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A Mechanism for Tn*5* Inhibition

CARBOXYL-TERMINAL DIMERIZATION*

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Lisa A. Mahnke Braam‡§, Igor Yu Goryshin, and William S. Reznikoff¶

From the Department of Biochemistry and the ‡M.D./Ph.D. Program, University of Wisconsin-Madison, Madison, Wisconsin 53706

Tn*5* is unique among prokaryotic transposable elements in that it encodes a special inhibitor protein identical to the Tn*5* transposase except lacking a short NH_2-terminal DNA binding sequence. This protein regulates transposition through nonproductive protein-protein interactions with transposase. We have studied the mechanism of Tn*5* inhibition *in vitro* and find that a heterodimeric complex between the inhibitor and transposase is critical for inhibition, probably via a DNA-bound form of transposase. Two dimerization domains are known in the inhibitor/transposase shared sequence, and we show that the COOH-terminal domain is necessary for inhibition, correlating with the ability of the inhibitor protein to homodimerize via this domain. This regulatory complex may provide clues to the structures of functional synaptic complexes. Additionally, we find that NH_2- and COOH-terminal regions of transposase or inhibitor are in functional contact. The NH_2 terminus appears to occlude transposase homodimerization (hypothetically mediated by the COOH terminus), an effect that might contribute to productive transposition. Conversely, a deletion of the COOH terminus uncovers a secondary DNA binding region in the inhibitor protein which is probably located near the NH_2 terminus.

Transposable DNA elements occur widely in nature and have important roles in evolution, chromosome mutagensis, and gene transfer. Common to all transposable elements is a need to fix transposition at low levels to avoid excessive mutagenesis of the host genome.

Transposition is a multistep process involving nucleoprotein intermediates which is catalyzed by an element-encoded transposase (Tnp).[1] The first step is the specific binding of Tnp to short transposon DNA end sequences that are inverted repeats. Synapsis follows such that Tnp-Tnp interactions bridge the ends. Subsequently, the DNA cutting and joining reactions of transposition occur (for a review, see Ref. 1). In the case of transposon Tn*5*, Tnp completely releases the element from the donor DNA molecule before inserting it into a target DNA site (2).

Tn*5* is a 5.8-kilobase composite transposon found in Gram-negative bacteria which consists of two insertion sequences, IS*50*R and IS*50*L (for reviews, see Refs. 3 and 4). The insertion sequences, 1.5 kilobases in length, are very nearly identical and are arranged in inverted orientation flanking a central region containing antibiotic resistance genes. The outermost ends of the Tn*5* transposon overall are 19-bp inverted repeats called outside ends (OE). IS*50*R encodes a 53-kDa Tnp protein, 476 amino acids in length, and a 48-kDa inhibitor protein (Inh), 421 amino acids in length.

Inh is encoded in the same reading frame as Tnp but is transcribed from a separate promoter and is translated from a distinct initiation codon such that Inh lacks the first 55 amino acids of Tnp. Inh, apparently, does not bind to OE DNA, as does Tnp (5), but it can participate in a three-molecule complex with an OE-bound Tnp monomer (5, 6). Whereas Tnp acts in *cis* (*i.e.* acts on local DNA sequences relative to its own gene), Inh is a *trans*-acting factor that functions post-translationally to form nonproductive complexes with Tnp (5). Tnp has also been shown to function as a self-inhibitor in *trans* (7, 8). Tnp and Inh are folded similarly in terms of gross secondary and tertiary structure (9), and thus the convention for numbering the primary amino acid sequence of Inh is based on the Tnp primary sequence (*i.e.* the initiating methionine of Inh is referred to as residue 56). Tnp and Inh share two distinct dimerization domains[2] (9, 10), one of which is located at the COOH terminus and the other near amino acids 114–314.

Correlating with the fact that Inh-DNA binding has not been detected, the NH_2 terminus of Tnp has been implicated in OE DNA recognition (9–11). A series of NH_2-terminal point mutations at residues 41, 47, and 54 in Tnp has been shown to enhance OE DNA binding affinity or specificity (11). Also, OE DNA can protect the first 113 amino acids of Tnp against proteolysis (9). Furthermore, the deletion of a very short part of the NH_2 terminus disrupts Tnp-DNA binding (10). Additionally, a predicted helix-turn-helix DNA binding motif is present in the NH_2-terminal region at residues 35–54.[3]

Tnp regions constituting a conserved catalytic domain have been predicted and verified within proteolysis-resistant segments spanning amino acids 93–217 (9) and 312–365 (12).[2] Part of the catalytic domain, therefore, overlaps with the more NH_2-terminal of the two known dimerization regions. *In vitro*, Tnp is the only protein necessary for the catalysis of transposition in the presence of divalent metal ions (2).

Many transposition systems are regulated negatively by repression of transcription, regulation of translation, innate Tnp instability or inactivity, or by accessory protein competition for DNA binding; however, transposase inhibition by nonproduc-

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§ Predoctoral trainee on National Institutes of Health Training Grant GM07215.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, 433 Babcock Dr., University of Wisconsin-Madison, Madison, WI 53706. Tel.: 608-262-3608; Fax: 608-262-3453; E-mail: reznikoff@biochem.wisc.edu.

[1] The abbreviations used are: Tnp, transposase; bp, base pair(s); OE, transposon outside end(s); Inh, transposase inhibitor; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

[2] D. R. Davies, L. A. M. Braam, H. Holden, W. S. Reznikoff, and I. Rayment, in preparation.

