Mutational analysis of the *att* DNA-binding domain of phage Mu transposase

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ABSTRACT

The transposase (A protein) of phage Mu encodes binding to two families of DNA sites, *att* sites located at the Mu ends and enhancer sites located internally. Separate subdomains in the N-terminal domain I of Mu A protein are known to be involved in recognition of the *att* and enhancer sites. We have delineated an ~135 aa region within domain I $\beta\gamma$ that specifies binding to Mu *att* sites. This peptide was overexpressed and its properties compared with that of the larger domain I $\beta\gamma$ as well as the intact Mu A protein. Extensive mutagenesis of residues around a putative helix-turn-helix DNA-binding motif within the I β domain identified several mutants defective in DNA transposition *in vivo*. Of these, Mu A(K157Q) was completely defective in *att* DNA-binding. Mu A(F131S) and Mu A(R146N) had a lower affinity for *att* DNA and low levels of transposition *in vitro*. Our results indicate that residues in the γ region are required for activity and that residues outside the $\beta\gamma$ region must also influence discrimination between the multiple *att* sites.

INTRODUCTION

The transposase (A protein) of phage Mu is responsible for cleaving the ends of Mu and joining them to target DNA (reviewed in 1–3). Execution of the chemistry of DNA transposition is dependent on a complex process of assembling a tetrameric form of Mu A at the ends of Mu (4,5). Assembly of the functional synaptic complex is assisted by *Escherichia coli* HU protein (6,7) and is dependent on Mu A recognition of multiple DNA sites [three *att* subsites located at each end of Mu DNA and two internally located enhancer sites (8–12)] present on supercoiled DNA (6,13). A domain sharing model for active site assembly within the Mu A tetramer has been recently proposed (14).

The multiple functions carried out by Mu A have been localized to different domains of this tri-domainal protein. Both the *att* and the enhancer DNA binding activities of Mu A map to the N-terminal domain I (Fig. 1A; 9,15). The first 64 residues in domain I α are sufficient for enhancer binding, while *att* binding is mediated through residues located in I $\beta\gamma$. An NMR structure of the enhancer binding peptide shows it to be a variation of the helix-turn-helix (HTH) secondary structure motif known as

‘winged helix’ (16). An HTH-like sequence can also be discerned in domain I β (17).

Mu A protein-*att* DNA recognition has many unusual features. At five of the six *att* subsites (L1–L3 at *att*L and R1–R3 at *att*R; Fig. 1B) a monomeric form of Mu A interacts with one face of the DNA helix over two consecutive major grooves, resulting in bending up to ~90° (Fig. 1C; 12,18). Binding specificity is achieved primarily through the major grooves. Strong contacts are found with three guanines which are conserved at five of the sites. Two of these guanines are missing in the weakest binding site (L2). DNA-bending studies using (+)-CC-1065 (a minor groove-specific DNA alkylating agent) as a structural probe have suggested that the minor grooves flanking the two contacted major grooves at *att* site L3 are compressed (19). These authors have proposed that Mu A protein straddles the central minor groove at each *att* site and penetrates into the back side of the adjacent major grooves, in order to compress the flanking minor grooves. A third bending site, possibly into the central minor groove is proposed, which, together with the two DNA bends in the flanking minor grooves, would likely result in DNA being bent towards the protein.

In order to eventually understand the unusual *att* DNA recognition displayed by Mu A, we have carried out deletion and site-directed mutation analyses of domain I $\beta\gamma$. We have identified an ~135 aa minimal *att* DNA-binding peptide, within which mutation of three amino acid residues affects DNA binding. Our results indicate that residues outside the $\beta\gamma$ domain also influence discrimination between the multiple *att* subsites.

MATERIALS AND METHODS

Plasmids

Plasmid pRA-L29 (15) containing Mu *att*L, pZA-R1 (12) containing Mu *att*R, pBRL3 (18) containing *att*L3 and mini-Mu plasmid pMK21 (20), were used in footprinting, DNA bending and DNA transposition assays.

