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Structural domains in phage Mu transposase: Identification of the site-specific DNA-binding domain

(transposable elements/gene rearrangements/limited proteolysis/protein structure/protein–DNA interaction)

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ABSTRACT **Limited proteolysis of phage Mu transposase with three proteases of differing specificities produced a common pattern of fragmentation. The fragments were mapped by using a combination of immunoblotting and amino acid sequence analysis. Our results suggest that the transposase molecule is organized principally into three domains: an amino-terminal domain of molecular mass 30 kDa, a core region of approximately 35 kDa, and a carboxyl-terminal domain of approximately 10 kDa. The amino-terminal domain has at least two additional sites that are partially accessible to proteases. Filter binding and nuclease protection studies were done to determine the functions of the isolated domains. Site-specific binding to Mu DNA was localized to the amino-terminal domain. The core domain showed nonspecific DNA-binding activity.**

Phage Mu transposase is a 75-kDa protein encoded by gene *A* (1). Together with the product of gene *B* (2) and several host proteins, the A protein is exceptionally proficient at transposing Mu DNA into random sites in many bacterial genomes (3). Proper expression of gene *A* is absolutely essential for both replicative and conservative transposition and for Mu DNA excision. In the absence of gene *B*, transposition frequencies are decreased to 1/100th (4) and transposition events become preferentially intramolecular (5, 6). Mu DNA excision can be observed in the absence of gene *B* (7).

Transposition is a multistep process (3). Obligate early events in transposition include specific recognition of the left and right ends of Mu DNA (*att*L and *att*R). *In vitro* analysis of Mu DNA transposition has shown that the A protein is responsible for the site-specific recognition of Mu DNA ends (8). Two other proteins, the transposase A of phage D108 and the immunity determinant or repressor c of phage Mu, share DNA-binding sites with Mu A protein. A comparison of the amino acid sequences of these three proteins has revealed regions of homology which, at the amino termini of the proteins, show resemblance to the bihelical DNA-binding structure found in other site-specific DNA-binding proteins (1). Besides site-specific DNA binding, transposition also requires binding to target DNA followed by nicking and strand-exchange reactions in which three DNA sequences participate: Mu *att*L and *att*R and the host target. The efficiency of this reaction is increased in the presence of the Mu B protein.

To understand the mechanism of Mu transposition, we have begun an analysis of the structure and function of the A and B proteins. By limited proteolysis of the A protein with trypsin, chymotrypsin, and *Staphylococcus aureus* V8 protease, we show here that the transposase polypeptide is organized principally into three domains with additional protease-accessible sites in the amino-terminal domain. Predictions of hydrophobicity and secondary structure support the conclusions from our proteolysis experiments. Both amino-terminal and core domains bind DNA. However, only the amino-terminal domain shows site-specific binding to Mu DNA.

MATERIALS AND METHODS

Enzymatic Digestion and Isolation of Fragments. A protein was purified as described (9). A sample of purified protein (1.2 mg/ml) in 0.3 M KCl/25 mM Hepes, pH 7.6/0.1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol was treated with trypsin (Sigma catalog no. T 8642), α-chymotrypsin (Sigma catalog no. C 3142), or *S. aureus* V8 protease (Sigma catalog no. P 8400), at a ratio of 3000:1 (wt/wt), and incubated at 30°C for various times. (Peptides generated by trypsin, chymotrypsin, and V8 protease are indicated by the prefixes T-, CT-, and V-, respectively.) Digestion was stopped with phenylmethanesulfonyl fluoride (added to a final concentration of 1 mM) and the samples were subjected to electrophoresis on a $NaDodSO_4$/12.5% polyacrylamide gel (10). Protein fragments were transferred to nitrocellulose (11) and analyzed with antibodies specific for three different regions at the amino terminus of the A protein. These antibodies were produced by immunization of rabbits with the synthetic peptides A-(1–22), A-(27–50), and A-(61–88), which represent fragments of the A-protein sequence deduced in ref. 1. These peptides were coupled to keyhole limpet hemocyanin (KLH) with *m*-maleimidobenzoyl *N*-hydroxysuccinimide ester as described (12).