[3] A. S. Silbergleit, V. A. Lanzov, and N. Benuch, personal communication.

tive multimerization may be unique to Tn*5*, especially in prokaryotic systems. Here, the molecular mechanism of Tn*5* inhibition is explored. We demonstrate a critical importance of the COOH terminus of Tnp/Inh for inhibition *in vitro*. Because the two proteins are nearly identical, the structure of the inactive complex bound to DNA could provide clues to the nature of functional transposition complexes. An alternative hypothesis separates the inhibitory and synaptic functions of the Tnp protein into distinct regions, inhibition at the COOH terminus and synapsis at an unknown location. Furthermore, we find that the NH_2- and COOH-terminal regions of either Tnp or Inh are in close contact or proximity, and we discuss possible functional consequences of this structural arrangement.

EXPERIMENTAL PROCEDURES

Purification of Inh, Tnp, and TnpEK54/LP372—The overexpression and purification of Inh and Tnp in *Escherichia coli* have been described previously (9). TnpEK54/LP372 was purified similarly except Heparin HyperD (BioSepra) was used during the heparin chromatography step. Based on Coomassie-stained SDS-polyacrylamide gels, Inh was >95% pure, Tnp was >90% pure, and TnpEK54/LP372 was >95% pure. The proteins were stored in 0.4 M NaCl TEG (20 mM Tris-HCl, pH 7.9, 1 mM EDTA, 10% glycerol) at −70 °C.

Purification of His_6 Fusion Proteins—Fusion protein vectors encoding a 41-amino acid leader sequence with a His_6 tag and a protein kinase recognition site were constructed for Inh, InhEQ451, InhAD466, InhΔ430 (a COOH-terminal deletion), and Tnp using tailed *Eco*RI-containing primers with *Pfu* polymerase (Stratagene) polymerase chain reactions (9). The template for the InhEQ451 construct was pRZ622 (13). For InhAD466, the A466D mutation was introduced as a mismatch in one of the primers. All oligonucleotides were synthesized by Research Genetics. The polymerase chain reaction products were digested with *Eco*RI and were ligated into the *Eco*RI site in pET33b(+) (Novagen). The fusion proteins were overexpressed in *E. coli* and were purified as described previously (9). His_6-Inh, InhEQ451, and InhAD466 protein samples were >95% pure based on Coomassie-stained SDS-polyacrylamide gels. His_6-InhΔ430 was partially purified. The proteins were stored in 0.4 M NaCl HEG (20 mM HEPES, pH 7.2, 1 mM EDTA, 10% glycerol) at −70 °C.

In Vitro Inhibition—The *in vitro* assay for Tn*5* transposition has been described (2); however, we used modified OE with mutations at positions 10, 11, 12, and 15 which are hyperactive (14) in conjunction with a modified DNA substrate useful for the detection of cleavage events. This 4-kilobase DNA substrate, pUC19 (15)-based pGRST2, contains 1.3 kilobases of "transposon" DNA consisting of the kanamycin resistance gene from Tn*903* flanked by the modified OE 19-bp ends. A unique *Xho*I restriction site is located 180 bp from one transposon end within the transposon. The functional version of Tnp used in this assay has a double mutation, E54K/L372P, which renders a hyperactive, *trans*-active transposition phenotype. Transposition reactions contained 0.1 M potassium glutamate, 25 mM Tris acetate, pH 7.5, 10 mM Mg^{2+}, 50 μg/ml bovine serum albumin, 0.5 mM β-mercaptoethanol, 2 mM spermidine, 100 μg/ml tRNA, 50 mM NaCl, 34 nM pGRST2 supercoiled plasmid DNA, 0.21 μM TnpEK54/LP372, and various concentrations of Inh, His_6-Inh, or His_6-InhAD466. The reactions were incubated at 37 °C for 1 h. *Xho*I (Promega) was added, and the reactions were incubated further at 37 °C for 1 h. SDS was added to 0.5%, and the reactions were heated to 68 °C for 15 min. Loading dyes were added, and the reactions were analyzed by 1% agarose gel electrophoresis in 1 × TAE electrophoresis buffer. The gels were stained with SYBR Green (Molecular Probes) and were analyzed by fluoroimaging.

Gel Filtration Analysis of Inh and Tnp—The potential for homodimerization of Tnp or Inh was assayed by gel filtration. In the presence of 10% glycerol, Inh was tested in 0.1 M and 0.4 M NaCl, whereas Tnp was tested in 0.4 M NaCl only because of its tendency to precipitate at lower salt concentrations. For these studies, a Toyopearl TSK HW 55 superfine (Supelco) 200-ml column was equilibrated with 0.1 M NaCl or 0.4 M NaCl TEG and was calibrated with molecular weight markers (Sigma). 1 ml of a 0.5 mg/ml protein sample was applied to the column, and each sample was chromatographed at 0.5 ml/min. An additional study was conducted in which Inh was chromatographed at low salt concentrations in the absence of glycerol. For this experiment, a Bio-Gel P-100 (Bio-Rad) 150-ml column was equilibrated with 0.1 M NaCl TE (20 mM Tris-HCl, pH 7.9, 1 mM EDTA) and was calibrated with molecular weight markers (Sigma). A 2-ml sample of 0.5 mg/ml Inh was chromatographed at 0.4 ml/min. Fractions were analyzed by Bradford assay, and logarithmic plots of elution volume/void volume *versus* peak fraction were used for molecular weight calculations.

