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Purification of TnsB, a Transposition Protein That Binds to the Ends of Tn7*

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We have purified TnsB, a transposition protein encoded by the bacterial transposon Tn7. The purification procedure involves three chromatographic steps (DNA-cellulose, norleucine-Sepharose, and phosphocellulose) and yields milligram quantities of highly purified protein. The apparent molecular mass of denatured TnsB protein is approximately 85 kDa. Gel filtration chromatography and sucrose gradient sedimentation studies indicate that in solution, native TnsB is a monomer of nonspherical shape. Using DNase I protection analysis, we established that TnsB is a sequence-specific DNA-binding protein that recognizes multiple sites in both ends of the transposon. The TnsB binding sites, three in the left end of Tn7 and four in the right end, are highly related in nucleotide sequence and are located in DNA segments that we have previously shown contain *cis*-acting sequences important for Tn7 transposition. Our results also show that one of the TnsB binding sites overlaps a proposed promoter for the transposition genes of Tn7. These studies suggest that the specific binding of TnsB to the ends of Tn7 mediates recombination and may also regulate the expression of Tn7-encoded transposition genes.

Transposable elements are DNA segments that can move from one genetic location to another. To promote this movement, transposons encode two types of information: transposition proteins, usually one but occasionally more, and *cis*-acting DNA sequences that are directly involved in transposition (for review, see Berg and Howe, 1989). In most transposons, these sequences are confined to the termini of the element. A key step in transposition is the recognition of the recombination sequences at the ends by transposition proteins. The interactions between transposition proteins and the transposon ends have been analyzed at the biochemical level for only a few transposons (Craigie *et al.*, 1984; Groenen *et al.*, 1987; Ichikawa *et al.*, 1987; Zerbib *et al.*, 1987; Gierl *et al.*, 1988; Wiater and Grindley, 1988; Kunze and Starlinger, 1989). We are interested in understanding the protein-DNA interactions that mediate the movement of the bacterial transposon Tn7 (Barth *et al.*, 1976; for review, see Craig, 1989).

Tn7 is unique among transposable elements because it encodes five transposition genes, *tnsABCDE*, that mediate two transposition pathways (Rogers *et al.*, 1986; Waddell and Craig, 1988; Kubo and Craig, 1990). *tnsABC* + *tnsD* mediate high frequency transposition to a specific site in the *Escherichia coli* chromosome, *attTn7*, whereas *tnsABC* + *tnsE* mediate low frequency transposition to apparently random target sites. Because *tnsABC* are required in both pathways, the products of these genes are good candidates for recombination proteins that interact directly with the transposon ends.

We established previously that the *cis*-acting DNA segments required for both Tn7 transposition pathways lie at the ends of the transposon (Arciszewska *et al.*, 1989). These segments are extensive and differ in length and sequence; about 150 bp[1] at the left end of Tn7 (Tn7L)[2] and about 100 bp at the right end of Tn7 (Tn7R) are necessary for efficient transposition. The Tn7 ends are also functionally distinct; miniTn7 elements containing two Tn7R ends are active in transposition whereas those containing two Tn7L ends are not. As in many other transposons, the extreme termini of Tn7 are very similar; the terminal 30 bp of Tn7 are imperfect inverted repeats. In addition, the ends are related by the presence of multiple copies of highly homologous 22-bp repeats that are arranged differently in each end (Lichtenstein and Brenner, 1982). Tn7L contains three directly oriented repeats separated by two segments of unrelated sequence. Tn7R contains four closely spaced repeats in inverted orientation with respect to those in Tn7L. Our *in vivo* analysis of the *cis*-acting transposition sequences at the Tn7 ends indicates that these 22-bp repeats are important for transposition (Arciszewska *et al.*, 1989).

Previously we detected in cell lysates a *tnsB*-dependent DNA binding activity that specifically recognizes both ends of Tn7 (McKown *et al.*, 1987). We demonstrate here that this activity reflects the specific interaction of TnsB protein with the *cis*-acting transposition sequences of Tn7. We report the purification of TnsB protein and show that it binds to multiple sites in both ends of the transposon; these binding sites encompass the 22-bp repeats. The specific recognition of the Tn7 ends by TnsB is consistent with the requirement for

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[1] The abbreviations used are: bp, base pair(s); BSA, bovine serum albumin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] Tn7 end segments are designated L (left end) or R (right end). The numbers denote the extent of Tn7 sequences; for example, R1-199 denotes a Tn7R segment extending from the terminal bp (R1) of the right end of Tn7 to position R199.

tnsB in both Tn7 transposition pathways (Rogers *et al.*, 1986; Waddell and Craig, 1988).

We also show that one of the TnsB binding sites overlaps a proposed *tns* gene promoter (Gay *et al.*, 1986). This observation supports the hypothesis (Rogers *et al.*, 1986) that TnsB can also play a regulatory role in Tn7 transposition by modulating the expression of the Tn7 transposition genes (Waddell and Craig, 1988; Flores *et al.*, 1990; Orle and Craig, 1990).

EXPERIMENTAL PROCEDURES

Bacteria and Plasmids

tnsB and *tnsABCDE* cell lysates were prepared from CAG456 (*E. coli* K12 $lacZ_{am}$ trp_{am} pho_{am} $supC_{ts}$ *rpsL* $htpR_{165}$; Baker *et al.*, 1984) carrying the indicated plasmids. TnsB protein was isolated from CAG456 carrying plasmids F′$lacI^q$ *lacZ*::Tn5 (Sauer *et al.*, 1988) and p*tac-tnsB*, a pBR322 derivative containing the *tnsB* gene downstream of the *tac* promoter. p*tac-tnsB* was constructed by inserting a 2.5-kilobase pair *Hin*dIII fragment containing *tnsB* from pCW12 (McKown *et al.*, 1987) into the *Pvu*II site of p*tac*12 (Amann *et al.*, 1983) by blunt end ligation. The *tnsB*-containing fragment extends from the *Bgl*II site approximately 900 bp from the right terminus of Tn7 (flanked by *Bam*HI to *Hin*dIII of the pUC19 polylinker) to the *Hin*dIII site located approximately 3,440 bp from the right terminus of Tn7 (Flores *et al.*, 1990; Waddell and Craig, 1988). *tnsABCDE* lysates were prepared from CAG456-carrying pCW4, a pACYC184 plasmid containing *tnsABCDE* (McKown *et al.*, 1987). Host lysates were prepared from CAG456. Cell lysates and protein fractions other than those containing TnsB which were used in the *in vitro* transposition assay were prepared from strains containing other *tns* plasmids as described in Bainton *et al.* (1991).

The Tn7L and Tn7R fragments used in the DNA binding assays were isolated from plasmids containing various Tn7 end segments. Plasmids pKS$^+$Ra and pKS$^+$La were constructed by Roland Bainton. The fragment containing R1–199 was isolated from pKS$^+$Ra, which contains the *Eco*RI-*Bam*HI Tn7R fragment of pLA28 (Arciszewska *et al.*, 1989) inserted between the *Eco*RI and *Bam*HI sites of Bluescript-KS (Stratagene). The fragment containing L1–166 was isolated from pKS$^+$La, which contains the *Eco*RI-*Bam*HI Tn7L fragment of pLA26 (Arciszewska *et al.*, 1989) inserted between the *Eco*RI and *Bam*HI sites of Bluescript-KS. The fragment containing L109–166 was isolated from pLA77, which was constructed by inserting an *Alu*I-*Hin*cII fragment, obtained by digestion of the *Eco*RI-*Hin*cII Tn7L fragment from pLA26, into *Eco*RV of Bluescript-KS; in pLA77, position L109 is adjacent to the vector *Eco*RI site. Plasmids used as substrates in the *in vitro* transposition assay are described elsewhere (Bainton *et al.*, 1991).