For functional studies, chymotryptic fragments were applied to a phosphocellulose column equilibrated with 0.1 M NaCl in HEDG (25 mM Hepes, pH 7.6/0.1 mM EDTA/1 mM dithiothreitol/10% glycerol) at 4°C. CT30 and CT35 were eluted with 0.3 M NaCl in HEDG. The eluate was adjusted to 0.1 M NaCl and applied to a DNA-cellulose column equilibrated with 0.1 M NaCl in HEDG. CT35 was recovered in the void volume fractions, while CT30 was eluted with 0.15 M NaCl in HEDG. Recovery of pure fragments was 5–10%. Bovine serum albumin was added to the final fractions to prevent loss during concentration and storage. The added albumin did not interfere with the activity of the purified fragments. CT28 copurified with CT30. Using immunoblotting, we judged contamination of purified CT30 or CT35 with native A protein or CT62 to be less than 0.5%.

Protein Sequence Analysis. The Mu A protein was cleaved with chymotrypsin or *S. aureus* V8 protease as described above. Chymotryptic peptides CT62, CT48, CT35, and CT30 were simultaneously purified and prepared for protein sequence analysis by the electroblotting method of Aebersold *et al.* (13) and then loaded directly into a gas-phase sequenator (14). Peptide CT15 was purified by $NaDodSO_4$/PAGE, Coomassie blue staining, and electroelution according to the

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method of Hunkapiller *et al.* (15). Peptides V56 and V39 were purified by NaDodSO$_4$/PAGE, visualizing with 4 M sodium acetate, and electroelution (15). Peptides purified by electroelution were spotted onto quaternary ammonium-modified glass fiber discs for sequencing (13). The resulting phenylthiohydantoin derivatives were analyzed essentially as described by Hunkapiller and Hood (16), except that 5% (vol/vol) tetrahydrofuran was included in the A buffer, which was adjusted to pH 5.1.

DNase I Protection Experiments. A 300-base-pair (bp) *Alu* I fragment containing Mu *att*L and flanking host DNA was cloned in a multiple cloning site vector by using *Sal* I linkers. The resulting plasmid pRA-L29 was cut with *Hin*dIII and labeled at the 5′ end by using polynucleotide kinase and [γ-^{32}P]ATP. After digestion with *Bam*HI, the labeled *att*L fragment was purified from a polyacrylamide gel. Approximately 1–10 nM end-labeled fragment was incubated with protein in DNase I protection experiments, which were performed essentially as described (8).

Filter Binding Assay. A 300-bp *Hin*dIII–*Bam*HI *att*L-containing fragment from pRA-L29 was used as a source of specific DNA, and a 351-bp *Cla* I–*Bam*HI fragment from pBR322 was used as a source of nonspecific DNA. ^{32}P-labeled DNA fragments (1–10 nM) were incubated in the assay buffer (25 mM Hepes, pH 7.5/75 mM KCl/1 mM $MgCl_2$/1 mM dithiothreitol) with various amounts of protein for 20 min at 37°C. Aliquots were filtered through a presoaked nitrocellulose filter and washed with assay buffer, and their radioactivities were measured in a toluene-based scintillant.