Protein-Protein Cross-linking—9 μl of His_6-protein samples of Inh, Tnp, InhEQ451, or InhAD466, 0.1 mg/ml in 0.4 M NaCl HEG each, were mixed with 1 μl of freshly diluted 0.1% or 1% glutaraldehyde (Sigma). The reactions were incubated at 25 °C for various times. 1 μl of 1 M Tris-HCl, pH 8.0, was added along with 3 μl of SDS-PAGE loading buffer, and the samples were incubated for 5 min at 68 °C. The samples were analyzed by SDS-PAGE. The gels were stained with SYPRO Orange (Bio-Rad) (analyzed by fluoroimaging) or Coomassie Blue.

GST-Tnp Pull-down Assays—A GST-Tnp fusion protein was overexpressed in *E. coli* DH5α using expression vector pRZ4779[4] based on pGEX-2T (Amersham Pharmacia Biotech). As a negative control, GST was expressed from pGEX-2T. 1-liter cultures containing 100 mg/ml ampicillin were grown at 37 °C to mid-log phase, and protein synthesis was induced with 300 μM isopropyl 1-thio-β-D-galactopyranoside for 1.5 h. Cells were harvested and resuspended in 10 ml of 0.3 M NaCl TEG and were lysed using a French Press at 16,000 p.s.i. Lysates were cleared by centrifugation. 0.5-ml samples of 50% glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), equilibrated in 0.4 M NaCl TEG, were added to 2.5 ml of the lysates, and the reactions were incubated at 25 °C for 30 min. The beads were washed five times with 0.1% milk powder, 0.4 M NaCl TEG. 100 μl of 1.0 mg/ml Inh was added to 50% bound-bead suspensions, and the reactions were incubated at 30 °C for 30 min. The beads were washed with 0.4 M NaCl TEG, resuspended in SDS-PAGE loading buffer, and boiled for 1 min. Supernatants were analyzed by SDS-PAGE, and the gels were stained with Coomassie Blue. For qualitative comparisons with and without DNA, His_6-Inh was labeled at the fusion kinase site with [γ-^{32}P]ATP as described previously (9). 200 μl of 50% suspensions of GST-Tnp-bound beads (approximately 5 μg/ml in GST-Tnp) equilibrated in 0.2 M NaCl TEG was mixed with 90 μl of 200 mM potassium glutamate. 10 μl of a 29-bp OE-containing DNA fragment (9) (0.2 mg/ml), calf thymus DNA (0.2 mg/ml), or H_2O was added. 3 μl of 0.1 mg/ml ^{32}P-labeled His_6-Inh was added, and the reactions were incubated at 25 °C for 1 h. 300 μl of 1% milk powder, 0.2 M NaCl TEG was added. The beads were washed twice with 0.2 M NaCl TEG, then they were resuspended with 100 μl of SDS-PAGE loading dyes and boiled for 1 min. Supernatants were analyzed with 12% SDS-PAGE, and the gels were dried, imaged, and quantitated by PhosphorImaging.

Gel Shift Assay—1 μl of His_6-Inh, His_6-InhAD466, or His_6-InhEQ451, 0.1 mg/ml each, or 1 μl of a partially purified His_6-InhΔ430 preparation (0.1 mg/ml in fusion protein) or mock preparation was incubated in 20-μl reactions containing 0.7 μg/ml of a ^{32}P-labeled, 60-bp, OE-containing DNA fragment (10) and 5 μg/ml Tnp or TnpEK54/LP372 in 10 mM HEPES, pH 7.2, 200 mM NaCl, 100 mM potassium glutamate, 0.5 mM EDTA, 5% glycerol at 30 °C for 30 min (final ratio is 1:1 Inh:Tnp). 4 μl of loading dye was added, and the samples were electrophoresed at 4 °C with 8% native PAGE in 1 × TBE electrophoresis buffer. The gels were analyzed by autoradiography.

RESULTS

In Vitro Inhibition—An *in vitro* assay for Tn*5* transposition has been developed which utilizes two point mutations in Tnp, E54K and L372P, to promote both general and *trans* activities of the otherwise inactive transposase *in vitro* (2), and we tested Inh for inhibitory activity in this system. The results of an *in vitro* transposition assay performed in the presence of various concentrations of Inh are shown in Fig. 1. We show that the addition of increasing amounts of Inh produces increasing inhibition. As the molar ratio of Inh:Tnp approaches 1, transposition, as measured by donor backbone excision, is near zero. Moreover, the inhibitory effect is strong; 50% inhibition is achieved with a molar ratio of approximately 1:3 to 1:4, Inh:Tnp. We know that the inhibition is not the result of nonspecific effects because a mutant Inh protein (InhAD466), added in equal concentrations relative to Inh, was found to have no inhibitory effect (this result is discussed below). If we assume that functional synapsis is required for cleavage and that synapsis is the result of one monomer of Tnp binding to each OE followed by Tnp-Tnp dimerization, then the inhibition experiment suggests inhibition before synapsis because one molecule

[4] Constructed by M. D. Weinreich.

