regions. The scale indicates the amino acid sequence number based on previous studies (17).

### Structural and Functional Domains of TnpX

| TnpX | Resolvase Domain | Charged Region | CCC Region | Leu-rich Region | Opp. Charged Region | Cys-rich Region |
|------|------------------|----------------|------------|-----------------|--------------------|-----------------|
|      |                   |                |            |                 |                    |                 |

### Experimental PROCEDURES

Cloning, Expression, and Purification of TnpX Constructs—The strains and plasmids used for this study are described in Table I. The six separate TnpX regions (Table I) were amplified by PCR from a plasmid carrying the relevant TnpX sequence. The oligonucleotides used for each construct (Table II) included an NdeI restriction site that incorporated a start codon and a XhoI site at the 3' end prior to the stop codon. PCR products were digested with NdeI and XhoI and cloned into a plasmid carrying the relevant TnpX sequence. The oligonucleotides were then transferred to 30 °C, and expression was induced with 0.5 mM isopropyl-

### Table I

| Strains          | Characteristics | Reference or source |
|------------------|-----------------|---------------------|
| DH5α             | F− φ80 lacZAM15ΔlacZYA-argF (169 endA1 recA1) | Invitrogen         |
| C43(DE3)         | BL21(DE3) with uncharacterized mutation(s) | Ref. 18            |
| plET22b+         |                 | Novagen             |
| pJIR639          | Ap′, pBlueScriptII (tnpX′ from Tn4451) | Ref. 3              |
| pJIR1998         | Ap′, pET22b + NdeI/XholI (pJIR639 12882/12873 PCR product, tnpX1-266) | Ref. 17             |
| pJIR1999         | Ap′, pET22b + NdeI/XholI (pJIR639 12882/13200 PCR product, tnpX1-597) | Ref. 17             |
| pJIR2531         | Ap′, pET22b + NdeI/XholI (pJIR639 17612/12873 PCR product, tnpX267-707) | Recombinant         |
| pJIR2532         | Ap′, pET22b + NdeI/XholI (pJIR639 17612/13200 PCR product, tnpX267-707) | Recombinant         |
| pJIR2533         | Ap′, pET22b + NdeI/XholI (pJIR639 19088/13200 PCR product, tnpX533-597) | Recombinant         |
| pJIR2534         | Ap′, pET22b + NdeI/XholI (pJIR639 19088/12873 PCR product, tnpX533-707) | Recombinant         |
| pJIR2535         | Ap′, pET22b + NdeI/XholI (pJIR639 19087/13200 PCR product, tnpX583-707) | Recombinant         |
| pJIR2461         | Ap′, pET22b + NdeI/XholI (pJIR639 12882/18353 PCR product, tnpX1-266) | Recombinant         |

### Table II

| Primer | Location and use |
|--------|------------------|
| 12882  | 5' end of tnpX gene |
| 12873  | 3' end of tnpX gene |
| 13200  | 3' end of tnpX1-597 |
| 13712  | 5' end of tnpX267-707 |
| 19088  | Recombinant |
| 19087  | Recombinant |
| 18383  | Recombinant |

Protein and DNA binding site was identified between residues 583 and 707. Finally, it was shown that a small polypeptide consisting of the aa 533–587 region was capable of binding specifically to the TnpX binding sites.
filtration column pre-equilibrated in 50 mM Tris-HCl (pH 7.2), 0.35 M NaCl, and 2 mM DTT. Elution was performed in the same buffer at 0.4 ml/min, in 1-ml fractions collected, and in 15-µl samples examined by Coomassie Blue staining of 12% SDS-PAGE gels. The column was calibrated using standard proteins (gel filtration kit, Bio-Rad). The apparent molecular size of each protein was determined by interpolation from a standard curve of log molecular size versus Kav.

Limited Proteolysis—Purified TnpX1–707 and TnpX1–597 (80–130 µg) were solubilized in 50 mM Tris-HCl (pH 7.2), 0.5 M NaCl, and 2 mM DTT and incubated with chymotrypsin or trypsin (Sigma) (0.025–0.07 µg) in the same buffer containing 0.1 mg of purified TnpX1–707 N-terminal T7 tag. The resulting solutions were incubated for 20 min at room temperature before the addition of 3× SDS sample buffer and the samples were boiled for 3 min. The proteolytic fragments were then separated by SDS-PAGE on 10 or 12% gels and either visualized by Coomassie Blue staining or immunoblotted using anti-His tag antibodies.