Proteins

Purification of Mu A and HU have been described (20). Expression levels and sizes of Mu A deletion variants were confirmed by visual evaluation of Western blots of crude lysates fractionated on 10% SDS polyacrylamide gels. DNA-binding activity of these variants was assayed in ammonium sulfate

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Figure 1. Sequence of the N-terminal domain I of Mu A protein and interaction with *att* sites. (A) The N-terminal domain I contains subdomains α , β and γ (as defined by limited proteolysis), which encode site-specific DNA-binding activity. α binds to Mu enhancer and $\beta\gamma$ (shaded sequence) binds to Mu *att* sites. A potential HTH motif (underlined; residues 128–147) has been identified in β . (B) Schematic arrangement of *att* and enhancer sites on Mu DNA (not drawn to scale). (C) Interaction of Mu A at *att* site L1. The ribbon diagram of DNA is drawn with 10.5 bp per helical turn. The direction of the long horizontal arrow matches the orientation of the *att* sites indicated in B. Mu A is thought to interact with consecutive major and minor grooves, on one face of the DNA. Backbone residues protected from hydroxyl radical attack are shown in black; protection of the flanking minor grooves is thought to be a result of minor groove compression rather than direct protein contact. DNaseI hypersensitive sites are indicated by curved arrows, guanines that show strong interference are circled and the site where Mu A nicks DNA is indicated by a diamond. See text for references and other details.

fractions of crude extracts (21). Mu A polypeptides derived from fusions to glutathione S-transferase (GST) were purified by affinity chromatography according to the procedure described by Smith and Johnson (22). DNA-binding mutants of Mu A generated by site-directed mutagenesis were expressed and purified after transfer to a T7 expression vector (pET158) as described (20). Concentrations of purified proteins were derived from the extinction coefficient $\epsilon_{1 \text{ mg/ml}} = 1.83$.

Deletion and site-directed mutagenesis

Plasmid pMJ211 Δ 17X encodes a Mu A variant (Mu A Δ 17X) in which the first eight N-terminal amino acid residues of the Mu Ner protein are fused to the 84th residue of Mu A Δ 17, a protein truncated at residue 607 in the C-terminal domain (21). DNA encoding this hybrid protein was targeted for further deletions within domain $\beta\gamma$, using PCR methodology (23). Engineering appropriate restriction sites into the PCR primers allowed selectively amplified DNAs to be ligated back into an appropriate Mu A variant clone to reconstruct the deletions into the whole

protein. These were next transferred to a T7 vector for expression (20).

DNA segments encoding various regions of the $\beta\gamma$ domain were amplified by PCR and cloned in-frame with (GST)-encoding DNA on vector pGEX-2T (Pharmacia), to generate GST-Mu A peptide fusions.

Site-directed mutagenesis of residues within the minimal *att* DNA-binding region of Mu A was done according to Kunkel (24), using reagents supplied in the Bio-Rad Muta-Gene™ kit. The mutations were transferred into the T7 expression vector pET158 (carrying the whole Mu A gene) by restriction fragment exchange.

In vivo assay for Mu DNA excision

The assay was done according to Bukhari (25) and is described in Kim *et al.* (20). Briefly, strain RH208 ($F' \text{ pro}^+ \text{ lac}::\text{mini-Mukan}/\Delta \text{prolac his met Str}^R$; 21) was transformed with plasmids harboring wild-type and mutant alleles of Mu A. The transformants were patched (1 cm²) on McConkey lactose indicator plates

and incubated at 30°C for 4 days to allow precise excision of Mu DNA, which was scored by counting the number of Lac⁺ papillae.

Gel retardation, DNaseI footprinting and DNA binding assays

An ~300 bp *Bam*HI-*Hind*III *att*L fragment of pRA-L29 served as a substrate in gel retardation assays. Binding reactions contained 0.1–0.2 ng of ³²P-labeled DNA, 30–120 ng of Mu A protein and 200 ng of sonicated calf thymus DNA, in buffer containing 25 mM Hepes/150 mM NaCl/1 mM DTT, pH 7.5 in a total volume of 20 µl as described (18). Reaction mixtures were incubated at 30°C for 15 min before electrophoresis on 5% polyacrylamide gels.