Preparation and Labeling of DNA Fragments

Plasmid DNA was digested with appropriate restriction enzymes and electrophoresed through 6% polyacrylamide gels. Slices containing the DNA fragments of interest were cut out from the gels, the DNA electrophoretically transferred onto a DEAE membrane (Schleicher & Schuell), and recovered as suggested by the manufacturer. Purified DNA fragments were labeled at their 3′ ends using DNA polymerase I Klenow fragment and appropriate [α-^{32}P]dNTPs (Tabor and Struhl, 1987). The *Eco*RI-*Bam*HI fragment (294 bp) from pKS$^+$Ra containing R1–199 was used for analysis of Tn7R; this fragment was labeled at *Eco*RI for analysis of the top strand[3] and at *Bam*HI for analysis of the bottom strand. Fragments from pKS$^+$La were used for analysis of L1–166, the *Sma*I-*Hin*dIII (270 bp) fragment labeled at *Hin*dIII for the top strand and the *Eco*RI-*Bam*HI (258 bp) fragment labeled at *Eco*RI for the bottom strand. For analysis of L109–166, the *Sma*I-*Hin*dIII (88 bp) fragment of pLA77 labeled at *Hin*dIII was used.

DNA Binding Assays

The band shift method (Fried and Crothers, 1981; Garner and Revzin, 1981) was used to evaluate the specific binding of TnsB fractions to Tn7 end fragments. Prior to assay, TnsB fractions were diluted in P buffer with 0.5 M NaCl (see below) containing 1 mg/ml BSA (Fraction V, Boehringer Mannheim) (except in the experiments in Table I, in which TnsB fractions were diluted in host cell lysates prepared from strains lacking TnsB). The binding reactions (20 μl) contained 1 μl of diluted TnsB, 13.8 mM Tris·HCl (pH 8.0), 1.05 mM EDTA, 2.05 mM DTT, 125 mM NaCl, 10.5% (v/v) glycerol, 350 μg/ml poly(dI-dC)·poly(dI-dC), 1.05 mg/ml BSA, and approximately 0.15 pmol of end-labeled DNA fragment. After incubation for 7 min at room temperature, the reaction mixtures were electrophoresed through 6% polyacrylamide gels (29:1 acrylamide/*N,N′*-methylenebisacrylamide) in Tris borate/EDTA buffer at 19 V/cm for 90 min. After drying, the gels were exposed to x-ray film. The amount of DNA fragment complexed with TnsB was evaluated by densitometric scanning of autoradiograms. One unit of TnsB binding activity is arbitrarily defined as the amount of protein required to shift about 50% of the L109–166 fragment under these conditions and is observed with reactions containing 1.3 μg/ml Fraction IV TnsB.

Buffers

CL buffer was 150 mM Tris·HCl (pH 8.0), 0.1 M NaCl, and 1 mM EDTA; P buffer was 25 mM Tris·HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, 10% (v/v) glycerol, and NaCl as indicated. S buffer was P buffer with 0.5 M NaCl but contained 25% glycerol.

Cell Growth and Recovery

Bacterial cultures used for protein purification were grown in LB broth (Miller, 1972) supplemented with carbenicillin at 100 μg/ml in a 60-liter fermentor at 30 °C. At A_{600} 1.2, the cells were induced by the addition of isopropyl-β-D-thiogalactoside to 230 μg/ml and grown for an additional 2 h (final A_{600} 2.0). The cells were harvested by centrifugation, washed in CL buffer, spun again, divided into approximately 40-g portions, and frozen in liquid nitrogen. Cultures for small volume lysates were grown at 30 °C without induction, harvested in exponential phase, washed, and frozen.

tnsABCDE and Host Cell Lysate Preparation

CL buffer was added to frozen cell pellets (3 ml/g of cells), and lysis was achieved through a cycle of freezing and thawing in the presence of lysozyme at 200 μg/ml. Cell debris was removed by a 20-min centrifugation at 48,000 × *g*. During the preparation of *tnsABCDE* lysate, DNA was removed by DNase I treatment using conditions as described for TnsB purification, and the collected lysate was dialyzed against P buffer with 0.1 M NaCl. Cell lysates and protein fractions used for the *in vitro* transposition assay were prepared as described elsewhere (Bainton *et al.*, 1991).

TnsB Purification

Unless otherwise indicated, all steps were carried out at 4 °C. The results of a typical preparation are presented in Table I and Figure 1.

Cleared Cell Lysate Preparation—40 g of frozen cell pellet was thawed in the presence of 1 volume of CL buffer (1 ml/g of cells). $MgCl_2$ (10 mM), $CaCl_2$ (2 mM), and pancreatic DNase I (10 μg/ml) were added to the cell suspension. The cells were disrupted by a single passage through a French press at 400 atm. After addition of another 2 volumes of CL buffer, the crude extract was centrifuged for 45 min at 48,000 × *g*. The concentrations of $MgCl_2$ (10 mM) and $CaCl_2$ (2 mM) were readjusted in the collected supernatant, and another portion of DNase I (10 μg/ml) was added followed by incubation at 16 °C for 30 min. The cell lysate was dialyzed extensively against P buffer with 0.1 M NaCl with three 6-h changes; the first change also contained 20 mM EDTA. After centrifugation at 90,000 × *g* for 80 min, a clear cell lysate was collected (Fraction I). The amount of TnsB in Fraction I was estimated by immunoblot analysis to be less than 0.5% of total protein (data not shown).

DNA-cellulose Chromatography—Fraction I was applied to a double-stranded DNA-cellulose column (40-ml packed volume equilibrated with P buffer with 0.1 M NaCl) at a flow rate of 8 ml/h. After a 160-ml wash with P buffer with 0.1 M NaCl (flow rate 20 ml/h), the column was eluted with a 10-column volume linear gradient of P buffer with 0.1–1.4 M NaCl (flow rate 40 ml/h). *tnsB*-dependent binding activity eluted from the column between 0.38 and 0.51 M NaCl, coincided with a peak of 280 nm absorbance, and comigrated with the 85-kDa TnsB polypeptide on SDS-PAGE. TnsB fractions were pooled to form Fraction II.

Norleucine-Sepharose Chromatography—Solid ammonium sulfate was added to Fraction II to 20% saturation. After centrifugation at 90,000 × *g* for 30 min, the supernatant was applied to a norleucine-Sepharose column (8-ml packed volume, equilibrated with P buffer

[3] The top and bottom strands are as shown in Fig. 6.

TABLE I

TnsB purification

Specific activities of the pooled fractions were compared using the band shift assay to evaluate TnsB binding to the L109-166 fragment. Prior to assay, aliquots of each fraction were diluted in crude lysates from cells lacking TnsB; the specific activity of the TnsB fractions was not different when the fractions were diluted and assayed in the presence of BSA, *i.e.* without the addition of *E. coli* crude lysate.