RESULTS

Proteolytic Cleavage of Mu A Protein with Chymotrypsin. Fig. 1*A* shows the time course of digestion of the 75-kDa Mu A protein with chymotrypsin. After a 5- or 10-min incubation, major fragments with masses of 62, 48, 35, 30, and 15 kDa are observed. With prolonged incubation, CT62 and CT48 diminish and CT35 accumulates. CT15 and CT35 appear to be stable. CT30 is also stable, although less so than are CT15 and CT35. Besides these major products, there are minor species with masses of 73, 60, 58, 56, 42, 28, 22, and 20 kDa. The protein fragments were resolved by NaDodSO$_4$/PAGE, transferred to nitrocellulose, and probed with serum specific for the peptide A-(27–50) (Fig. 1*B*). The 75-kDa native A protein and CT62 and CT30 fragments react with this probe, and hence the fragments are derived from the amino terminus. Note that all three amino-terminal species appear as doublets with a molecular mass difference of 2 kDa. The reason they are equally intense on the immunoblot (Fig. 1*B*) but not on the Coomassie blue-stained gel (Fig. 1*A*) is saturation of the fragments with antibody. The amino-terminal-containing chymotryptic doublets were further analyzed by using serum specific for the A-(1–22) peptide (Fig. 1*C*). Only the larger fragment of each of the three doublets reacts with this probe, indicating that a chymotrypsin cleavage site occurs about 20 residues in from the amino terminus. When probed with serum specific for the A-(61–88) peptide (not shown), the minor doublet species CT58 and CT22 react. From the pattern of appearance of the chymotryptic fragments, we postulated that CT15 was derived from the carboxyl terminus and CT35 from the middle of the protein. These assignments were confirmed by direct sequencing of the peptide fragments, as seen in Fig. 2. Thus, the stable products of chymotrypsin digestion are CT30 (amino terminus), CT35 (core), and CT15 (carboxyl terminus).

Proteolytic Cleavage of Mu A Protein with Trypsin. The time course of digestion with trypsin is shown in Fig. 3*A*. At early times the major fragments are T63, T50, T45, T39, T34, T29, T21, and T10. As digestion continues, T45 diminishes and T34 accumulates. This behavior parallels that of chymotrypsin digestion, in which CT48 diminishes and CT35 accumulates. T70, T63, T50, T39, and T29 are unstable intermediates. An immunoblot of this digest (Fig. 3*B*) with serum specific for the A-(27–50) peptide shows that T63, T29, and T21 are the amino-terminal fragments. T21 is a stable amino-terminal species. T63, T34, and T29 thus correspond to CT62, CT35, and CT30, respectively. T29 is cleaved further to give T21. We postulate that T10 is derived from the carboxyl terminus and corresponds to CT15.

Proteolytic Cleavage of Mu A Protein with V8 Protease. Digestion of Mu A protein with *S. aureus* V8 protease is shown in Fig. 3*C*. Major fragments with masses of 64 and 56 kDa appear first. The 64-kDa fragment disappears as time progresses, while 39-, 18-, and 12-kDa fragments begin to accumulate. The 56-kDa fragment appears to be quite stable. Immunoblot analysis shows that V64 and V12 contain the amino terminus of the A protein (Fig. 3*D*). The amino acid sequences of V56 and V39 are shown in Fig. 2. V39 corresponds to CT35 and T34. V56 is actually a mixture of two peptides produced by cleavages at two positions, seven residues apart, near the amino terminus. These cleavages also result in the production of the V12 fragment(s). We have not determined whether V12 has a seven-residue ragged end, as would be predicted from these results. We postulate that V56 may be further cleaved to produce V18 and V39. The faint band around 10 kDa may correspond to the carboxyl-

