

# An ATP–ADP switch in MuB controls progression of the Mu transposition pathway

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**MuB protein, an ATP-dependent DNA-binding protein, collaborates with Mu transposase to promote efficient transposition. MuB binds target DNA, delivers this target DNA segment to transposase and activates transposase's catalytic functions. Using ATP-bound, ADP-bound and ATPase-defective MuB proteins we investigated how nucleotide binding and hydrolysis control the activities of MuB protein, important for transposition. We found that both MuB–ADP and MuB–ATP stimulate transposase, whereas only MuB–ATP binds with high affinity to DNA. Four different ATPase-defective MuB mutants fail to activate the normal transposition pathway, further indicating that ATP plays critical regulatory roles during transposition. These mutant proteins fall into two classes: class I mutants are defective in target DNA binding, whereas class II mutants bind target DNA, deliver it to transposase, but fail to promote recombination with this DNA. Based on these studies, we propose that the switch from the ATP- to ADP-bound form allows MuB to release the target DNA while maintaining its stimulatory interaction with transposase. Thus, ATP-hydrolysis by MuB appears to function as a molecular switch controlling how target DNA is delivered to the core transposition machinery.**

**Keywords:** ATP-binding protein/ATP hydrolysis/DNA binding/molecular switch/protein–DNA interaction

## Introduction

Spatial and temporal information can be communicated by molecular switches controlled by nucleotide binding and hydrolysis. Nucleotide-dependent regulation is used, for example, to ensure proper formation of intermediates during multi-step reactions. Classic examples of this type of control occur during GTP-regulated signal transduction (for review, see Bourne *et al.*, 1991), and recent characterizations suggest that ATP-regulated switches also exist to control multi-step processes (Laurent *et al.*, 1993; Pérez-Martín and Lorenzo, 1996; Gradia *et al.*, 1997; Klemm *et al.*, 1997). A potential candidate for such a switch protein is the MuB protein, a regulator of bacteriophage Mu transposition.

Mu transposition occurs via a recombination mechanism similar to that used by many transposable elements (Mizuuchi, 1992a; Craig, 1995). The recombination pathway is also mechanistically related to retroviral integration

(Mizuuchi, 1992b; Andrade and Skalka, 1996) and the early steps of V(D)J recombination (Craig, 1996; van Gent *et al.*, 1996). Mu encodes two transposition proteins: the transposase, called MuA, and its activator protein, MuB. The central DNA cleavage and joining reactions required during Mu transposition are catalyzed by a stable tetramer of MuA (Surette *et al.*, 1987; Lavoie *et al.*, 1991; Baker and Mizuuchi, 1992; Savilahti *et al.*, 1995), whereas MuB is an ATP-dependent DNA-binding protein that interacts with MuA to stimulate both assembly of the MuA–DNA complex and catalysis of the recombination reactions (Maxwell *et al.*, 1987; Baker *et al.*, 1991; Surette and Chaconas, 1991; Surette *et al.*, 1991; Mizuuchi *et al.*, 1995; Naigamwalla and Chaconas, 1997). MuB also dramatically affects the choice of DNA target sites during transposition (see below).

Transposition proceeds through a series of stable protein–DNA complexes called transpososomes. First, MuA assembles into a tetramer on sequences near the ends of the Mu genome, bringing the two ends together to form the stable synaptic complex (SSC, or type 0 complex; Mizuuchi *et al.*, 1992). MuA then catalyzes the two chemical steps of recombination, introducing a single-stranded cut at each end of the Mu DNA to generate the cleaved donor complex (CDC, or type 1 complex), and then promoting attack of these cleaved 3' ends into a new DNA site (a process called DNA-strand transfer) to generate the strand transfer complex (STC, or type 2 complex) (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Lavoie *et al.*, 1991; Mizuuchi, 1992b; Mizuuchi *et al.*, 1992). MuB can interact with all of these MuA–DNA complexes and the most efficient recombination pathway probably occurs when MuB–MuA contact initiates during transpososome assembly (Baker *et al.*, 1991; Mizuuchi *et al.*, 1992; Naigamwalla and Chaconas, 1997). This MuA–MuB interaction allows for two types of MuB-dependent regulation of transposition: target site selection (Maxwell *et al.*, 1987; Reyes *et al.*, 1987; Adzuma and Mizuuchi, 1988; Darzins *et al.*, 1988), and protection of correctly assembled transpososomes from premature disassembly by the host chaperone, ClpX (Levchenko *et al.*, 1997).