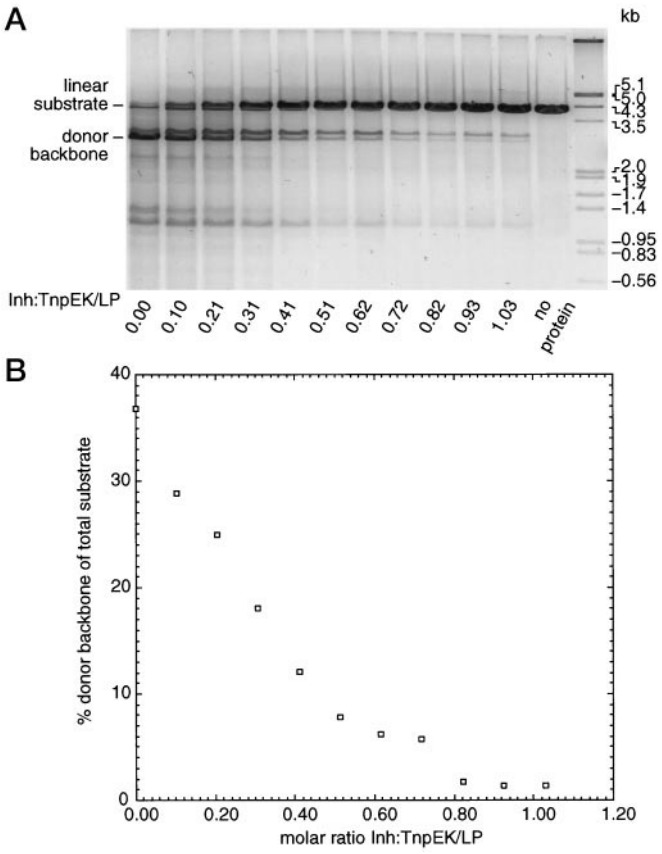

FIG. 1. ***In vitro* inhibition of transposition.** *Panel A*, transposition reactions were performed using a supercoiled plasmid DNA substrate and TnpEK54/LP372 with added Inh at various molar ratios, Inh:Tnp. After 1 h, the reactions were digested with *Xho*I to linearize all DNA molecules, and the reactions were analyzed using 1% agarose gel electrophoresis and fluoroimaging of SYBR Green-stained gels, which were quanititated. The full-length linear substrate and excised donor backbone fragment are indicated, although other transposition products are apparent at low molar ratios. *Panel B*, percent donor backbone DNA, a measure of transposon double-ended cleavage, was calculated, and the results were plotted against the molar ratio, Inh:Tnp, for each lane in *panel A*. 50% inhibition is taken as 18.5% donor backbone release because the uninhibited reaction reached 37% donor backbone release.

is capable of inhibiting multiple molecules of Tnp. For example, one Inh monomer should be sufficient to prevent the formation of one synapse if it can heterodimerize with one Tnp monomer that is bound to one transposon end, even if the other end is bound by another Tnp monomer. This hypothesis assumes that the nonproductive complex is relatively strong, and it does not rule out Inh-Tnp interactions prior to Tnp-OE binding which might contribute to inhibition.

Tnp or Inh Gel Filtration and Chemical Cross-linking—The potential for homodimerization of Inh or Tnp was addressed by both gel filtration and covalent protein-protein cross-linking to probe the role of multimerization in transposition or inhibition. The results of gel filtration experiments conducted under various solution conditions are presented in Table I. We have found that in the presence of glycerol (with buffer containing 0.1 M NaCl) Inh can homodimerize, as has been shown previously (5). However, in the absence of glycerol (with buffer containing 0.1 M NaCl) Inh is predominantly monomeric. At higher salt concentrations (with buffer containing 0.4 M NaCl and glycerol) Inh homodimerizes, whereas Tnp is predominantly monomeric. Glutaraldeyde cross-linking was used to confirm the homodimerization of Inh. Fig. 2 demonstrates that in the presence of 0.01% glutaraldehyde (with buffer containing 0.4 M NaCl and glycerol) His_6-Inh cross-links into homodimers,

TABLE I

Gel filtration results of Inh or Tnp analyzed under various conditions

The expected molecular masses are as follows: Inh monomer, 47.6 kDa; Inh dimer, 95.2 kDa; Tnp monomer, 53.3 kDa; Tnp dimer, 106.6 kDa.

	Tris-HCl buffer	Major peak	Result
		kDa	
Inh	0.1 M NaCl	52	Monomer
Inh	0.1 M NaCl, 10% glycerol	109	Dimer
Inh	0.4 M NaCl, 10% glycerol	110	Dimer
Tnp	0.4 M NaCl, 10% glycerol	54	Monomer