Southwestern and Far-Western Analyses—Purified TnpX1–707 and TnpX1–597 proteins were digested with trypsin as described above. Proteolytic fragments were separated by SDS-PAGE on 12% gels and electroblotted onto nitrocellulose. The blot was incubated with hybridization buffer (20 mM MES (pH 7), 0.1 mM EDTA, 1 mM DTT, 1% skim milk, 0.04% Tween 20, 15% glycerol, 0.1 mM ZnCl2, 1 mM MgCl2, 75 mM KCl, 200 mM NaCl) overnight at 4 °C. For Southwestern analysis (20), the blot was probed for 4 h using the same buffer containing a32P-labeled atT7 fragment and rinsed, dried, and visualized by autoradiography. For Far-Western analysis (21), the blot was probed for 4 h using the same buffer containing 0.1 mg of purified TnpX1–707 N-terminal T7 tag. The blot was then rinsed three times for 5 min and probed with anti-T7 tagged antibodies (Novagen).

Protein Sequence Determination—Purified TnpX1–597 was digested with chymotrypsin and proteolytic fragments separated by 12% SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and stained with Coomassie Blue. The desired bands were then excised, and the N-terminal amino acid sequence was determined by automated Edman degradation performed at the Protein Chemistry Facility, La Trobe University.

Circular Dichroism Spectroscopy—Circular dichroism measurements were performed on a Jasco 810 spectropolarimeter using a 0.1-cm path length cuvette at 25 °C. TnpX derivatives were examined in the far-UV range between 200 and 250 nm in 50 mM Tris-HCl (pH 7.2), 0.35 M NaCl, and 2 mM DTT. Each spectrum represents the average of 5–10 scans processed for base-line subtraction and smoothing using software provided by the manufacturer.

Electrophoretic Gel Mobility Shift Assays—For gel mobility shift assays, TnpX constructs were mixed with binding buffer (20 mM Heps (pH 7.6), 1 mM EDTA, 10 mM (NH4)2SO4, 1 mM DTT, 0.2% Tween 20, 50 mM KCl, 2 mM MgCl2) in a total volume of 20 µl containing nonspecific DNA carrier poly(dI-dC) (1 µg), polylysine (0.1 µg), and 10,000 cpm of the32P-labeled 360-bp DNA substrate (attL). The mixtures were incubated for 20 min at room temperature before the addition of 3 µl of loading buffer (0.25× Tris borate-EDTA buffer (60%); glycerol (40%)). Samples were loaded onto a native 6.5 or 8% polyacrylamide gel containing 0.5% Tris borate-EDTA, and gels were run and analyzed as described previously (17).

**RESULTS**

**TnpX Is Organized into Discrete Domains**—Computer analysis of the primary structure of TnpX led to the suggestion that TnpX is a multidomain protein (17). To investigate the structural organization of TnpX, limited proteolytic digestion experiments using chymotrypsin were performed on purified full-length TnpX1–707 and a truncated version of the protein, TnpX1–597, which has been shown to be fully functional in vivo (17). The results (Fig. 2) revealed a clear digestion pattern with a relatively small number of chymotryptic fragments, indicating that many of the Phe, Tyr, Leu, and Trp residues of TnpX are not readily accessible to the protease. For each protein, digestion yielded three major products with molecular masses of 64, 52, and 46 kDa for TnpX1–707 (Fig. 2A) and 51, 39, and 33 kDa for TnpX1–597 (Fig. 2B). The difference in masses of the fragments generated from TnpX1–707 and TnpX1–597 corre-

**Fig. 2.** Limited proteolysis of TnpX1–707 and TnpX1–597. TnpX1–707 (A) and TnpX1–597 (B) were treated with limited amounts of chymotrypsin. Samples were incubated at 30 °C, and the reactions were stopped at the indicated times. Reactions products were analyzed by SDS-PAGE and stained with Coomassie Blue (top panel) or immunoblotted using anti-His tag antibodies (bottom panel). Molecular sizes are indicated (in kDa).