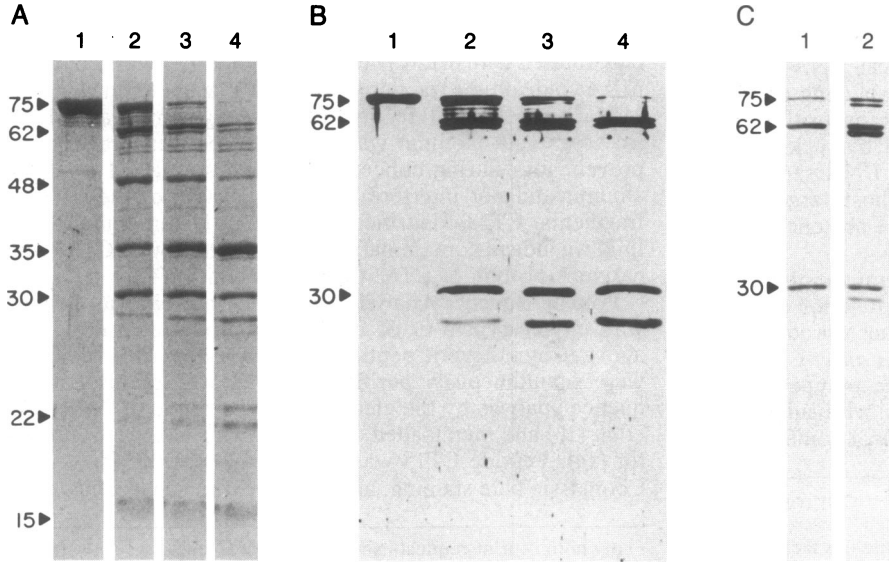

FIG. 1. NaDodSO$_4$/PAGE and immunoblot analysis of Mu transposase chymotryptic peptides. At various times 10-μl aliquots of the reaction mixture were electrophoresed in polyacrylamide gels, then either stained with Coomassie blue or blotted onto nitrocellulose and treated with specific antibodies. Molecular masses are given in kilodaltons. (*A*) Coomassie blue staining. Lanes 1–4, 0-, 10-, 20-, and 40-min digests, respectively. (*B*) Immunoblot analysis with antiserum to peptide fragment A-(27–50) of Mu A protein. Lanes 1–4 as in *A*. (*C*) Immunoblot analysis of sample from a 10-min incubation with chymotrypsin. Lanes 1, treated with antiserum to A-(1–22) peptide; lane 2, treated with antiserum to A-(27–50) peptide.

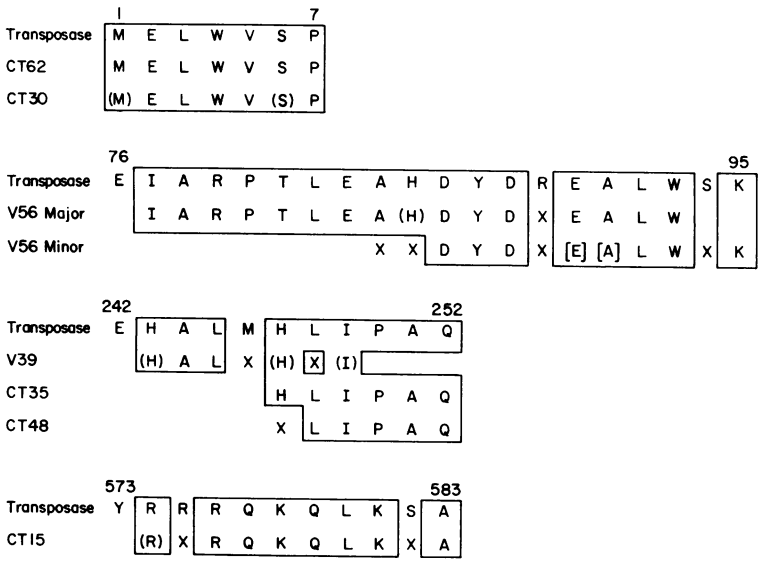

FIG. 2. Protein sequence analysis of transposase peptides generated by limited proteolysis. The regions of the transposase molecule from which each peptide was derived were determined by alignment of the peptide sequences with the published sequence of the native Mu A protein (1). Numbers correspond to positions in this native molecule. Homologous sequences are boxed. The symbols for residues yielding low signals are enclosed by parentheses. The standard one-letter symbols are used for residues; unidentified residues are signified by X. Bracketed symbols are tentative assignments arising because two peptides were sequenced simultaneously and at these positions each peptide contained the same amino acid.

terminal fragments CT15 and T10. Thus, three proteases with different, and nonoverlapping, specificities produce similar patterns of cleavage fragments.

DNA-Binding Activity of Mu A-Protein Fragments. Site-specific recognition of the left and right ends of Mu DNA is one of the earliest steps in transposition, a function carried out by A protein (8). To localize this function to a specific region of the protein molecule, we carried out preliminary gel retardation and DNA "footprinting" studies with Mu *att*L- and *att*R-containing DNA fragments and a mixture of CT30 and CT35. The data thus obtained (not shown) suggested that CT30 bound Mu *att*L and *att*R specifically and that CT35 bound DNA nonspecifically. To confirm this observation, the CT30 and CT35 domains were separated from each other (Fig. 4*A*), then their DNA-binding activities were studied in filter binding assays. Both fragments bound *att*L-containing DNA and pBR322 DNA with approximately the same avidity (Fig. 4*B*), CT30 having a dissociation constant (K_d) of approximately 2.3×10^{-7} M and CT35 having a K_d value of 3.3×10^{-7} M. By comparison, native A protein bound DNA more efficiently than did either subunit alone, as indicated by its K_d value of 1.0×10^{-7} M.