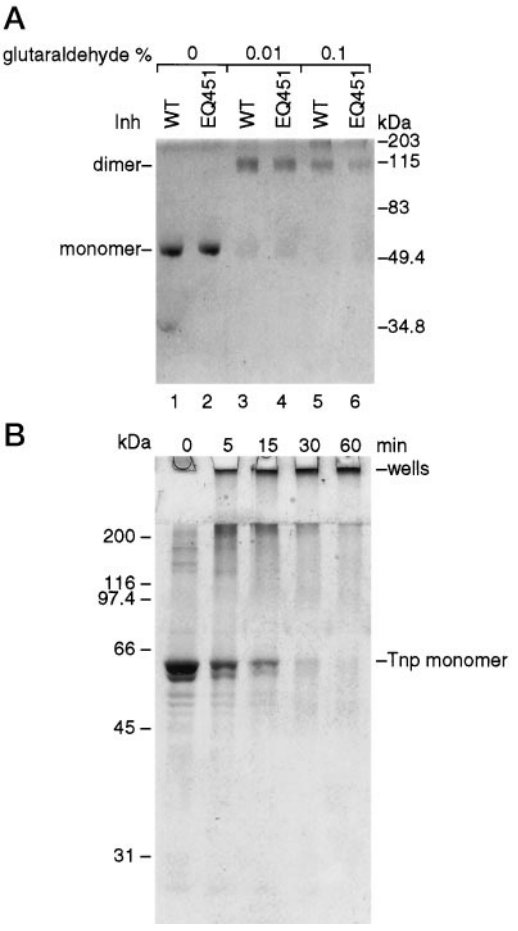

FIG. 2. **Glutaraldehyde protein-protein cross-linking.** *Panel A*, His_6-Inh or His_6-InhEQ451 in 0.4 M NaCl HEPES buffer with 10% glycerol was reacted with 0.01 or 0.1% final concentrations of glutaraldehyde for 5 h at 25 °C. The reactions were analyzed by 12% SDS-PAGE, and the gel was stained with Coomassie Blue. The expected molecular masses of His_6-Inh or His_6-InhEQ451 monomers and dimers are 52.0 and 104 kDa, respectively. *Panel B*, His_6-Tnp in 0.4 M NaCl HEPES buffer with 10% glycerol was reacted with 0.01% gluaraldehyde for various times, and the reactions were analyzed by 9% SDS-PAGE. The gel was stained with Coomassie Blue. Expected molecular masses are as follows: His_6-Tnp, 57.8 kDa, monomer, and 115.6 kDa, dimer.

whereas in 0.1% glutaraldehyde, His_6-Inh cross-links into a mixture of homodimers and higher order multimers (Fig. 2*A*, *lanes 3* and *5*). Next, we tested His_6-Tnp in a glutaraldehyde cross-linking experiment and found that, in contrast to His_6-Inh, His_6-Tnp did not cross-link into dimers and remained relatively monomeric while exhibiting some aggregation, as deduced by the appearance of His_6-Tnp in the wells of the polyacrylamide gel (Fig. 2*B*). The relative instability of Tnp and its propensity for aggregation have been observed by us and by others and correlates with a tendency toward precipitation at lower salt concentrations. In summary, both the gel filtration

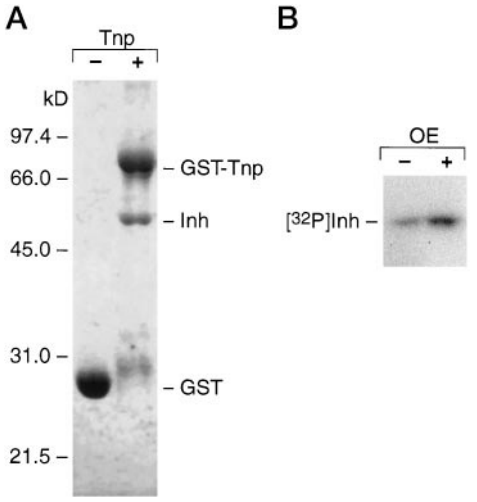

FIG. 3. **GST-Tnp pull-down assay for heterodimerization.** *Panel A*, GST-Tnp or GST, immobilized on glutathione beads, was incubated with Inh, and the beads were washed. Retained proteins were analyzed by 12% SDS-PAGE, and the gel was stained with Coomassie Blue. *Panel B*, GST-Tnp bound to glutathione beads was incubated with ^{32}P-labeled His_6-Inh with or without OE DNA, and the beads were washed. Retained ^{32}P-labeled His_6-Inh was analyzed by 12% SDS-PAGE; the gel was imaged by PhosphorImaging and was quantitated.

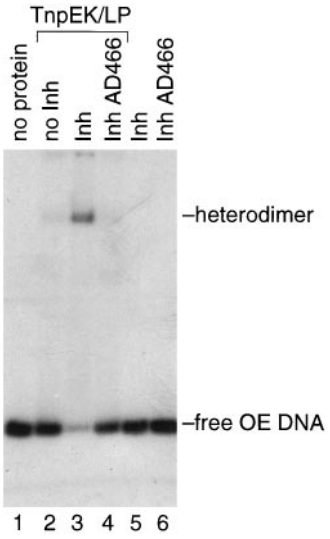

FIG. 4. **Gel shift assay; DNA-bound heterodimers.** His_6-Inh or His_6-InhAD466 was added to OE DNA binding reactions in the presence or absence of TnpEK54/LP372; the reactions were analyzed by 8% native PAGE and autoradiography.

and cross-linking experiments indicate that Inh homodimerizes whereas Tnp is monomeric. Furthermore, the dimerization of Inh is facilitated by glycerol and is relatively insensitive to salt, suggesting that hydrophobic effects may be important at the dimer interface. Finally, because Inh lacks a short part of the Tnp NH_2 terminus, we speculate that the NH_2-terminal region of Tnp interferes with Inh-like homodimerization.

GST-Tnp Pull-down Assay with Inh—Interactions between Inh and Tnp have been reported using a gel shift assay in which Tnp is bound to one molecule of OE DNA (5, 6); here, we have sought any heterodimer interactions that might occur in the absence of DNA. We used a pull-down assay in which GST-Tnp was immobilized onto glutathione beads before incubation with Inh. Then, the beads were washed and analyzed for retained protein. The assay was performed under the same conditions in which Inh is known to dimerize, whereas Tnp is monomeric (0.4 M NaCl with glycerol). Fig. 3*A* shows an SDS-PAGE analysis of proteins recovered from this assay. We find that Inh is immobilized only in the presence of GST-Tnp, and we conclude that Inh heterodimerizes with Tnp.