For DNaseI footprinting, the *Bam*HI-*Hind*III *att*L DNA from pRA-L29 and *Eco*RI-*Hind*III *att*R DNA from pZAR1 were end-labeled at one or the other end using Klenow enzyme in the presence of 100 µM dGTP, dCTP, dTTP and 50 µCi [α -³²P]dATP. 0.5 ng of labeled DNA was incubated for 15 min at 30°C with indicated amounts of Mu A variants in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 10 mM MgCl₂ and 10 µg/ml sonicated calf thymus DNA in final volume of 20 µl. 0.5 U of RQ DNaseI (Promega) was added to the reactions and incubation continued for 1 min. Reactions were stopped by the addition of 100 µl of stop mix (20 mM EDTA, 0.3 M sodium acetate, 40 µg/ml yeast tRNA), the DNA precipitated with ethanol and resuspended in formamide-dye solution. Samples were electrophoresed on 6% denaturing polyacrylamide gels, dried and autoradiographed.

DNA bending measurements were carried out by monitoring the relative gel mobilities of protein–DNA complexes formed with labeled DNA fragments in which the *att*L3 site was located either in the middle or at the end of the fragment, as described in Kuo *et al.* (18).

In vitro assay for strand cleavage

Type I assays were performed as described by Surette *et al.* (26). Negative films of ethidium bromide-stained gels were scanned with a BioRad video densitometer and DNA bands were quantitated using software supplied with the system.

RESULTS

Delineation of a minimal *att* DNA-binding region of Mu A

Previous studies had mapped the *att* DNA-binding activity of Mu A to domain Iβγ (see Fig. 1A) within residues 63–242 (9,15). This region was therefore subjected to further deletion analysis, in order to define the minimal residues required to bind *att* DNA. The results are presented in Figure 2. DNA-binding activity was measured by a gel-retardation assay using ³²P-labeled *att*L DNA (encoding L1, L2, L3 subsites) as substrate. Initial analysis of Mu AΔ17X (encoding Mu A residues 84–607) suggested that the N-terminal boundary of the *att*-binding region lay between residues 99–109 in domain Iβ (see 4th and 5th schematic for Mu AΔ17X in Fig. 2). To test if residues in Iβ were sufficient for *att* binding, two C-terminal deletions that terminated at residue 167 in Iβ and at 176 in the Iβ–Iγ junction were generated (2nd and 3rd schematic for Mu AΔ17X in Fig. 2). The former was inactive in the binding assay, while the latter showed very poor activity. Thus, residues in the Iγ region are also required for DNA binding.

Based on this information, we made a set of DNA constructs which encode various segments of the Iβ–Iγ domain. The DNA fragments were fused in-frame to the 3' end of the GST gene in order to allow a two-step affinity purification of the Mu A peptides (see Materials and Methods). As expected, the βγ domain (aa 77–245) showed almost wild-type levels of *att* DNA binding, while neither the β (aa 84–176) nor γ (aa 177–245) domain-encoding polypeptides were active. Mixing the β and γ peptides did not restore binding (not shown). The C-terminal boundary of the DNA-binding region on the βγ peptide was mapped between residues 225 and 235. A minimal peptide encoding residues 99–235 retained DNA binding activity, although less so than a peptide encoding residues 99–245. We therefore define the minimal *att* DNA-binding Mu A peptide as aa 99–235.

DNA binding properties of domain Iβγ variants

From gel-retardation experiments we estimated that the affinity of the minimal peptide for *att*L DNA was ~20- to 50-fold lower, while that of the βγ domain was ~2- to 5-fold lower than that of the wild-type protein (not shown). However, the DNA affinity of the GST-minimal peptide fusion was similar to that seen with the βγ domain, suggesting that GST moiety in the fusion contributes to the stability of the minimal peptide on DNA; thus the lower affinity of the minimal peptide does not stem from the absence of specific DNA contacts.