Fraction	Volume	Total protein	Total activity	Specific activity	Purification	Yield
	ml	*mg*	*units*	*units/mg*	*-fold*	*%*
I Cleared lysate	100	2,350	587,000	250	(1)	(100)
II DNA-cellulose	25	16	380,000	23,700	95	65
III Norleucine-Sepharose	18.5	3.7	245,000	66,300	267	42
IV Phosphocellulose	4.8	2.6	100,000	38,500	154	17

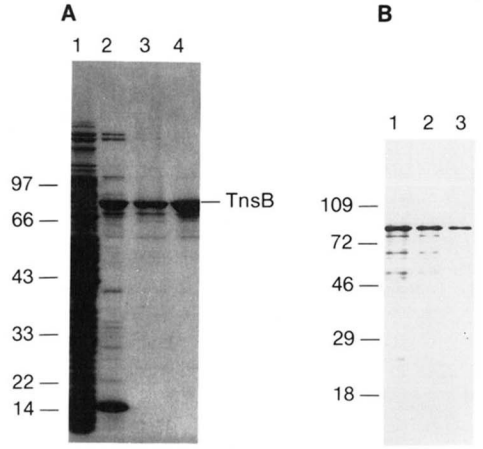

FIG. 1. **Protein composition of TnsB fractions.** *Panel A,* SDS-PAGE analysis of TnsB fractions. Each fraction was separated by SDS-PAGE on a 5–15% gradient gel and stained with Coomassie Blue R-250. *Lane 1,* Fraction I, 75.2 μg; *lane 2,* Fraction II, 10.4 μg; *lane 3,* Fraction III, 3.2 μg; *lane 4,* Fraction IV, 8.8 μg. The *numbers* at the *left* indicate the positions of molecular weight markers. *Panel B,* immunoblot analysis of purified TnsB protein. The indicated amounts of TnsB protein (Fraction IV) were separated by SDS-PAGE on a 7.5% gel and detected with anti-TnsB antibodies. *Lane 1,* 320 ng; *lane 2,* 60 ng; *lane 3,* 13 ng. The *numbers* at the *left* indicate the positions of molecular weight markers.

with 0.5 M NaCl + 20% $(NH_4)_2SO_4$) at a flow rate of 4 ml/h. The column was washed with 18 ml of P buffer with 0.5 M NaCl + 20% $(NH_4)_2SO_4$ and then eluted with 48 ml of P buffer with 0.5 M NaCl + 10.3% $(NH_4)_2SO_4$ at a flow rate of 16 ml/h. TnsB fractions were pooled to form Fraction III.

Phosphocellulose Chromatography—Fraction III was diluted 2-fold with P buffer with 0.5 M NaCl (to avoid protein precipitation upon dialysis into low salt buffer), dialyzed against P buffer with 0.25 M NaCl with two 6-h changes and applied to a phosphocellulose column (5-ml packed volume, equilibrated with P buffer with 0.25 M NaCl) at a flow rate of 5 ml/h. The column was washed with 15 ml of P buffer with 0.25 M NaCl and then eluted stepwise with 15 ml of P buffer with 0.39 M NaCl and then with 15 ml of P buffer with 0.52 M NaCl. TnsB eluted from the column with the second step. TnsB fractions were pooled to form Fraction IV.

Storage—Purified protein was dialyzed against S buffer, divided into aliquots, frozen in liquid nitrogen, and stored at −80 °C.

Resins—DNA-cellulose was prepared from CF11 cellulose (Whatman) and calf thymus DNA (Pharmacia LKB Biotechnology Inc.) as described by Alberts and Herrick (1971). Norleucine-Sepharose was prepared as described in Morris *et al.* (1979). Phosphocellulose (Whatman) was precycled as suggested by the manufacturer.

Nuclease Test

Circular single-stranded *M13mp18(+)* phage DNA (5 μg/ml) and supercoiled Bluescript KS^+ plasmid DNA (3.5 μg/ml) were used as substrates in 50-μl reactions in 15 mM Tris·HCl (pH 8.0), 1.1 mM EDTA, 2.1 mM DTT, 150 mM NaCl, 15 mM $MgCl_2$, 11% (v/v) glycerol, and 0.8 mg/ml BSA. TnsB (final concentration of Fraction IV = 5.5 μg/ml and of Fraction III = 2.0 μg/ml) was incubated with DNA for 15 min at 30 °C. The reactions were stopped by the addition of 2 ml of 0.2 M EDTA. DNA was subjected to electrophoresis on 0.7% agarose gels and visualized by ethidium bromide staining.

Gel Filtration Chromatography

A Sephadex G-150 column (98 × 1.6 cm) equilibrated with 25 mM Tris·HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, and 0.5 M NaCl was run at a flow rate of 4 ml/h. Protein standards (Pharmacia) at 2 mg/ml and TnsB (Fraction IV) at 40 μg/ml were run separately; the elution volumes for the standards and TnsB were determined by protein content and DNA binding activity, respectively.

Sucrose Gradient Sedimentation

5 ml of 5–20% (w/v) sucrose gradients were prepared in 25 mM Tris·HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, and 0.5 M NaCl. Protein standards (Pharmacia) at 2 mg/ml and TnsB (Fraction IV) at 140 μg/ml were layered onto the gradients and centrifuged in a 50.1 (Spinco) rotor at 40,000 rpm for 15 h. Fractions were collected from the bottoms of the tubes. Positions of the protein standards were determined by protein content, and the position of TnsB was determined by evaluation of DNA binding activity.

DNase I Protection Experiments

Protection assays were performed using the procedure of Galas and Schmitz (1978). The reactions (100 μl) contained 15 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 1.1 mM DTT, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 11% (v/v) glycerol, 2 ng/ml poly(dI-dC)·poly(dI-dC), 250 μg/ml BSA, and approximately 0.15 pmol of 3′ end-labeled DNA fragments. After incubation for 20 min at room temperature in the presence of TnsB, 10 μl of 5 μg/ml pancreatic DNase I (Worthington) was added, and the incubation was continued for 2 min. Digestion was stopped by the addition of 100 μl of 0.65 M ammonium acetate, 0.1 M EDTA, and 50 μg/ml of poly(dI-dC)·poly(dI-dC). DNA was precipitated with 3 volumes of ethanol, washed in 0.3 M sodium acetate, reprecipitated, and resuspended in formamide gel loading solution. DNA sequencing was performed using the chemical degradation method (Maxam and Gilbert, 1980).

In Vitro Transposition Assay

In vitro assays were carried out as described by Bainton *et al.* (1991). Reactions (100 μl) were performed in 26 mM Hepes (pH 7.5), 2 mM Tris·HCl (pH 7.5), 2.5 mM KPO_4, 128 mM KCl, 15 mM NaCl, 0.1 mM EDTA, 2.2 mM DTT, 0.8% (v/v) glycerol, 15 mM magnesium acetate, 2 mM ATP, and 5% polyvinyl alcohol in the presence of 10 ng of donor plasmid containing a miniTn7 element and 2 μg of an *attTn7* target plasmid. The reactions also contained approximately 40 μg of *tnsA* cell lysate, 17 ng of TnsB (Fraction IV), 10 μg of *tnsC* cell lysate, and 4 μg *tnsD* fraction (Stage III). After preincubation in the absence of magnesium acetate for 7 min at 30 °C, the reactions were incubated for 15 min at 30 °C, stopped, and the DNA purified from the other reaction components. Recovered DNA was linearized by restriction enzyme digestion, electrophoresed through 0.7% agarose, transferred to Nytran (Schleicher & Schuell), and analyzed by Southern hybridization using as a probe a DNA fragment specific for the miniTn7 element.