Because two modes of heterodimerization have now been observed (free and DNA-bound heterodimers), we repeated the pull-down assays to reveal any differences between dimerization in the presence of OE DNA and dimerization in the absence of DNA. To accomplish this, immobilized GST-Tnp was incubated with or without a 3-fold molar excess of OE DNA before the addition of ^{32}P-labeled His_6-Inh. The amount of resulting immobilized ^{32}P-labeled His_6-Inh was then measured and was found to be consistently higher in the presence of OE DNA (Fig. 3*B*) (variability was observed between experiments probably because of differential washing times; the error in independent experiments conducted in duplicate was 3%). Nonspecific DNA also increased the immobilization of ^{32}P-labeled His_6-Inh (data not shown) although to a lesser extent than OE DNA. In summary, because more Inh was found to bind to GST-Tnp in the presence of DNA, we hypothesize that the DNA-bound heterodimer is relatively more stable than the free heterodimer.

OE DNA Gel Shift Assay—To address whether Inh could heterodimerize with TnpEK54/LP372 (the mutant protein used in the *in vitro* transposition/inhibition assay), a gel mobility shift experiment was performed in which Inh was added to Tnp-DNA binding reactions (the Inh was added last, but the order of addition has no effect on complex formation[5] In such assays, Tnp exhibits little or no monomeric binding, except in cases in which a COOH-terminal truncation of Tnp is present (6, 8, 10). However, the addition of Inh is known to promote strongly the binding of Tnp into a heterodimeric complex that contains one DNA molecule (5, 6). In Fig. 4, the results of a gel shift assay with radiolabeled OE DNA are presented. The heterodimeric complex bound to one molecule of DNA is stimulated in the presence of the Inh fusion protein (compare *lanes 2* and *3*, Fig. 4). This experiment confirms that Inh heterodimerizes with TnpEK54/LP372 in a DNA-bound complex, and we suspect that this complex may be important for inhibition. The addition of Inh also appears to increase the complexes remaining in the well. This is a variable result that is thought to be an artifact of Tnp aggregation.

InhEQ451—A hypertransposing mutation in Tnp and Inh, E451Q, has been identified previously *in vivo* (13) which, because of its location near one of the known dimerization domains, was predicted to function through a reduction in inhibitory regulation of Tn*5* transposition (9). We studied this mutation in the context of His_6-Inh for dimerization and for inhibitory activity. Gel shift analysis with TnpEK54/LP372 (data not shown) revealed that His_6-InhEQ451 could heterodimerize with Tnp-DNA as efficiently as His_6-Inh. In addition, cross-linking experiments indicated that His_6-InhEQ451 could homodimerize (Fig. 2*A*, *lanes 4* and *6*). Furthermore, His_6-InhEQ451 was found to inhibit TnpEK/LP *in vitro* to the same extent as His_6-Inh (data not shown). Thus, residue 451 is apparently not involved in either Inh-like homodimerization, Inh-Tnp-DNA heterodimerization or inhibition, as thought previously. However, recent structural studies of Inh may explain why E451Q does not disrupt dimerization. These studies reveal a COOH-terminal dimerization domain in which Glu-451 is not part of the dimerization interface itself but resides near the beginning of a long helix whose end forms the basis of the interface (discussed further below).[2] We hypothesize that the E451Q mutation in Tnp influences transposition during some step other than regulatory inhibition, perhaps during transpo-

[5] D. York and W. S. Reznikoff, unpublished data.

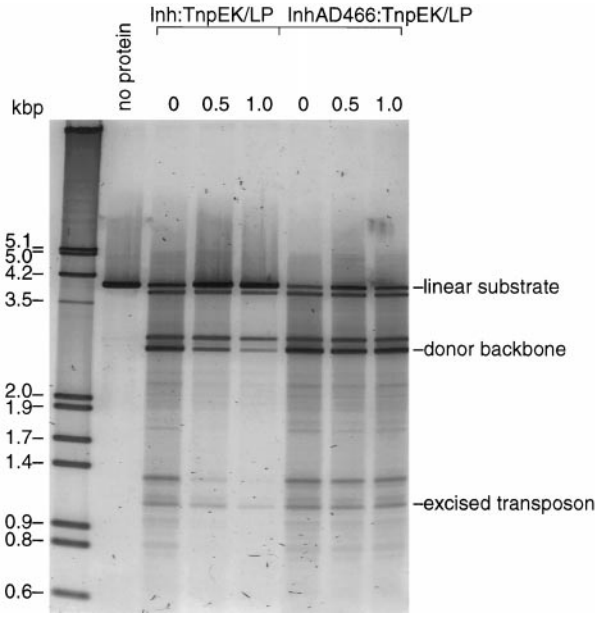

FIG. 5. ***In vitro* inhibition of transposition.** His_6-Inh or His_6-InhAD466 was added to transposition reactions at molar ratios of 0, 0.5, or 1.0 His_6-protein:TnpEK54/LP372. The reactions were digested with *Xho*I and were analyzed using 1% agarose gel electrophoresis with ethidium bromide staining. The inhibitory effect of His_6-Inh is slightly reduced compared with Inh as in Fig. 1.