**Fig. 3.** Far-Western analysis of chymotrypsin-digested TnpX1–707 and TnpX1–597. The reaction products were separated on SDS-PAGE and blotted onto nitrocellulose. The blot was probed with N-terminal T7-tagged TnpX1–707 and detected with anti-T7 antibodies.
sponded to the absence of the last 110 C-terminal amino acids in the latter protein. All of these fragments were shown to be C-terminal fragments, because they reacted with anti-His tag antibodies (Fig. 2, bottom panels).

To determine whether the different proteolytic fragments were generated by cleavage at sites that were also highly accessible to other proteases, trypsin was used to digest both TnpX1–707 and TnpX1–597. Both proteins yielded digestion products very similar to those obtained with chymotrypsin (data not shown).

Identification of the N-termini of the Stable Proteolytic Fragments—Two proteolytic fragments generated from TnpX1–597, corresponding to the 39- and 33-kDa bands (Fig. 2B), were submitted for N-terminal sequencing so that the chymotrypsin digestion site could be mapped. The N-terminal sequences of these fragments were KLHKRK and GTHSNR, respectively, indicating that cleavage had occurred before Lys-267 and Gly-312. We could not generate sufficient amount of the 51-kDa product for sequencing, but based on the size of this TnpX fragment, we suggest that cleavage occurred between residues 165 and 170. Because the proteolytic pattern obtained with TnpX1–707 was comparable to that obtained with TnpX1–597, it was concluded that the 46-kDa proteolytic fragment generated from TnpX1–707, resulted from cleavage at the same site. Note that all of the chymotrypsin cleavage sites were located within putative random coil regions according to secondary structure predictions carried out using PSIPRED (22).

Based on these data, it appeared that TnpX was organized into three major domains: 1) an N-terminal domain (aa 1–165/170) that included the catalytic resolvase domain; 2) a middle domain from aa 165/170 to 266; and 3) the C-terminal domain (from aa 267), which could be further digested at residue 312. This last product was very stable and had a predicted α-helical structure.
structure. The middle domain contained two regions of unknown function that were conserved within the large serine recombinase family (7), namely, residues 177–203 (predicted secondary structure: β-strand-α-helix) and residues 240–263 (predicted secondary structure: α-helix-β-strand).

The Stable Proteolytic Fragments TnpX267–707 and TnpX267–597 Are Correctly Folded and Are Still Capable of Dimerization—We have recently shown by gel filtration that TnpX1–707 is a dimer in solution (17). Structural predictions indicated that there was a high probability that the C-terminal domain consisted of a significant amount of α-helical structures and multiple coiled-coil regions. As a consequence, we asked whether the C-terminal region of TnpX was involved in the dimerization process. Proteolytic fragments generated from TnpX1–707 and TnpX1–597 were assayed for their ability to multimerize using Far-Western analysis. The products of chymotrypsin digests were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were renatured by incubation of the membrane under conditions that allowed the recovery of activity of the undigested protein. The membrane was then probed with N-terminal T7-tagged TnpX1–707 before incubation with anti-T7 antibodies. The protein. The membrane was then probed with N-terminal T7-tagged TnpX1–707 before incubation with anti-T7 antibodies. The protein.

When a partial chymotryptic digest of TnpX1–597 was examined under these conditions (Fig. 5). These results implied that a major DNA binding domain to the aa 492–597 region (17). When a partial chymotryptic digest of TnpX1–597 was examined in the same way, TnpX1–597 and each of the C-terminal proteolytic fragments generated from TnpX1–707 and TnpX1–597 were tested for DNA binding activity by Southwestern blotting using a specific 32P-labeled TnpX267–707, and TnpX312–707 were still able to bind DNA under these conditions (Fig. 5). These results implied that a DNA binding domain was located in the C-terminal region of the protein as expected from previous studies that localized the major DNA binding domain to the aa 492–597 region (17). When a partial chymotryptic digest of TnpX1–597 was examined in the same way, TnpX1–597 and each of the C-terminal proteolytic fragments were also shown to bind DNA but with reduced affinity (data not shown). These data suggested that either TnpX1–597 was incorrectly refolded after renaturation or that a DNA binding domain was also located between residues 598 and 707.