In a nuclease protection assay, however, only CT30 showed site-specific recognition of Mu DNA ends (Fig. 4*C*). The pattern of protection was the same as that observed with the native A protein. DNA-binding activity of the fragment varied with the particular preparation. In the experiment in Fig. 4*C*, approximately 3- to 5-fold more fragment was required to achieve the same degree of protection as was achieved by using the native A protein. The protection cannot be due to contamination with native protein, which was less than 0.5%. CT35 failed to protect *att*L (not shown). We conclude that the site-specific DNA-binding function of the A protein resides in the amino-terminal domain.

DISCUSSION

To understand how the complex process of DNA transposition occurs, we have begun structure/function analyses of phage Mu transposase, the product of the Mu *A* gene and the

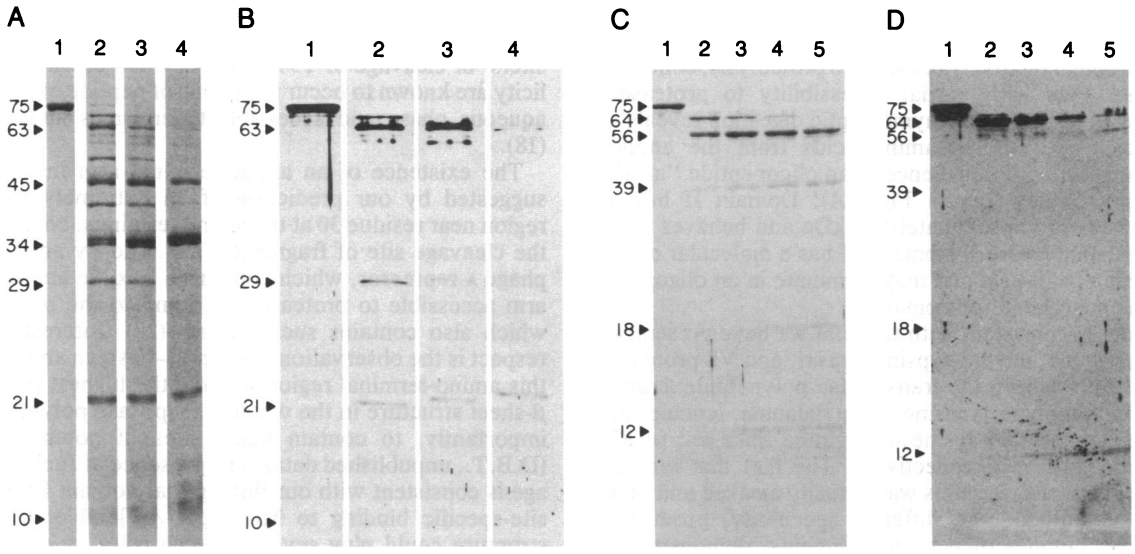

FIG. 3. $NaDodSO_4$/PAGE and immunoblot analysis of Mu transposase tryptic and V8 peptides. At various times 10-μl aliquots of the reaction mixture were electrophoresed in polyacrylamide gels, then either stained with Coomassie blue or blotted onto nitrocellulose and treated with specific antibodies. Molecular masses are given in kilodaltons. (*A*) Coomassie blue-stained trypsin digest. Lanes 1–4, 0-, 10-, 20-, and 40-min digests, respectively. (*B*) Immunoblot analysis of trypsin digests with antiserum to A-(27–50) peptide. Lanes 1–4 as in *A*. (*C*) Coomassie blue-stained V8 protease digest. Lanes 1–5, 0-, 30-, 60-, 90-, and 120-min digests, respectively. (*D*) Immunoblot analysis of V8 protease digest with antiserum to A-(27–50) peptide. Lanes 1–5 as in *C*.