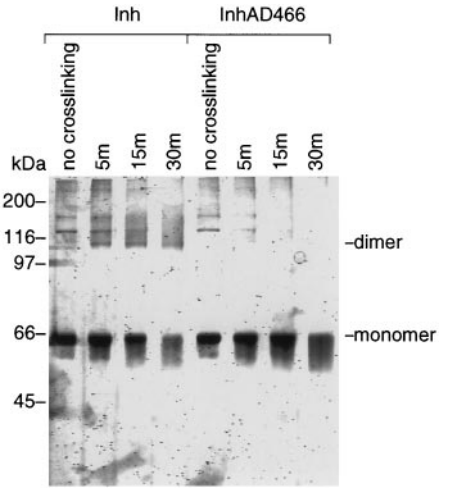

FIG. 6. **Glutaraldehyde protein-protein cross-linking.** His_6-Inh or His_6-InhAD466 was cross-linked for various times in 0.01% glutaraldehyde. The reactions were analyzed by 9% SDS-PAGE; the gels were stained with SYPRO Orange (Bio-Rad) and were analyzed by fluoroimaging.

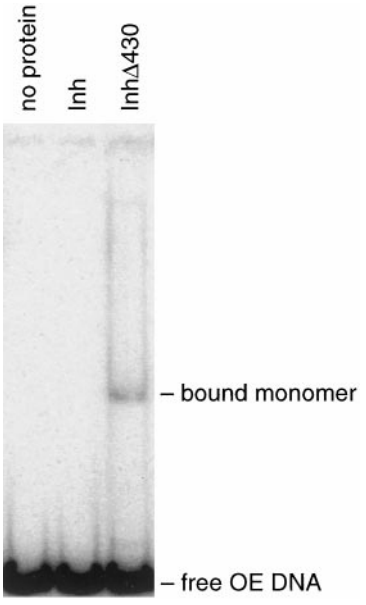

FIG. 7. **Gel shift assay; Inh truncation.** His_6-Inh or His_6-InhΔ430 was incubated with OE DNA, and the reactions were analyzed by 8% native PAGE. The gel was imaged by autoradiography.

son DNA end binding or during the capture of target DNA.

InhAD466—We wished to mutagenize the COOH-terminal dimerization domain of Inh at the recently discovered dimer interface[2] to test for functional effects. The dimer interface is formed predominantly by the close interaction of two helices in which the β-carbon of alanine 466 faces the interface, adjacent to the corresponding β-carbon of residue 466 in the dimer partner.[2] Thus, the substitution of aspartic acid for alanine at this position should disrupt the close interaction of the dimer helices by steric occlusion and electrostatic repulsion. We introduced the Ala → Asp mutation at position 466 by site-directed mutagenesis and found the overexpressed His_6-InhAD466 protein to be stable, as judged by relative expression levels and the absence of detectable degradation fragments during purification (data not shown). His_6-InhAD466 was compared with His_6-Inh for inhibitory activity and, as shown in an *in vitro* transposition assay in Fig. 5, was found to be inactive for inhibition at all protein concentrations tested. We note that the His_6-Inh protein was slightly less active for inhibition than Inh itself (this may reflect NH_2-terminal fusion and COOH-terminal domain interactions; the importance of the COOH terminus for inhibition and evidence for the close proximity of NH_2 and COOH termini is discussed below). Furthermore, His_6-InhAD466 did not homodimerize when tested in a cross-linking experiment (Fig. 6), nor did it heterodimerize in gel shift assays with DNA-bound Tnp (data not shown) or TnpEK54/LP372 (Fig. 4, *lane 4*), in contrast to His_6-Inh. We conclude that the A466D mutation disrupts the dimer interface at the COOH terminus of the protein and that this interface is critical for inhibition.

InhΔ430—To study further the effects of mutating the COOH terminus of Inh, a truncation was introduced which deletes all residues COOH-terminal to residue 430. His_6-InhΔ430 was found to have relatively low expression levels in *E. coli* compared with His_6-Inh. We tested partially purified preparations in DNA binding assays (in the absence of Tnp). Because Inh has not been previously reported to bind to DNA, we were surprised to find that His_6-InhΔ430 could bind to OE DNA (Fig. 7). Mock preparations did not show OE DNA binding activity (data not shown), nor did His_6-Inh (Fig. 7). The shifted complex consists of a bound His_6-InhΔ430 monomer, as judged by comparison with the monomeric binding of a previously characterized protein, TnpΔ387 (6, 10) (data not shown). Thus, the COOH-terminal deletion in Inh has apparently exposed a previously unknown DNA binding region.

DISCUSSION

Inh Inhibits Synapsis—Here we find that purified Inh strongly inhibits Tn*5* transposition *in vitro* such that one molecule of Inh apparently inhibits multiple molecules of TnpEK54/LP372. This effect suggests that inhibition occurs prior to synapsis because the present model of transposition requires two or more functional Tnp molecules for every synapse, and therefore the nonproductive complexation of one Tnp molecule at one transposon end would block synapsis and effectively

inactivate a second Tnp molecule at the other end. Corresponding to our *in vitro* data, Inh has been demonstrated to inhibit TnpEK/LP *in vivo* whereas InhAD466 (see below) does not.[6]