Other Methods

Preparation of affinity-purified anti-TnsB antibodies and immunoblot analysis using conjugated alkaline phosphatase for detection were carried out as described elsewhere (Orle and Craig, 1990). SDS-

PAGE was carried out as described in Smith (1987). Protein content was assayed by the Bradford method (Bradford, 1976). The amino acid sequence of the N terminus of TnsB was determined by the Protein Structure Laboratory, HSRL, Davis, CA, on an AB1470 Gas Phase Sequencer.

RESULTS

We reported previously (McKown *et al.*, 1987) that crude *E. coli* lysates prepared from cells carrying Tn7 or a *tnsABCDE* plasmid contain a *tnsB*-dependent DNA binding activity that specifically recognizes DNA fragments containing the *cis*-acting transposition sequences at the ends of Tn7 but does not recognize DNA fragments lacking these sequences. Here we report the fractionation of this binding activity and show that TnsB is a sequence-specific DNA-binding protein that recognizes the ends of Tn7.

In our previous studies of the *tnsB*-dependent DNA binding activity we used crude lysates from cells containing all the *tns* genes. We began our current characterization of the *tnsB*-dependent binding activity by establishing that *tnsB* is the only transposon-encoded gene required for specific end recognition as judged by the band shift assay method (Fig. 2; Fried and Crothers, 1981; Garner and Revzin, 1981). The *tnsB*-dependent binding activity produces several protein-DNA complexes when the intact Tn7 ends are used as substrates, which presumably reflect the presence of several protein binding sites in each end (McKown *et al.*, 1987). To simplify the assay for the purpose of monitoring the purification of TnsB, we selected a DNA fragment that contains only a single binding site: the Tn7L fragment containing L109–166 (see below). Our previous *in vivo* studies have shown that this region is critical for transposition (Arciszewska *et al.*, 1989).

Purification of TnsB

TnsB protein was purified from *E. coli* containing a pBR322 derivative in which *tnsB* is located downstream of an inducible *tac* promoter. As a host, we used an *htpR*$^-$ strain that is known to be defective in proteolysis (Baker *et al.*, 1984) because we observed substantial proteolytic degradation of TnsB in *htpR*$^+$ hosts (data not shown; Orle and Craig, 1990). Fractionation of the *tnsB*-dependent binding activity was monitored by band shift assays using the L109–166 DNA fragment as a substrate, and protein composition was followed by SDS-PAGE. The purification procedure (described in detail under "Experimental Procedures") involves three chromatography steps: DNA-cellulose, norleucine-Sepharose, and phosphocellulose. The *tnsB*-dependent DNA binding activity and the 85-kDa TnsB polypeptide (Orle and Craig, 1990) copurified at all steps during fractionation. The results of a typical preparation are presented in Table I and Fig. 1. This purification procedure yields about 2.5 mg of nearly homogeneous TnsB protein from 40 g of cells with recovery of about 20% of the original binding activity present in the cleared cell lysate. The specific DNA binding activity, as measured by band shift assays, increased about 150-fold (Table I).

The predominant species in Fraction IV is an 85-kDa polypeptide which is intact TnsB protein (Fig. 1*A*; Orle and Craig, 1990). However, several smaller polypeptides, ranging in size from 55 to 85 kDa, can also be seen. These smaller polypeptides are specifically recognized by anti-TnsB antibodies (Fig. 1*B*). We estimate that at least 90% of Fraction IV is intact TnsB polypeptide; the shorter TnsB polypeptides result from proteolytic degradation (data not shown).

We also evaluated the purity of the TnsB fractions by testing for the presence of nonspecific single- and double-stranded DNA endonucleases; the DNA substrates in these assays lacked Tn7 sequences. No endonucleolytic activity was detectable in Fraction IV or even Fraction III upon incubation of these TnsB fractions with DNA at protein concentrations severalfold higher than those used in other biochemical experiments (see "Experimental Procedures").

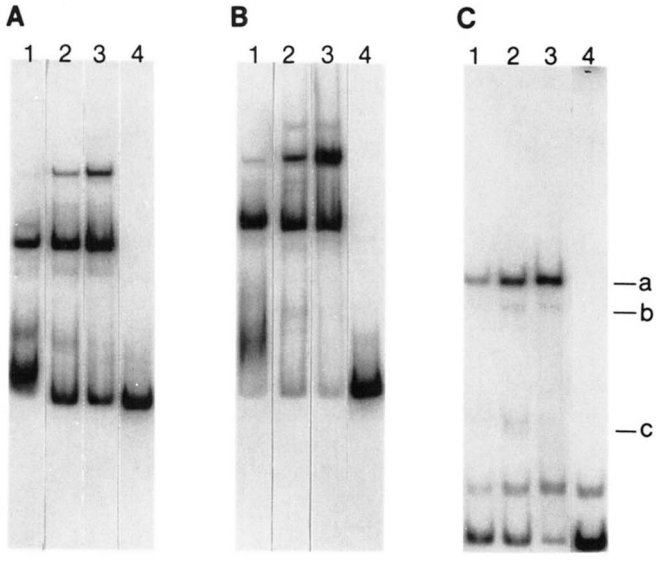

FIG. 2. **TnsB binds to ends of Tn7.** The DNA binding activity of various TnsB-containing fractions was evaluated by band shift assays using different Tn7 end fragments as substrates. *Panel A,* L1–166; *panel B,* R1–199; *panel C,* L109–166. *Lane 1, tnsABCDE* crude cell lysate, 280 μg/ml final concentration in reaction mixture; *lane 2, tnsB* crude cell lyste (Fraction I), 50 μg/ml in *A* and *B* and 120 μg/ml in *C; lane 3,* purified TnsB (Fraction IV), 1 μg/ml in *A* and *B* and 2.5 μg/ml in *C; lane 4,* no addition. In *C,* the complex labeled *a* is thought to contain intact TnsB whereas those labeled *b* and *c* are proposed to contain TnsB derivatives generated by proteolytic degradation.

Properties of TnsB

N-terminal Amino Acid Sequence—The amino acid sequence of the first 12 amino acids of Fraction IV TnsB protein was determined to be X-X -Gln-Ile-Asn-Glu-Val-Val-Leu-Phe-Asp-Asn. A nucleotide sequence encoding this amino acid sequence is present at the 5′ end of the *tnsB* gene (Flores *et al.*, 1990; Orle and Craig, 1990).

Native Molecular Mass—The apparent molecular mass of denatured TnsB is approximately 85 kDa as determined by SDS-PAGE (Fig. 1*A*; Orle and Craig, 1990). This value is in good agreement with the value of 81 kDa calculated from the DNA sequence of the gene (Flores *et al.*, 1990). Characterization of the native molecular mass of TnsB by gel filtration chromatography and sucrose gradient sedimentation analysis suggests that TnsB is a monomer in solution.