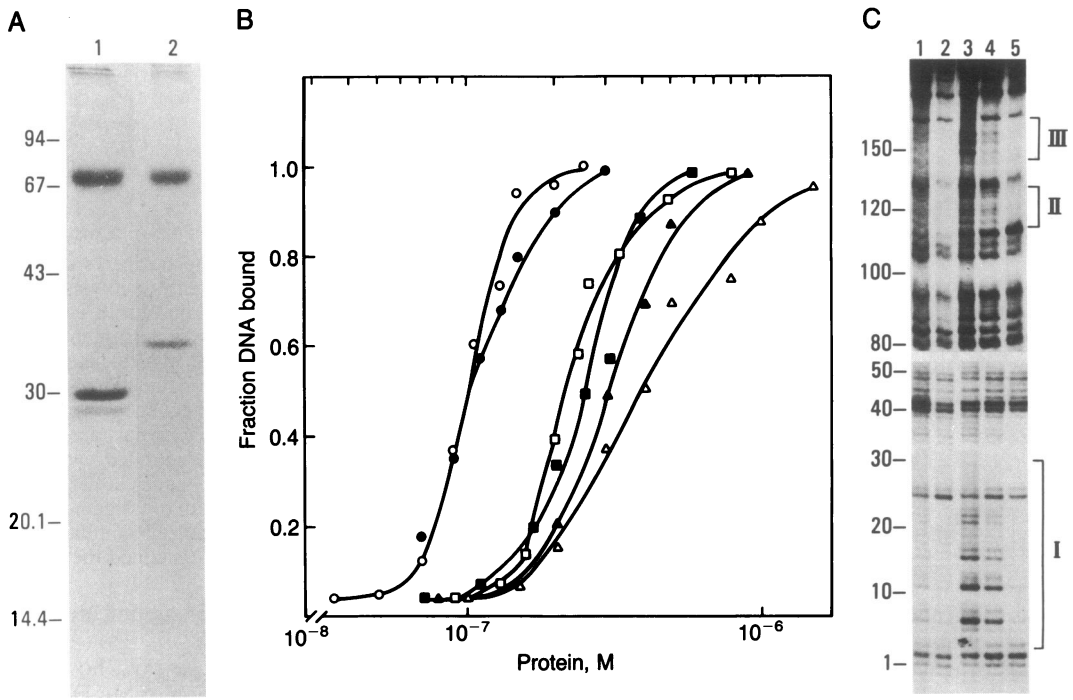

FIG. 4. DNA-binding experiments with purified CT30 and CT35. (*A*) NaDodSO$_4$/PAGE analysis of purified CT30 (lane 1) and CT35 (lane 2). The 68-kDa band in both lanes is bovine serum albumin. Molecular masses of standards (in kDa) are indicated on the left. (*B*) Nitrocellulose filter binding of DNA. ^{32}P-labeled *att*L-containing DNA fragments (open symbols) or pBR322 DNA fragments (closed symbols) were mixed with A protein (○), CT30 (□), or CT35 (△) and passed through nitrocellulose filters. (*C*) DNase I protection of Mu *att*L by CT30. Prior to DNase I digestion, the Mu DNA fragment was incubated with native A protein or CT30. Lanes 1 and 2, A protein at 0.27 and 2.7 μM, respectively; lane 3, no protein; lanes 4 and 5, CT30 at 0.2 and 2.0 μM, respectively. I, II, and III refer to A-protein binding sites L1, L2, and L3 on *att*L (8). The numbers on the left refer to nucleotide positions from the left end of the Mu genome.

primary effector of DNA transposition during the phage life cycle. The results we have obtained from limited proteolysis of the A protein, immunoblotting, amino acid sequencing, and hydrophobicity predictions are summarized in Fig. 5 *B*, *C*, and *D* and provide the experimental basis for a model of the organization of structural domains in the A protein (Fig. 5*A*).