Figure 3 compares the DNaseI footprint of these polypeptides on *att*L and *att*R DNA, on both the top and bottom strands. Although the overall pattern of protection of the six *att* subsites (L1–L3 on *att*L and R1–R3 on *att*R) was similar for the three polypeptides, several differences in the pattern of recognition of the individual subsites were evident. At *att*L, where the binding affinities of wild-type Mu A follow the order L3 > L1 > L2 (18), the smaller polypeptides showed equal affinities for L3 and L2 and a lower affinity for L1 (Fig. 3A and B). At L1, the minimal peptide failed to generate the DNaseI hypersensitive site on the top strand, but produced new DNaseI hypersensitive sites flanking L2 (Fig. 3A). The βγ domain produced a pair of strong new hypersensitive sites on the top strand at L2, corresponding to the similar but weaker pair produced by the minimal peptide (Fig. 3A). Both the βγ domain and the minimal peptide failed to produce the DNaseI hypersensitive site on the bottom strand of L1 and L3 seen with wild-type protein (Fig. 3B). Other differences in the enhancement of DNaseI cleavage sites on sequences between the three *att*L subsites were also evident.

At *att*R, the minimal peptide showed weaker binding to R3 compared to wild-type (order of affinities of wild-type Mu A is R2 > R1–R3; 18), while the βγ domain showed a similar pattern as wild-type (Fig. 3C and D). The βγ domain did not produce the strong DNaseI hypersensitive sites at R3 (Fig. 3C, top strand) and R2 (Fig. 3D, bottom strand) seen with wild-type Mu A. However, the minimal peptide produced a stronger DNaseI hypersensitive site on the bottom strand of R1 (Fig. 3D), as well as a pair of new sites on the bottom strand of R2.

Wild-type Mu A is able to bend most *att* sites by 80–90° (18). Using the same circular permutation assay employed in the earlier studies (18), we estimated the bending angle produced by the βγ domain to be ~60° at *att*L3 (data not shown). The affinity of the minimal peptide for the single *att* site was too low to allow its bend angle to be estimated.

Figure 2. Defining the minimal *att* DNA-binding peptide of Mu A. N- and C-terminal deletions within domain I of Mu A were used to delineate the minimal residues required for *att* DNA binding. A gel-retardation assay using *attL* DNA as substrate scored the binding activity of the deleted polypeptides (see Materials and Methods). Protein domains in wild-type Mu A are shown at the top. For Mu AΔ17X derivatives, hatched areas represent the deleted regions and grey boxes Mu Ner residues. Peptides drawn below the Mu AΔ17X derivatives were expressed as GST fusions. -, +/-, ++ and +++ represent undetectable, 1–5%, 5–20% and 20–100% of wild-type activity, respectively. See text for details.

Site-directed mutagenesis of minimal *att* DNA-binding peptide

Comparison to a master set of 10 DNA-binding proteins had identified a putative helix-turn-helix (HTH) motif within domain Iβ (Fig. 1A, aa 128–147; 17). Gly-136 occupied the turn region of this putative structure. We therefore targeted the region around this helix (aa 114–157) for extensive mutagenesis. Hydrophilic residues were changed to hydrophobic or non-conservative hydrophilic ones to disrupt possible polar interactions with DNA. Hydrophobic amino acids were mutated to hydrophilic ones to disturb helix stability. The mutants were analyzed *in vivo* for precise excision of Mu DNA inserted in the *lac* operon, an assay that provides a functional test for Mu transposition, hence *att* binding (Table 1;20). Of the 34 residues targeted for change, mutation of only four produced a significant drop in excision activity: G136 is the glycine in the turn region; F131 and R146 are within the HTH motif, while K157 lies outside this region. Western blotting of cell lysates revealed a low concentration of Mu A(G136E) protein, reflecting either poor expression, low stability or low solubility of the mutant protein (data not shown). This mutant was therefore not studied further. The other three mutations that affected Mu DNA excision were moved into a T7 expression vector for purification and further characterization of the mutant proteins.

In vitro activities of site-directed Mu A mutants

In vitro gel-shift assays for DNA binding showed that Mu A(F131S) and Mu A(R146N) were still capable of binding DNA although with a lowered affinity and altered banding (bending?) patterns (Fig. 4). Mu A(K157Q) on the other hand, was completely defective in DNA binding.