The observed Stokes radius of TnsB, as determined by gel filtration chromatography, is 5.4 nm (Fig. 3*A*). This value corresponds to a spherical protein of approximately 190 kDa. However, the sedimentation coefficient of TnsB as determined by sucrose gradient sedimentation is 4.3 s (Fig. 3*B*), a value similar to that observed for 67-kDa bovine serum albumin. The native molecular mass of TnsB is calculated as 96 kDa (Siegel and Monty, 1966). The calculated frictional ratio f/f_0 is 1.8. These results suggest that TnsB is monomeric in solution and that it is a protein of markedly nonspherical shape.

TnsB Binds Specifically to the Ends of Tn7

We used the band shift assay to examine the interaction of purified TnsB protein with the ends of Tn7 (Fig. 2). Several

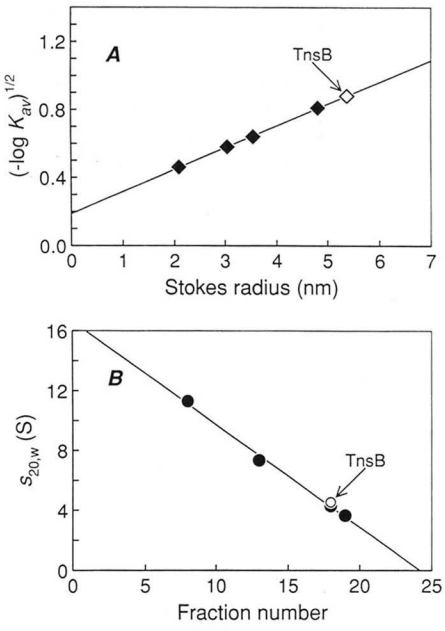

FIG. 3. **Determination of the native molecular weight of TnsB.** *Panel A*, gel filtration chromatography. The Stokes radius of TnsB was determined to be 5.4 nm by comparison of its observed elution position (*open square*) relative to the observed elution positions and known Stokes radii (*filled squares*) of the protein standards during gel filtration chromatography: aldolase, 4.81 nm; BSA, 3.55 nm; ovalbumin, 3.05 nm; and chymotrypsinogen A, 2.09 nm. *Panel B*, sucrose gradient sedimentation. The sedimentation coefficient of TnsB was determined to be $s_{20,w}$ 4.3 by comparison with its observed sedimentation position (*open circle*) relative to the observed sedimentation positions and known $s_{20,w}$ values (*filled circles*) of the protein standards during sucrose gradient sedimentation: catalase ($s_{20,w}$ 11.3), aldolase ($s_{20,w}$ 4.31), and ovalbumin ($s_{20,w}$ 3.66).

discrete complexes are observed with DNA fragments containing Tn7L (*panel A*) and Tn7R (*panel B*). The protein-DNA complexes formed with purified TnsB (Fig. 2, *A* and *B*, *lane 3*) are identical in mobility to complexes formed with crude cell lysates from a *tnsABCDE* strain (Fig. 2, *A* and *B*, *lane 1*) and a *tnsB* strain (Fig. 2, *A* and *B*, *lane 2*). No such protein-DNA complexes are observed with DNA fragments that lack Tn7 end sequences with either lysates from cells lacking *tnsB* (McKown *et al.*, 1987) or purified TnsB (data not shown). These results suggest that the specific interaction of TnsB with DNA underlies the *tnsB*-dependent complexes observed with the crude cell lysates. As considered in more detail below, both the Tn7L and Tn7R DNA segments contain several TnsB binding sites. A reasonable explanation for the several TnsB·DNA complexes observed with both Tn7L and Tn7R is that they reflect various distributions of TnsB among the multiple TnsB binding sites. Direct analysis of these complexes provides experimental support for this view.[4] Although our results indicate that TnsB is a monomer in solution, we have no information about the oligomeric state of TnsB at each TnsB binding site.

When a fragment of Tn7L containing a single TnsB binding site, L109–166, is used as a substrate in a band shift assay with crude *tnsABCDE* lysate, *tnsB* lysate and purified TnsB (Fig. 2*C*), a single major complex (marked *a*) of retarded mobility is observed. The fact that complexes of identical mobility are observed with all the protein sources supports the view that TnsB mediates the *tnsB*-dependent DNA binding activity. Close inspection of the L109–166 complexes reveals the presence of several minor species (marked *b* and *c*); other minor species are also present with the intact Tn7 ends (*panels A* and *B*). We suspect that these complexes contain smaller TnsB polypeptides that result from proteolytic degradation. Our TnsB preparation does contain smaller TnsB species, and these "minor" TnsB·DNA complexes increase in amount when TnsB fractions containing more of the shorter TnsB polypeptides are used in such assays (data not shown). The major TnsB-derived polypeptides that we have observed (apparent molecular mass between 55 and 85 kDa, Fig. 1*A*) copurify with intact TnsB, making preparation of the homogeneous, intact protein difficult.

Several lines of evidence suggest that TnsB protein binds directly to DNA. TnsB is, by far, the major protein in our purified fraction (Fig. 1*A*). Moreover, we have observed that the TnsB polypeptide copurifies with the specific Tn7 end binding activity throughout the purification procedure. As shown in Fig. 4, for example, the amount of shifted complex detected using the L109–166 fragment as a substrate in a band shift assay parallels the amount of TnsB polypeptide when fractions resulting from the stepwise elution of TnsB from a phosphocellulose column are examined. The binding activity of purified TnsB protein is not different when assayed in the presence or absence of host crude lysate (data not shown), and complexes of apparently identical mobility are observed with *tnsB*-containing crude lysates and purified TnsB protein (Fig. 2).

Identification of TnsB Binding Sites by DNase I Protection Analysis

To identify directly the positions of TnsB binding within the ends of Tn7, we carried out DNase I protection experiments with end-labeled DNA fragments containing Tn7L or Tn7R. The results of this analysis are shown in Fig. 5 and summarized in Fig. 6.

In Tn7L, three regions of protection, designated α, β, and γ, were observed in the presence of TnsB. Each of these

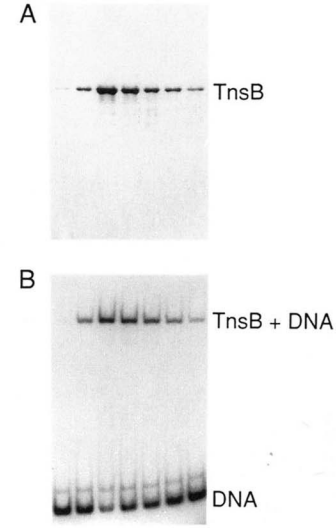

FIG. 4. **Specific binding to the end of Tn7 copurifies with TnsB.** 1.1 mg of a Fraction III TnsB preparation was subjected to phosphocellulose chromatography as described under "Experimental Procedures," and fractions containing TnsB protein eluted by a P buffer with 0.52 M NaCl step were evaluated for their protein content by SDS-PAGE (*panel A*) and their binding activity by a band shift assay (*panel B*). *Panel A*, 16 μl of each fraction was separated by SDS-PAGE on a 5–15% gradient gel and stained with Coomassie Blue R-250. *Panel B*, 0.1 μl of each fraction was assayed using the L109–166 fragment as a substrate; no addition was made to the *leftmost lane*.

[4] Arciszewska, L. K., and Craig, N. L., *Nucleic Acids Res.*, in press.