DNA-bound Heterodimerization Is Likely the Predominant Inhibitory Mechanism—Inh and Tnp heterodimers were detected both free in solution and within a DNA-bound complex, raising the possibility that either of these heterodimeric complexes may be important for inhibition. Because the DNA-bound heterodimer is more abundant than the free heterodimer in immobilization assays we suspect that the DNA-bound complex is highly stable. Correspondingly, we confirmed that Inh heterodimerizes with TnpEK54/LP372 in a gel shift experiment. Although the shift experiment and our *in vitro* inhibition experiments were performed under different conditions using different OE DNA substrates (short OE-containing fragments and mutated OE-containing DNA supercoiled circles, respectively), we are able to correlate DNA-bound heterodimerization with inhibition. Further evidence that the DNA-bound heterodimer is more stable than the free heterodimer has been found in gel shift experiments in which the DNA-bound heterodimer product was neither reduced nor enhanced by premixing Tnp and Inh.[5] Therefore, it appears that monomers of Tnp bind to DNA, that heterodimerization occurs after DNA binding, and that free heterodimerization has only minimal effects on DNA-bound heterodimer complex formation. In summary, we suspect that DNA-bound heterodimers may be the major mediators of inhibition.

In support of the hypothesis described above, we find that monomers of Tnp are abundant. In gel filtration studies, Tnp was found to be predominantly monomeric, whereas Inh could homodimerize. In addition, no Tnp-Tnp dimers were detected in cross-linking studies although Inh-Inh dimers were found. As discussed above, monomer-DNA binding is probably prerequisite to dimer complex formation with Inh and could also be important for synapsis, even though monomeric, untruncated Tnp apparently binds slowly or with low affinity to OE DNA.

The COOH-terminal Domain of Inh/Tnp Is Critical for Inhibition—Mutation of the recently discovered dimerization interface in Inh at the COOH terminus by site-directed mutagenesis abolishes not only *in vitro* inhibition but also homodimerization and OE-bound Tnp-Inh heterodimerization. We conclude that this domain represents the critical dimerization main for inhibition. The point mutation, A4660D, is not predicted to expose unfavorable side chains to solvent upon disruption of the dimer interface,[2] and we correspondingly found that the mutant protein was stable during overexpression and purification. Evidence that the COOH-terminal region can be disrupted without disturbing other functional regions in Inh is also found in our studies of InhΔ430 because this protein can bind to DNA. A second COOH-terminal mutated protein, InhEQ451, was found to be active for inhibition and could homodimerize and also heterodimerize with OE-bound Tnp. Although residue 451 is located near the COOH-terminal dimerization domain, this result confirms that the critical dimer interface is located at the end of the long COOH-terminal helices of the dimer.[2] In summary, Inh COOH-terminal homodimerization reflects the mechanism of Tn*5* inhibition, and this mechanism likely occurs through Inh heterodimerization with Tnp, probably via DNA-bound complexes.

The NH_2 and COOH Termini of Tnp and of Inh Are in Close Proximity—The COOH-terminal regulatory dimerization exerted by Inh is a functional consequence of the deficiency of the first 55 amino acids of Tnp, perhaps uncovering the COOH-terminal domain and making it available for dimerization. This hypothesis explains the monomeric nature of Tnp in contrast to Inh and suggests that the NH_2- and COOH-terminal regions of Tnp are in close proximity or close contact. In much the same manner, dominant-negative mutants of the maize *Activator* transposase have been found by mutating the NH_2-terminal DNA binding region of the protein; these mutants act by nonproductive oligomerization with functional transposase (16). There could be a purpose for the interaction of NH_2 and COOH termini in Tn*5* Tnp and, perhaps, other transposases, at least in bacterial cells; usually the NH_2 terminus is associated with DNA binding, and because it is produced first during translation it might bind to DNA before the inhibitory COOH terminus can be produced. Perhaps such self-regulation contributes to the *cis* phenotype of transposases in prokaryotic cells, including that of Tn*5*, where transcription and translation are coupled, and thus the nascent protein is physically close to transposon end sequences. In fact, the COOH terminus of Tn*5* Tnp does appear to inhibit DNA binding activity, as discussed below.

Interactions between NH_2- and COOH-terminal regions probably also occur in Inh, even though Inh is missing a short part of the corresponding Tnp NH_2 terminus. When we removed 46 amino acids from the Inh COOH terminus we uncovered a secondary DNA binding region that we suspect may be located near the NH_2 terminus of the protein. In fact, structural studies show that the NH_2- and COOH-terminal regions are spatially close in a three-dimensional structure of Inh,[2] and nearby is a disordered region containing a predicted helix-turn-helix, 107–124 amino acids.[3] We can also expect the NH_2 terminus of Tnp to be situated near this region, and it contains a second predicted helix-turn-helix DNA binding region, amino acids 35–54.[3] If this arrangement exists, it may resemble the dual helix-turn-helix DNA binding motifs that have been identified in protein-DNA structural studies of the Tc3 transposase (17) and the MuA transposase (18).

Just as a COOH-terminal deletion uncovered a DNA binding function in Inh, such deletions have also been found to stablize monomeric Tnp binding to OE DNA (5, 7, 9), presumably via the known NH_2-terminal DNA binding domain. Thus, the COOH terminus of Tnp partially interferes with DNA binding. Tnp-DNA binding is also stabilized by heterodimerization with Inh (5), which could be caused by removal of a hinderance of the COOH terminus via COOH-terminal heterodimerization. A somewhat similar situation is found in the *Drosophila* transposable P element system in which a KP repressor, an internal deletion of transposase, binds DNA more readily than the transposase and utilizes dimerization for high affinity binding (19).

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