DNaseI footprinting experiments showed a similar pattern of protection for Mu A(F131S) and Mu A(R146N) compared to wild-type. However, the affinity of both mutant proteins was lower, Mu A(R146N) showing the lowest affinity (Fig. 5). The enhancer-binding activity of these mutants was not affected, as determined by gel-shift experiments (data not shown).

Under standard assay conditions with a supercoiled mini-Mu donor substrate, Mu A can nick *attL* and *attR* to yield the cleaved donor complex (type I complex), the first chemical step of transposition. The ability of the mutant proteins to cleave Mu ends in this assay is shown in Figure 6. The lower binding affinity and altered *att* DNA interaction of the two mutants was reflected in low levels of the cleaved or type I complex generated by these mutants. While Mu A(F131S) showed 20–30% type I activity compared to wild-type, Mu A(R146N) was <5% as active.

Figure 3. DNaseI cleavage patterns on Mu A polypeptide-bound *attL* and R DNA labeled at the top (A, C) and bottom (B, D) strands. DNA-protein complexes were formed as described in Materials and Methods and electrophoresed on denaturing polyacrylamide gels. The first two lanes contain Maxam-Gilbert chemical cleavage reactions, followed by DNA digested with DNaseI in the absence of protein (lane 3) or in the presence of increasing amounts (60, 120, 240 ng) of either wild-type Mu A (lanes 4-6), $\beta\gamma$ domain (lanes 7-9) or minimal peptide (lanes 10-12).

Figure 4. *Att* DNA-binding properties of domain I β mutants assayed by gel-retardation. Lane 1 is *attL* DNA control without added protein, followed by increasing amounts (13, 40, 120 ng) of either wild-type Mu A (lanes 2–4), Mu A(R146N) (lanes 5–7), Mu A(F131S) (lanes 8–10) and Mu A(K157Q) (lanes 11–13). Complex I, II and III refer to occupation of one, two and three sites on the *attL* DNA fragment with wild-type Mu A.

Table 1. *In vivo* excision activity of domain I β mutants of Mu A

Mu A allele	Activity	Mu A allele	Activity
WT MuA	+++	V134S	+++
None	–	G136E	–
V114S	+++	Y138D	++
Q115L	++++	Q139S	+++
A116S	+++	S141A	+++
A117S	+++	S143A	+++
D118V	+++	T144A	+++
E119L	+++	L145S	+++
M120S	+++	R146N	–
N122V	+++	D147A	+++
Q123L	+++	K148Q	+++
S126A	+++	Y149S	+++
T127A	+++	Y150F	+++
K128Q	+++	Q151L	+++
T129A	+++	V152S	+++
A130S	+++	K154Q	+++
F131S	–	K157Q	–
T133A	+++		

Mutant alleles of Mu A were tested for their ability to excise a mini-Mu *kan* element inserted into the *lac* operon (see Materials and Methods). +++, ++, + and – refer to excision activity that is 100–150%, 50–100%, 20–50% and <10% of the wild-type Mu A allele, respectively. An average of 55 Lac⁺ papillae-per patch was given a value of 100% for the wild-type allele.

DISCUSSION

This study has focused on defining the *att* binding region of Mu A (Fig. 1A). The *att* family includes six sites, three at each Mu DNA end (Fig. 1B). They are arranged asymmetrically at the two

Figure 5. DNaseI footprints of DNA binding mutants on *attL*. DNA was labeled on the bottom strand and footprinted as described in Figure 3. The first four lanes are Maxam–Gilbert sequencing reactions. The following lanes contain DNA digested with DNaseI in the absence of protein (lane 5) or in the presence of increasing amount (30, 60, 120 ng) of either wild-type Mu A (lanes 6–8), Mu A(R146N) (lanes 9–11) or Mu A(F131S) (lanes 12–14).

ends, and their DNA sequence is similar but not identical (8). Although the overall mode of interaction at all six sites is similar (Fig. 1C; 12), Mu A distinguishes between the sites as evidenced by their different affinities and degrees of bending (18). This distinction must play an important role during the assembly of the functional tetrameric form of Mu A, a process that also requires interaction with the enhancer sites.