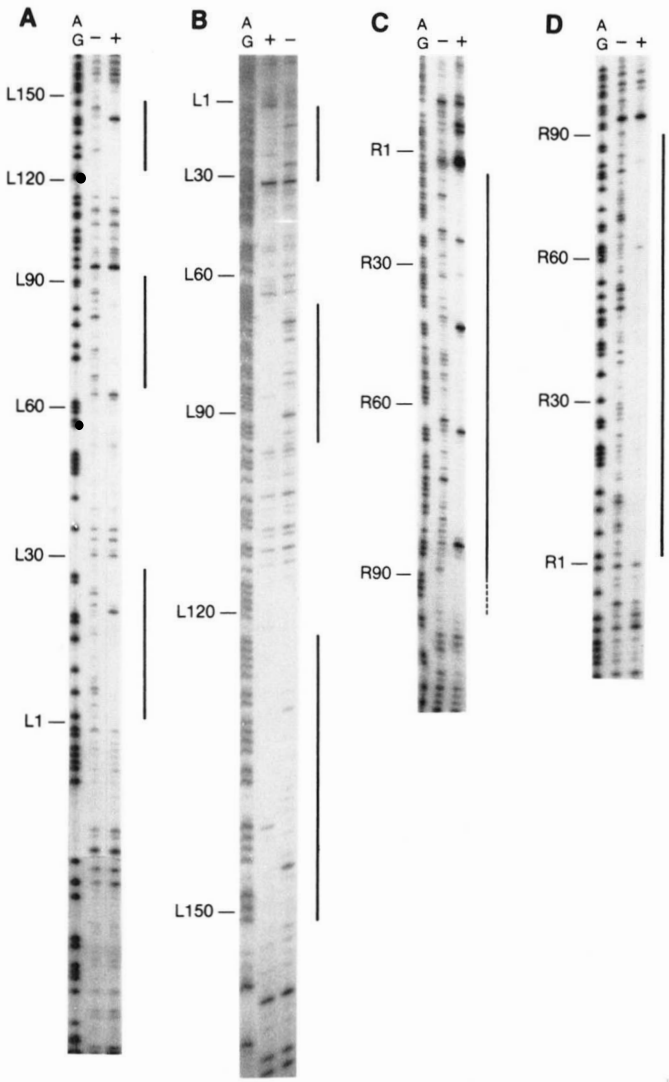

FIG. 5. **DNase I footprinting analysis of the interaction of TnsB with the ends of Tn7.** DNase I attack was performed in the absence (−) or presence (+) of 1.25 μg/ml (*panel A*) or 2.5 μg/ml (*panels B–D*) of Fraction IV TnsB to evaluate the interaction of TnsB with the indicated DNA strands. Products of A + G chemical sequencing reactions were used as markers. The nucleotide positions at the Tn7 ends are indicated, L1 being the terminal nucleotide in Tn7L, and R1 being the terminal nucleotide in Tn7R. The *vertical lines* indicate the regions protected by TnsB. Top and bottom strands are as shown in Fig. 6. *Panel A,* bottom strand of Tn7L; *panel B,* top strand of Tn7L; *panel C,* bottom strand of Tn7R; *panel D,* top strand of Tn7R.

protected regions is about 30 bp in length, and they are separated by 20–30 bp of unprotected sequences. We note that the L109–166 segment used in our band shift assays encompasses a single region of TnsB protection. Also evident are positions of enhanced sensitivity to DNase I on both strands within the protected regions. Alignment of the sequences of the protected regions (Fig. 7) reveals that they are highly related in nucleotide sequence and that the positions of enhanced DNase I cleavage are similarly located within each protected region. Each of the α, β, and γ regions encompasses a copy of the highly conserved 22-bp sequence that is repeated several times in both ends of Tn7 (Lichtenstein and Brenner, 1982). These results are consistent with our previous observation (McKown *et al.,* 1987) that a DNA fragment containing a single 22-bp repeat is specifically recognized by the *tnsB*-dependent binding activity in crude cell lysates.

In Tn7R, one continuous region of protection, approximately 90 bp in length, was observed. We propose that this region contains four TnsB binding sites designated φ, χ, ψ, and ω. The presence of four TnsB binding sites in this region is consistent with the nucleotide sequence of the protected region, *i.e.* it contains four sequences similar to the TnsB binding sites in Tn7L including four 22-bp repeats (Fig. 6). Furthermore, several positions of enhanced DNase I cleavage are evident within the Tn7R-protected region whose pattern corresponds to the pattern of DNase I cleavage enhancements in the Tn7L binding sites. However, several slight deviations from the conserved positioning of the enhanced DNase I cleavage sites are observed. In φ, the enhancement is shifted by 1 bp on one strand; and in ψ, partial protection, rather than enhancement, is observed on the same strand. These irregularities may reflect slightly different interactions of TnsB with the φ and ψ sites whose nucleotide sequences are very similar to each other and distinct from the other TnsB sites, or they may be caused by the close apposition of the TnsB binding sites in Tn7R.

Comparison of the seven proposed TnsB binding sites reveals that they share considerable sequence similarity (Fig. 7). The 22-bp repeats are prominent features of the TnsB binding sites, although some sequence similarities extend beyond the repeats. It is important to note that because of the apparent overlapping of the TnsB binding sites in Tn7R, the identity of some nucleotides may have multiple constraints. Thus, firm identification of the actual recognition determinants for TnsB binding must await mutational analysis.

An interesting feature of the TnsB binding sites is the presence in each site of 4-bp imperfect inverted repeats that flank a 3-bp region (Fig. 7, positions 21–23). These 3-bp regions are bracketed by the positions of increased DNase I cleavage. Interestingly, the most prominent sequence differences between the binding sites in Tn7L (α, β, γ) and in Tn7R (φ, χ, ψ, ω) occur within these 3-bp sequences (Fig. 7); the sites in the left end contain KGG whereas those in the right contain NTA, where K is T or G and N is any nucleotide. The functional significance of these structural differences remains to be determined.

Another feature of the DNase I protection patterns invites comment. The pattern of TnsB protection at the extreme termini of Tn7 appears to be slightly different than that at the internal binding sites. Two positions of considerably enhanced sensitivity to DNase I cleavage, adjacent to the 5′ ends of the transposon, are evident at the edges of the terminal α and ω binding sites (Figs. 5 and 6). Such enhancements are not obvious at comparable positions in the β and γ sites. (In Tn7R, comparable positions in the internal TnsB sites cannot be evaluated clearly because of the considerable overlap of these binding sites.) The enhancements at the terminal sites may represent a distinctive interaction between TnsB and the transposon ends. The sequences of the α and ω sites are slightly different from the other TnsB sites; an obvious difference is that they are very GC rich between positions 1 and 8 (Fig. 7). These terminal DNase I cleavages could reflect a change in DNA structure promoted by the binding of TnsB to these sites (Gartenberg and Crothers, 1988). Unique interactions of TnsB with the terminal α and ω binding sites could play an important role in defining the junctions between each transposon end and flanking donor site DNA.

Activity of TnsB in Tn7 Transposition in Vitro

During purification, the activity of TnsB was measured by this protein's ability to bind specifically to the ends of Tn7.