We postulate that the transposase polypeptide is organized principally into three globular domains (I, II, and III in Fig. 5*A*) separated by nonglobular, linker regions (open arrows in Fig. 5*A*). Domain I has a molecular mass of approximately 30 kDa and, although relatively resistant to proteolysis, contains two internal sites with partial accessibility to proteases (closed arrows in Fig. 5*A*). Chymotryptic digestion reveals a potential cleavage site 20 amino acids from the amino terminus, suggesting the presence of an oligopeptide "arm" in this region (wavy line in Fig. 5*A*). Domain II has a molecular mass of approximately 35 kDa and behaves as a protease-resistant "core." Domain III has a molecular mass of approximately 10 kDa and may terminate in an oligopeptide arm, as postulated for domain I.

This model is consistent with the data we have presented here. For example, chymotrypsin, trypsin, and V8 protease can theoretically cleave the transposase polypeptide at any one of 129 tryptophan, tyrosine, phenylalanine, leucine, or methionine linkages; 97 lysine or arginine linkages; or 87 glutamic acid linkages, respectively. The fact that only a small percentage of these sites was actually cleaved and that each enzyme, although of differing specificity, produced highly similar families of peptide fragments, demonstrates that these cleavage sites are clustered in regions accessible to all three enzymes. The simplest structural corollary for these regions is a nonglobular interdomain oligopeptide (signified with open arrows in Fig. 5*A*) connecting globular domains. Hydrophobicity predictions, done according to Kyte and Doolittle (17), are consistent with this structure (Fig. 5*B*). Especially noteworthy are the occurrence of particularly hydrophilic oligopeptide regions around residue 572, which coincides precisely with the cleavage site of fragment CT15 and the predicted sites of cleavage of fragments T10 and V10, and around residues 630 and 655, near the carboxyl terminus of the polypeptide, where a hydrophilic arm may be located. Similar very hydrophilic regions near residues 80 and 170 coincide with the cleavage sites of fragments V56 and T21, respectively. Additionally, a local hydrophobic minimum occurs near residue 241, which coincides with the interdomain cleavage sites of V39, CT35, CT48, and the predicted site(s) of cleavage of T34 and T45. Such areas of hydrophilicity are known to occur in regions of peptide exposed to the aqueous phase and accessible to enzymes and antibodies (18).

The existence of an amino-terminal arm in domain I is suggested by our prediction of an extremely hydrophilic region near residue 30 at the amino terminus, coinciding with the cleavage site of fragment CT28, and by analogy to the phage λ repressor, which contains a flexible amino-terminal arm accessible to proteases (19), and to the *trp* repressor, which also contains such an arm (20). Interesting in this respect is the observation that Chou–Fasman analysis shows this amino-terminal region to have the highest potential for β-sheet structure in the whole transposase polypeptide and, importantly, to contain many sites of potential β-bends (D.B.T., unpublished data). The presence of such an appendage is consistent with our finding that domain I functions in site-specific binding to Mu ends. A flexible or extended structure could play several special roles in such protein–DNA interactions. In the case of λ repressor, the arm wraps around the DNA helix, helping to determine sequence specificity and contributing a large portion of the binding energy (21).

The core domain shows nonspecific DNA binding. This domain could function in binding or cleaving the random