By deletion analysis we have identified a minimal peptide encompassing residues 99–235 within the $\beta\gamma$ region of the N-terminal domain I (Fig. 2). The affinity of this peptide for *att* DNA is 20- to 50-fold lower than that of the wild-type protein, but can be improved by an order of magnitude (to levels seen with the larger $\beta\gamma$ domain; 77–245 aa) when expressed as a fusion to GST, suggesting that the lower affinity of the minimal peptide is not due to absence of specific DNA contacts, but perhaps to loss of stabilizing charge interactions in the smaller peptide. The DNaseI protection pattern observed with the smaller peptides is similar to that seen with the full-length protein, in that a large region of DNA is protected at most sites (Fig. 3). However, the smaller peptides show altered binding affinities for the six *att* sites, as well as differences in the patterns of DNaseI hypersensitive sites. These results suggest that residues outside the $\beta\gamma$ domain must also, directly or indirectly, influence Mu A protein discrimination between the *att* sites.

In earlier studies there appeared to be a correlation between the appearance of DNaseI hypersensitive sites and a larger estimated

Figure 6. Type I assays with DNA binding mutants. Wild-type Mu A and the DNA binding mutants were assayed for type I complex formation using mini-Mu plasmid pMK21 as the donor. Lane 1 is control without added protein. The following lanes contain increasing amount (60, 120, 240, 480 ng) of either wild-type Mu A protein (lanes 2–5), Mu A(R146N) (lanes 6–9) or Mu A(F131S) (lanes 10–13). OC and SC refer to open circular and supercoiled forms of the donor plasmid.

bend angle at Mu A bound *att* sites. For example, the five strong *att* sites were all bent by 80–90° and all generated DNaseI hypersensitive sites upon Mu A binding (12,18). At the weakest site L2, where only 13 bp are contacted, no DNaseI hypersensitive sites were found and a 60° bend was estimated. We have shown in this study that the $\beta\gamma$ domain produces a smaller bend at the L3 site. Interestingly, no DNaseI hypersensitive sites were seen upon $\beta\gamma$ binding to L3.

Site-directed mutagenesis of the minimal *att*-binding peptide was targeted around a putative HTH motif identified in the β region (aa 128–147; 17). Of the 34 Mu A mutants generated, only four showed diminution of *in vivo* activity. Of these, one was not tested further due to low protein recovery, while the remaining three showed loss or diminution of *in vitro* DNA binding and transposition activities (Table 1, Figs 4–6). While two of the mutated residues (F131 and R146) lie within the 128–147 stretch of the putative HTH motif, one (K157) lies outside. That the vast majority of amino acid changes created did not affect DNA binding raises the possibility that the aa 128–147 region is not a true HTH motif. A longer helical wheel projection of the second helix region (aa 141–157), however, shows that this helix has an amphipathic character, where hydrophobic amino acids are clustered on one side and hydrophilic amino acids predominate on the other side. R146 and K157, mutations of both of which affect DNA binding, lie close together on the same side of helix, suggesting that these two residues could be part of a DNA recognition helix.

We have not targeted the γ region for site-directed mutagenesis in this study since there was no rationale that would guide the selection of residues to be targeted. Residues in this region are very likely to participate in DNA recognition since the Mu A contact region at each *att* site is large and includes two major and an intervening minor groove. We expect that structure determination of the minimal peptide will serve to direct the design of future mutagenesis of this region.

The mutants described in this study should provide powerful tools in understanding the structure and assembly of the

functional Mu A tetramer. For example, a recent domain sharing model for active site assembly suggests that a monomer of Mu A does not harbor a functional active site, which is built by sharing of specific domains by more than one transposase monomer (14). One key feature of the model is that not all four Mu A monomers within the active tetramer need interact with the enhancer, whose role may be to specify domain contributions. The model suggests that within a tetramer competent in strand cleavage, the relative position of Mu A monomers involved in enhancer recognition is necessarily fixed. With isolation of the *att* binding mutants it becomes possible to address the domain contributions of the individual Mu A monomers. For example, we are currently using these mutants as starting points for the isolation of altered specificity mutants of Mu A. The novel *att* site/Mu A reagents thus generated should prove invaluable in designing experiments to reveal the pathway of assembly of the functional Mu A tetramer.

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