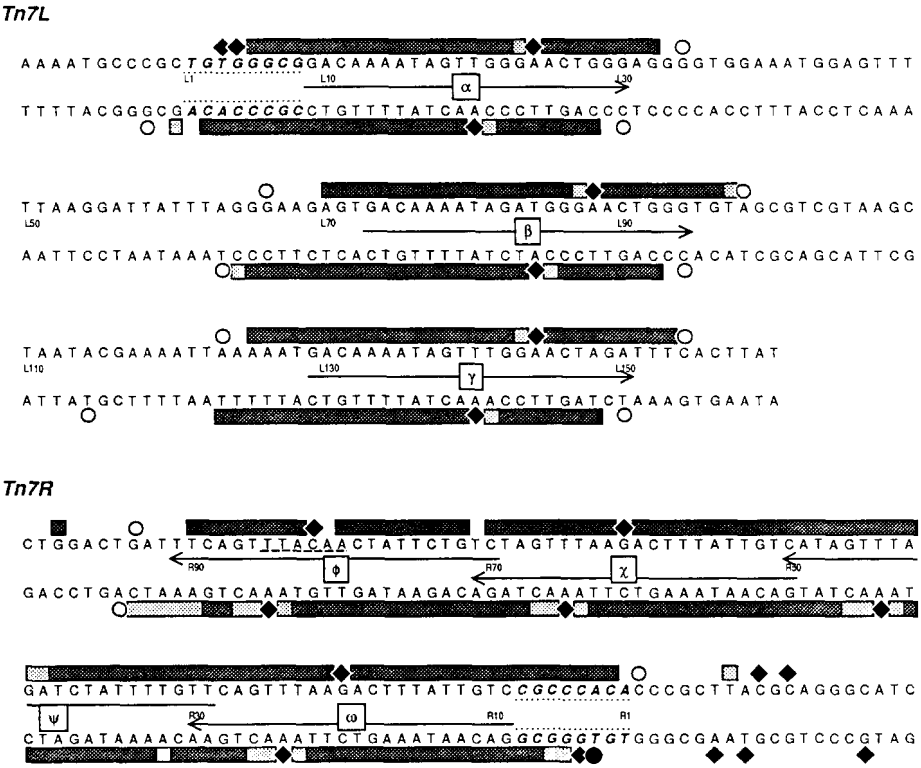

FIG. 6. **Nucleotide sequences of the Tn7 ends and of the regions protected by TnsB protein against DNase I attack.** The nucleotide sequences of Tn7L and Tn7R are shown. The terminal Tn7 nucleotides are L1 and R1, and numbers increase toward the inside of Tn7; the sequences flanking the ends are from *attTn7* (Lichtenstein and Brenner, 1982; Gay *et al.*, 1986; McKown *et al.*, 1988). The *arrows* mark the 22-bp repeat sequences; the nucleotides of the terminal 8 bp of each end of Tn7, which together with the adjacent 22-bp repeats form Tn7's imperfect terminal inverted repeats, are denoted with *bold italics* and marked with *dotted lines*. The −35 sequence of the putative *tnsAB* promoter in Tn7R is indicated by a *broken line*. The regions of the Tn7 ends protected by TnsB and the proposed TnsB binding sites, α, β, and γ in Tn7L and ϕ, χ, ψ, and ω in Tn7R, are marked. The *dark gray boxes* indicate bases whose 5′ phosphodiester bonds are protected from DNase I attack; the *light gray boxes* indicate partially protected regions; the *diamonds* indicate positions of enhanced cleavage; and *circles* mark positions that are not protected from attack by the presence of TnsB. In some cases, the status of regions between the protected and unprotected regions cannot be evaluated because these areas are not markedly sensitive to DNase I attack in the absence or presence of TnsB.

We also tested the activity of purified TnsB in Tn7 transposition. For this purpose we used a cell-free transposition system (Bainton *et al.*, 1991) in which a miniTn7 element transposes from a donor plasmid to *attTn7* in a target plasmid in the presence of lysates derived from cells carrying *tnsABC + tnsD*, which are the genes required for *in vivo* Tn7 insertion into *attTn7* (Rogers *et al.*, 1986; Waddell and Craig, 1988). Bainton *et al.* (1991) demonstrated that partially purified TnsB (Fraction II) can provide the *tnsB* function required in this system for *in vitro* Tn7 transposition.

We determined that highly purified TnsB protein (Fraction IV) is active in transposition *in vitro* (Fig. 8). Comparison of the *in vitro* transposition activity of various dilutions of *tnsB* cell lysates and purified TnsB protein (Fraction IV) suggests that the transposition activity of the TnsB polypeptide is similar before and after purification. Both sources provided similar levels of transposition at equivalent concentrations of TnsB (data not shown). Thus, our purification procedure yields TnsB that is active both in sequence-specific DNA binding and in Tn7 transposition.

DISCUSSION

In this paper we reported the purification of the Tn7-encoded transposition protein TnsB. The purification is relatively simple and yields highly purified protein in quantities sufficient for most biochemical studies. Two pieces of evidence demonstrate that the polypeptide we have purified is the product of the *tnsB* gene. (i) The purified polypeptide is specifically recognized by anti-TnsB antibodies; and (ii) the sequence of the first 12 N-terminal amino acid residues of the purified polypeptide is in agreement with that predicted from the nucleotide sequence of the *tnsB* gene (Flores *et al.*, 1990; Orle and Craig, 1990). Our work has determined directly that the TnsB coding sequence begins at the first of three possible initiation codons that are present in the 5′ region of the gene. The TnsB coding sequence overlaps the end of the coding sequence of the preceding gene, *tnsA* (Flores *et al.*, 1990; Orle and Craig, 1990), suggesting the possibility that expression of these genes may be translationally coupled.

We have established here that TnsB is a sequence-specific DNA-binding protein that recognizes sequences at the ends of Tn7 which are required for transposition. The involvement of TnsB in transposon end recognition is consistent with the requirement for *tnsB* in all Tn7 transposition reactions (Rogers *et al.*, 1986; Waddell and Craig, 1988). The DNase I protection studies described here show that TnsB binds specifically to several sites in each end of Tn7; three binding sites were found in Tn7L, and four are proposed in Tn7R. These binding sites lie within DNA segments we have established in genetic studies (Arciszewska *et al.*, 1989) to be

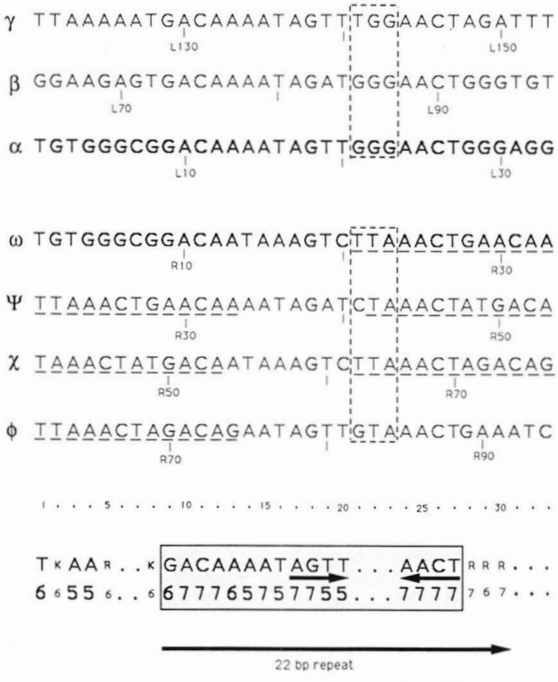

FIG. 7. **Sequence comparison of TnsB binding sites.** The sequence of one strand of each of the proposed TnsB binding sites is shown, the top strand of α, β, and γ in Tn7L and the bottom strand of ϕ, χ, ψ, and ω in Tn7R (as shown in Fig. 6). The terminal α and ω sites are shown in *bold type*. The 3-bp regions enclosed by the *boxes* are bracketed by the positions of increased DNase I cleavage in each binding site, the 23–24 junction in the upper strand being hypersensitive, and the 20–21 junction in the lower strand being hypersensitive. The *underlined* positions indicate regions of overlap between the Tn7R sites. The *bottom two lines* summarize the frequency of occurrence of particular nucleotides at each position with the protected regions; K is T or G, R is A or G. The *arrows* indicate a 4-bp inverted repeat within the sites. The nucleotide positions enclosed by the *gray box* are the most highly conserved in sequence identity among the sites. The *bottom arrow* indicates the 22-bp repeats noted by Lichtenstein and Brenner (1982).