To confirm the data obtained by Southwestern analysis and to further define the DNA binding regions, DNA binding experiments were conducted on purified TnpX267–707, TnpX267–597, and TnpX312–597 using TnpX1–707 and TnpX1–597 as positive controls. The specific binding of each of the purified proteins at similar concentrations was determined by gel mobility analysis using a radioactively labeled 360-bp attL fragment and a 405-bp attCI fragment. Only TnpX1–707, TnpX1–597, and TnpX267–707 interacted with these fragments (Fig. 6, A and B, lanes 2–4). To confirm that TnpX267–707, which does not contain the catalytic resolvase domain, was binding the DNA specifically, competitive gel mobility shift assays were performed. The addition of an excess of unlabeled attL DNA completely abolished the binding of both TnpX1–707 and TnpX267–707, whereas the addition of the same amount of nonspecific DNA (an unrelated pfoA gene fragment.
from Clostridium perfringens) had no effect (Fig. 6C). Finally, in the absence of the N-terminal resolvase domain, the removal of the last 110 aa had a dramatic effect on DNA binding since TnpX533–597 and TnpX312–597 only bound with very low affinity to attL and attCI (Fig. 6, A and B, lanes 5 and 6).

The Charged Domain (aa 533–583) and the Cysteine-rich Domain (aa 583–707) Are Both Involved in DNA Binding—These experiments and those of the previous study (17) provide evidence that the regions from aa 493 to 597 and aa 598 to 707 are both involved in DNA binding. The amino acid region of 493–597 contains two strings of oppositely charged amino acids, whereas the C-terminal cysteine-rich region (aa 598–707) contains two putative FCS (consensus sequence C\(X_2\)C\(X_{2-3}\)FCS\(X_2\)C\(X_{3-4}\)(F/Y)) zinc-coordinating domains (24), which are located between residues 591–616 and 639–664.

To provide more direct evidence that the oppositely charged region and the FCS motifs were involved in DNA binding, fragments encoding aa 533–597, aa 533–707, and aa 583–707 were cloned into pET22b and the resultant proteins were overexpressed and purified as before. The level of expression for TnpX583–707 was very low, leading to a poor yield of purified TnpX583–707 protein. After Talon chromatography, TnpX533–597 and TnpX533–707 were over 95% pure and showed an apparent molecular mass of 20 and 15 kDa, respectively, as observed by Tris-Tricine SDS-PAGE. The CD spectra of these polypeptides indicated that they were both folded with TnpX533–597 showing the double minimal characteristic of an \(\alpha\)-helical conformation (data not shown). In addition, gel filtration experiments revealed that both TnpX533–597 and TnpX583–707 were monomers in solution (data not shown). Gel mobility shift assays were then conducted on purified TnpX533–597, TnpX533–707, and the small amount of TnpX583–707 that was available. Each of these proteins was able to form a complex with attL DNA (Fig. 7A). The addition of excess, unlabeled attL DNA abolished the binding of TnpX533–597 and reduced the binding of TnpX533–707 (Fig. 7, B and C, lanes 4 and 5), whereas the addition of the same amount of nonspecific DNA had no affect (Fig. 7, B and C, lanes 6).

**Fig. 7. Gel mobility shift analysis of purified TnpX533–597, TnpX533–707, and TnpX583–707.** A, a fixed amount (0.1 pmol) of a 360-bp \(^{32}\)P-labeled attL fragment was incubated with varying amounts of purified TnpX533–597 (72.5, 50.75, and 29 pmol), TnpX533–707 (22, 14.5, and 5.5 pmol), and TnpX583–707 (23.75 and 11.9 pmol). The resultant protein-DNA complexes were analyzed on a 6% polyacrylamide gel. B and C, competition gel mobility shift assays were performed with purified TnpX533–597 (B) and purified TnpX533–707 (C). Both proteins were incubated at the highest and lowest protein concentrations used above with \(^{32}\)P-labeled attL (0.1 pmol), an excess (5.1 pmol) of the same unlabeled fragment, or an excess of unlabeled nonspecific DNA (pfo fragment, 5.1 pmol). The first lane in each series is the no protein control.
Based on these results, it is concluded that TnpX contains at least two DNA binding regions consisting of residues 533–597 and 598–707. Only the amino acid region of 533–597 is essential for biological activity (17).