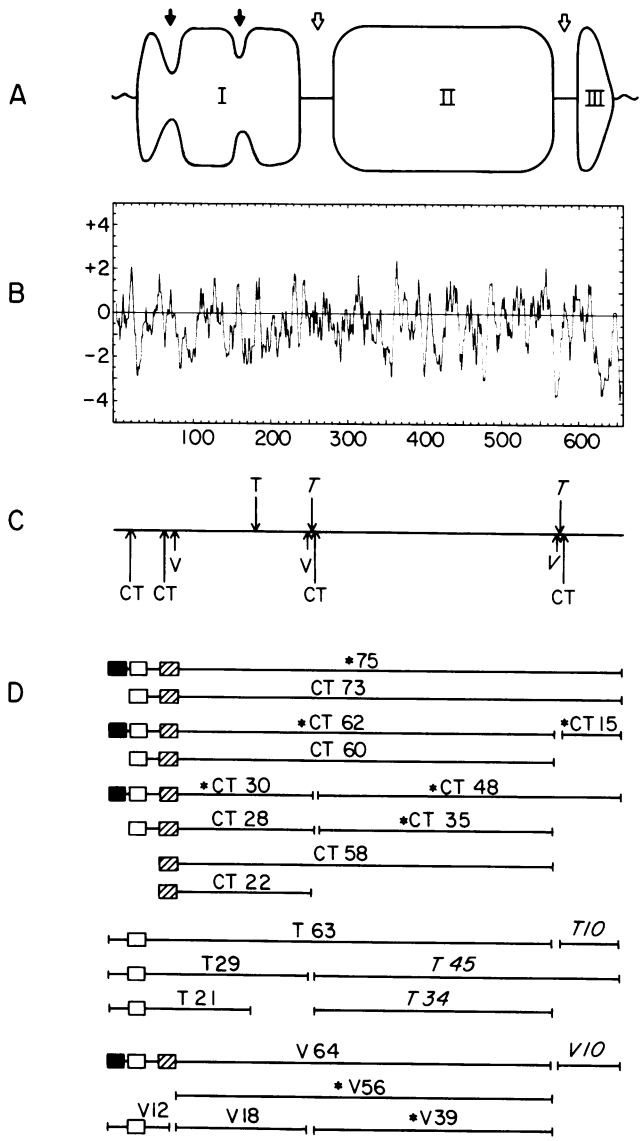

FIG. 5. Domain structure of Mu transposase. The amino terminus is always to the left. (*A*) Postulated globular domains (I, II, III) are separated by, and may contain (I), regions of polypeptide accessible to proteolytic enzymes. Open arrows signify accessible interdomain regions. Filled arrows signify accessible intradomain regions. We postulate that amino- and carboxyl-terminal "arms" (wavy lines) extend from domains I and III, respectively. (*B*) Hydrophobicity prediction was done according to the method of Kyte and Doolittle (17), using a window of seven residues. The hydrophobicity statistic (ordinate) was plotted versus the residue number in the A protein (abscissa). The more positive the statistic the more hydrophobic is the seven-residue survey region. (*C*) Proteolytic cleavage map. Sites of cleavage by chymotrypsin (CT), trypsin (T), and V8 protease (V) are noted with arrows. Sites with italicized labels are predicted on the basis of peptide mapping and immunoblot analysis but have not been confirmed by protein sequence analysis. (*D*) Major peptide fragments observed after limited proteolysis. Numbers indicate molecular masses in kilodaltons. Filled, empty, and hatched rectangles show sites of binding by antibodies specific for peptides A-(1–22), A-(27–50), and A-(61–88), respectively. It has not been determined whether A-(1–22)-specific antibodies bind to T63, T29, T21, or V12. Asterisks denote peptides whose positions have been confirmed by protein sequence analysis. The positions of peptides with italicized labels have been assigned on the basis of peptide map data alone.

target sites into which Mu integrates. We speculate that the carboxyl-terminal domain of A protein is involved in protein–protein interactions, especially with the B protein. We have observed that a deletion of 144 nucleotides from the 3′ end of gene *A* results in a truncated A protein that behaves as if it were "blind" to the presence of Mu B protein (22).

Several site-specific DNA-binding proteins are organized into distinct functional domains. Examples include the *lac* repressor, λ repressor, *434* repressor, cAMP-binding protein, and γδ resolvase (for a review, see ref. 23). They all have a bipartite organization, with one domain responsible for DNA binding and the other for protein–protein interactions. These proteins are involved in many aspects of cellular control, with the exception of γδ resolvase, which in addition catalyzes DNA strand exchange (24). Mu A protein shares with these proteins the function of sequence-specific DNA recognition and DNA strand exchange, but it is different and more complex in that the strand exchange reactions occur between three DNA sites and interaction with other proteins is necessary. The tripartite modular organization of Mu A protein probably reflects this diversity of required functions.

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