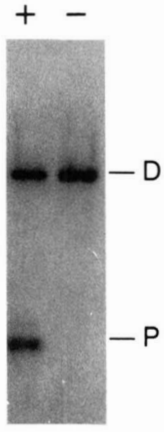

FIG. 8. **Purified TnsB is active in Tn7 transposition *in vitro*.** *In vitro* Tn7 transposition reactions were carried out as described under "Experimental Procedures" in the presence (+) and absence (−) of TnsB protein (Fraction IV) at 0.17 μg/ml. After incubation, DNAs were recovered, linearized by digestion with a restriction enzyme, separated by gel electrophoresis, and detected by southern hybridization with a miniTn7-specific probe. Transposition was evaluated by following the translocation of miniTn7 element from a donor plasmid (position of migration = *D*) to an *attTn7* target plasmid (position of migration = *P*).

directly involved in and required for transposition. These studies also suggested that the transposition sequences of Tn7 would contain multiple protein binding sites. The binding of a transposition protein to multiple sites at the ends of the element has also been observed in bacteriophage Mu (Craigie *et al.*, 1984) and several plant transposons (Gierl *et al.*, 1988; Kunze and Starlinger, 1989). The presence of several binding sites at each transposon end suggests the possibility that TnsB may be involved in the formation of a higher order nucleoprotein structure during the transposition reaction (Pato, 1989; Stark *et al.*, 1989; Thompson and Landy, 1989).

The TnsB binding sites observed by protection against DNase I attack in Tn7L are about 30 bp in length; in Tn7R, the four proposed sites are closely juxtaposed and are not individually distinct. Comparison of the sequences of the sites in Tn7L and proposed sites in Tn7R reveals that they share considerable sequence similarity (Fig. 8). Each of the proposed TnsB binding sites includes the 22-bp repeat sequence originally noted by Lichtenstein and Brenner (1982) upon their sequencing of the ends of Tn7. The actual contribution of particular nucleotides within and outside these repeats to TnsB binding and function can only be determined through mutational analysis. A prominent feature of the interaction of TnsB with all the Tn7 end binding sites is the presence of positions of enhanced DNase I sensitivity at comparable positions within each binding site (Fig. 6). This suggests that a common mode of TnsB-DNA interaction, perhaps a local distortion in DNA structure, occurs at all sites. Internal enhancements in protein binding sites have also been observed with Tn3 transposase (Ichikawa *et al.*, 1987) and MuA protein (Craigie *et al.*, 1984). Furthermore, there seems to be a unique interaction between TnsB and the very termini of Tn7 as reflected by additional, distinctive DNase I cleavage patterns at these positions.

A key issue in unraveling the mechanism of a transposition reaction is understanding the DNA strand cleavages that disconnect the transposon from flanking DNA in a donor site and identification of the polypeptide that mediates this step. The edges of two of the TnsB binding sites, α in Tn7L and ω in Tn7R (as defined by protection against DNase I attack), are adjacent to the transposon termini. The junctions of the 3′ transposon ends with flanking donor DNA are very close to (or, in Tn7L, perhaps at) the edges of the TnsB-protected region. By contrast, positions within the transposon near the junctions of the 5′ transposon ends with the flanking donor DNA are hypersensitive to DNase I attack. We note that the transposition protein MuA does not obviously protect the very terminal nucleotides of bacteriophage Mu on linear DNA fragments against DNase I cleavage (Craigie *et al.*, 1984), and yet it does perform strand cleavage at these positions during transposition (Craigie and Mizuuchi, 1987; Surrette *et al.*, 1987). The role of TnsB in cutting the transposon ends away from the donor site remains to be determined. We have been unable to detect a TnsB strand cleavage activity (data not shown). Perhaps certain interactions of TnsB with the termini would be evident only on an authentic transposition substrate, *i.e.* a supercoiled DNA molecule containing both Tn7 ends.

It is important to note that TnsB may not be the only protein that acts at the ends of Tn7. The possibility that even our most purified TnsB fractions contain a host protein involved in TnsB end binding which is not readily detectable by the staining methods we have used is, of course, difficult to exclude. If present, such a host component is not limiting for end binding as we observe no obvious differences in TnsB activity in the presence or absence of host extract. Although

perhaps unlikely, trace amounts of an enzymatic activity that modifies TnsB could also present in our purified preparations (Prives, 1990). Furthermore, we know that two other Tn7-encoded genes, *tnsA* and *tnsC*, are required in addition to *tnsB* in all transposition reactions (Rogers *et al.*, 1986; Waddell and Craig, 1988), and we suspect that the products of these genes participate directly in transposition (Bainton *et al.*, 1991). It is possible that TnsA or TnsC may mediate the strand breakages at the transposon termini. One role of TnsB binding adjacent to the transposon termini may be to facilitate the interaction of TnsA, TnsC, or even a host protein with the ends. The ends of many transposable elements are known to contain binding sites for several proteins (reviewed in Berg and Howe, 1989).

In addition to its direct role in transposition, TnsB also appears to play a regulatory role by controlling the expression of at least two transposition proteins, TnsA and itself. *tnsA* and *tnsB* form an operon whose transcription initiates near the right terminus of Tn7 (Waddell and Craig, 1988; Gay *et al.*, 1986). The −35 sequence of the proposed *tnsAB* promoter is embedded within the ϕ TnsB binding site (Fig. 6). The binding of TnsB to this site could explain the observation that the expression from this promoter is negatively regulated in the presence of *tnsB* (Rogers *et al.*, 1986).

We have observed considerable degradation of TnsB by host proteases. This proteolysis, which is markedly diminished in an *htpR*$^-$ strain, is manifested by the presence of several shorter TnsB-derived polypeptides. The relevance of such TnsB instability to Tn7 transposition is unclear; we observe TnsB degradation only when this protein is expressed at high level in the absence of the other *tns* genes (Orle and Craig, 1990). Some of the shorter species retain specific DNA binding activity but are inactive in Tn7 transposition *in vitro* (data not shown). Transposase-related polypeptides are known to play inhibitory roles in regulating transposition in other systems, such as Tn5 (Johnson and Reznikoff, 1984), IS1 (Zerbib *et al.*, 1990), and P elements (Robertson and Engles, 1989; Misra and Rio, 1990). Future studies will be required to reveal the role(s) that TnsB and its derivatives may play in both mediating Tn7 transposition and modulating its frequency.

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