TnpX1–266 and TnpX267–597 Interact and Can Cooperate in DNA Binding—Since TnpX1–597 but not TnpX267–597 was capable of binding specifically to the DNA, we decided to analyze the ability of the N-terminal resolvase domain to restore DNA binding after intermolecular association. Therefore, a TnpX1–266 derivative was cloned into pET22b, the resultant protein purified as before and shown by CD spectroscopy to be folded (data not shown).

To determine whether there was any interaction between the N- and C-terminal domains of TnpX, gel filtration experiments were conducted with purified TnpX1–266 and TnpX267–597. The elution patterns for TnpX1–266, TnpX267–597, and a TnpX1–266–TnpX267–597 mixture were monitored by analyzing each fraction by SDS-PAGE. Purified TnpX1–266 primarily eluted with an apparent molecular of 23.3 kDa (Fig. 8), corresponding to a monomer. Purified TnpX267–597 eluted as described previously with an apparent molecular mass of 95.5 kDa, corresponding to a dimer. When both fragments (with an excess of TnpX267–597) were preincubated for 15 min at room temperature before loading onto the gel filtration column, TnpX1–266 was displaced to a high molecular form and co-eluted with TnpX267–597 with an apparent molecular mass of 132 kDa corresponding to the size of a TnpX1–597 dimer binding to two TnpX1–266 monomers. These data suggested that there were protein-protein interactions between these two parts of the TnpX protein.

To see whether the interaction between these two domains was sufficient to restore DNA binding, gel shift experiments were carried out. The individual TnpX1–266 and TnpX267–597 proteins were not able to form a complex with attL DNA (Fig. 9, lanes 3 and 4). However, when these polypeptides were mixed in equimolar amounts and added to attL DNA, we observed a protein-DNA complex that migrated to the same position as the protein-DNA complex obtained with TnpX1–597 (Fig. 9, lane 5). In addition, the gel filtration-purified TnpX1–266–TnpX267–597 complex (Fig. 8) also had the ability to bind DNA (Fig. 9, lane 6). These data provide good evidence that intramolecular interactions occur between the N- and C-terminal fragments of TnpX and that these interactions can restore the ability of truncated derivatives to form a protein complex that binds to the specific TnpX binding site. Although we could restore the full DNA binding activity by incubating TnpX1–266 with TnpX267–707, no in vitro or in vivo recombination activity could be detected (data not shown), indicating that covalent linkage is required for biological activity.

**DISCUSSION**

TnpX is the only protein responsible for the precise excision of Tn4451 and the only transposon-encoded protein required for integration (13). TnpX-mediated recombination requires the binding of TnpX, presumably as a dimer, to each of its target sites followed by synapse formation to allow correct alignment of the GA-dinucleotides at the cleavage site, which is presumably mediated by TnpX-TnpX interactions. The final steps in recombination involve strand cleavage, strand trans-
fer, and religation. In the excision reaction, TnpX binds with equal affinity to the \textit{attL} and \textit{attR} ends of the transposon and strand exchange leads to the formation of a non-replicating circular intermediate (5, 17). By contrast, integration involves the binding of TnpX to the \textit{attCI} site at the joint of the circular intermediate, significantly weaker binding to an insertional target site, \textit{attT}, and subsequent strand exchange (17).

Prior to this study, little was known regarding the domain structure of TnpX. We have now shown that TnpX consists of three major domains (I, II, and III), each separated by exposed linker regions (Fig. 10). Each of the regions sensitive to proteolytic digestion was located within regions that, according to secondary structure predictions, consisted of random coils (17). These regions were located primarily in the N-terminal part of the protein (around residue 170, at residue 266, and at residue 312).

The N-terminal domain, domain I, was found to extend to a region around residue 170 and encompassed the catalytic resolvase region (aa 1–104), which is highly conserved among serine recombinases (7) and has been shown to be essential for TnpX function (5, 16). Domain II extended from aa 170 to 266 and contained two regions that were conserved between members of the large serine recombinase group (7). Finally, domain III stretched from residues 267 to 707 and was found to be highly resistant to proteolytic digestion. This domain contained a weakly conserved zinc finger-like motif (from residues 318 to 360) and two FCS-zinc finger motifs.

Gel filtration of the chymotryptic TnpX fragments showed that the 51-kDa fragment (residues 170–597) remained tightly associated with purified TnpX\textsubscript{267–597} and TnpX\textsubscript{312–597}. Furthermore, when the N- and C-terminal fragments were produced as the recombinant proteins TnpX\textsubscript{1–266} and TnpX\textsubscript{267–597}, they were found by gel filtration analysis to interact strongly (Fig. 8), providing evidence for intramolecular interactions between these TnpX domains.

Previous studies have shown that TnpX\textsubscript{1–597} and TnpX\textsubscript{1–492} exist as dimers, whereas TnpX\textsubscript{1–356} exists in two forms, both a dimer and monomer (17). In this study, it was shown that TnpX\textsubscript{1–266} behaved as a monomer, whereas TnpX\textsubscript{312–597} formed a dimer. Together these data suggest that the dimerization site lies at least partially within the amino acid region of 312–356. This region corresponds to a weak C4 zinc finger motif (residues 324–360) that has a predicted secondary structure also consistent with this type of motif (2–3 \beta-strands and an \alpha-helix). Zinc finger motifs from many proteins have been shown to mediate both homodimerization and heterodimerization depending on the protein (25). However, further mutagenesis studies are required to confirm the role of this region in TnpX dimerization.

Although gel mobility shift assays conducted on TnpX\textsubscript{1–266} and TnpX\textsubscript{267–597} demonstrated that neither protein was capable of DNA binding, the complex formed when they were pre-incubated together was shown to have the same DNA binding properties as native TnpX\textsubscript{1–597} (Fig. 9). These data indicated that the complex had the correct structural conformation in solution and that, not only were both domains intimately connected to each other by strong intramolecular interactions, this interaction was required for DNA binding (Fig. 10). However,
Despite the fact that the DNA binding properties were fully restored and the complex exhibited a similar pattern on gel filtration, no recombinase activity was evident either in vitro or in vivo. We conclude that, whereas specific DNA binding can tolerate a discontinuity in the peptide backbone, the catalytic activity of TnpX cannot.

Recent studies that utilized truncated derivatives of TnpX indicated the presence of an essential DNA binding region between aa 492 and 597 (17). We have now confirmed this conclusion by the cloning and purification of a fragment localized to this region and have shown that it was properly folded and was able to bind DNA specifically. Why was this polypeptide (TnpX1–597) active in DNA binding while TnpX267–597 had very poor DNA binding activity? It would seem that the formation of a dimer in the absence of the 1–266 region inhibits DNA binding, possibly because the putative DNA binding site is buried and is not accessible to the DNA. Conformation change brought about by intramolecular interaction between the N- and C-terminal domains appears to be required for DNA binding that is mediated by the functional dimeric form. This process may be required for specific synapase formation prior to recombination. It is clear that no such constraints are placed on the much smaller TnpX533–597 protein, which can bind efficiently as a monomer.

The C-terminal 110 aa of TnpX are not essential for biological activity, although their deletion does yield a TnpX enzyme with altered biological activity (17). Unexpectedly, the region between residues 583 and 707 was shown to bind specifically to TnpX target DNA. This region contains two FCS motifs (residues 583–707) that are known to be zinc binding domains in various chromatinic proteins and transcription factors (24). DNase I protection studies had previously indicated the presence of an essential DNA binding region (25). Though deletion resulted in a TnpX enzyme that was able to bind DNA specifically, why was this polypeptide (TnpX533–597) active in DNA binding while TnpX1–266 showed very poor DNA binding activity? It would seem that the formation of a dimer in the absence of the 1–266 region inhibits DNA binding, possibly because the putative DNA binding site is buried and is not accessible to the DNA. Conformation change brought about by intramolecular interaction between the N- and C-terminal domains appears to be required for DNA binding that is mediated by the functional dimeric form. This process may be required for specific synapase formation prior to recombination. It is clear that no such constraints are placed on the much smaller TnpX533–597 protein, which can bind efficiently as a monomer.

In summary, in this study, we have identified two regions that were important for DNA binding, neither of which contained the catalytic resolvase domain. The DNA binding region from residues 533 to 597 could bind DNA independently and was essential for the biological activity of TnpX. This region clearly represents the major DNA binding site. The second DNA binding region, residues 583–707, was not essential for biological activity. In addition, the dimerization domain of TnpX was localized to a region in close proximity to a putative C4 zinc finger domain. Finally, TnpX was shown to contain three major domains with intramolecular association between the N- and C-terminal domains required for TnpX dimers to bind specifically to the DNA.

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