Although Mu exhibits only a weak preference for target sites based on sequence (Mizuuchi and Mizuuchi, 1993), there is a strong preference for target sites at least 10–15 kb away from the original Mu insertion (reviewed in Craig, 1997). This phenomenon, called 'target immunity', is recapitulated in a simple *in vitro* reaction containing MuA, MuB, HU protein (a DNA-binding protein involved in transpososome assembly), Mg<sup>2+</sup>, ATP and two distinct DNA molecules, known as the 'donor' and 'target' plasmids (Craigie *et al.*, 1985). The donor plasmid has the Mu sequences necessary for transposition, whereas the target DNA can be any plasmid that lacks MuA–

binding sites. Thus, there are three potential classes of target sites: (i) sites on the same donor DNA molecule cleaved by MuA resulting in unimolecular recombination; (ii) sites on a different donor DNA molecule, such that MuA covalently joins two donor DNA molecules; and (iii) sites on the target plasmid, resulting in the joining of one donor DNA molecule to one target plasmid. In the absence of MuB or ATP, intramolecular target sites are used exclusively (Maxwell *et al.*, 1987; Baker *et al.*, 1991). However, when MuB and ATP are present, the target plasmid is the predominant target. This preference derives from a biased accumulation of MuB on target DNA plasmids, and exclusion of MuB from the donor DNA (Adzuma and Mizuuchi, 1988). This unequal distribution of MuB is established because, when MuA and MuB are bound to the same DNA molecule, MuA stimulates MuB to hydrolyze ATP and promotes its dissociation from DNA. Thus, MuB is 'cleared' from donor plasmids and accumulates on target plasmids. These MuB-bound target DNAs are then used as the preferred target sites because MuB, in turn, stimulates the recombinase activities of MuA (Baker *et al.*, 1991; Surette *et al.*, 1991).

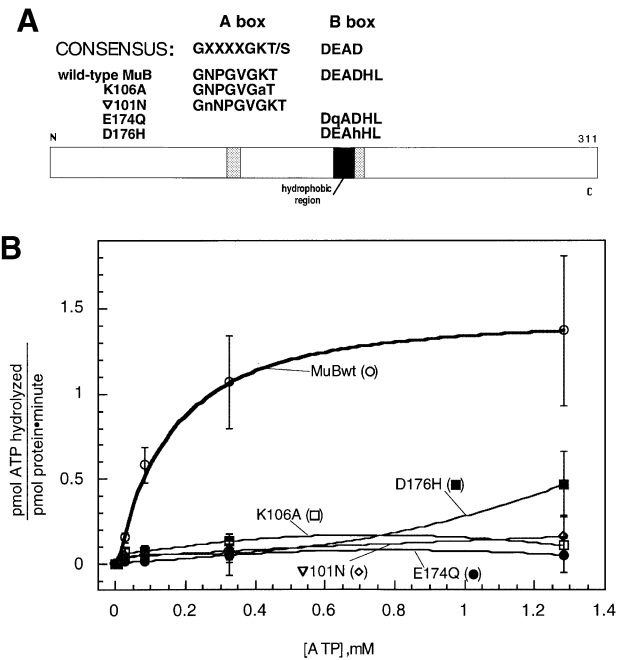
Recent analysis suggests that MuB has a second role in controlling the transposition pathway. Following strand transfer, Mu DNA replication initiates on the strand transfer product after a remodeling step in which the *Escherichia coli* ClpX chaperone destabilizes the MuA tetramer to allow recognition of the recombination site by the host DNA synthetic machinery (Levchenko *et al.*, 1995; Nakai and Kruklitis, 1995; Kruklitis *et al.*, 1996; Welty *et al.*, 1997). Both MuB and ClpX interact with the C-terminal domain of MuA (Levchenko *et al.*, 1997). The protein sequence determinants critical for ClpX to recognize MuA are imbedded in the region bound by MuB. As a consequence of these overlapping protein-protein interaction determinants, MuB inhibits the remodeling of MuA-DNA complexes by ClpX. MuB is therefore an attractive candidate for explaining how early transposition intermediates avoid being disassembled by ClpX prior to completing recombination.

Here we investigate the role of ATP binding and hydrolysis in modulating the activities of MuB protein required during transposition. These studies provide evidence for an ATP hydrolysis switch whereby the ATP- and ADP-bound forms of MuB play distinct roles in recombination. Analysis of transposition complexes generated using either ATPase-defective MuB mutant proteins or ADP-bound wild-type MuB indicates that this switch occurs after the target DNA is delivered to the transpososome, but before MuB stimulates catalysis of recombination by MuA.

## Results

### Isolation of MuB ATPase mutants

To generate ATPase-defective MuB proteins, site-directed changes were introduced into nucleotide-binding motifs (Walker *et al.*, 1982) within the MuB gene (Miller *et al.*, 1984; Teplow and Harshey, 1988) (Figure 1A). Four MuB mutants were obtained: K106A, E174Q, and D176H had single amino acid substitutions whereas  $\nabla$ 101N had an asparagine inserted between glycine 100 and asparagine 101. K106A and  $\nabla$ 101N changed the A box sequence



**Fig. 1.** Site-directed alterations within the MuB gene. (A) A schematic of the MuB gene with consensus nucleotide binding motifs shown above and specific alterations shown below. Two proteins have changes in the A box, K106A and  $\nabla$ 101N. Two other proteins have changes in the B box or DEAD box. (B) Wild-type MuB and variants were assayed for their abilities to hydrolyze ATP (see Materials and methods). Rate of hydrolysis (pmol ATP hydrolyzed/pmol protein•min) versus ATP concentration (mM) is shown.

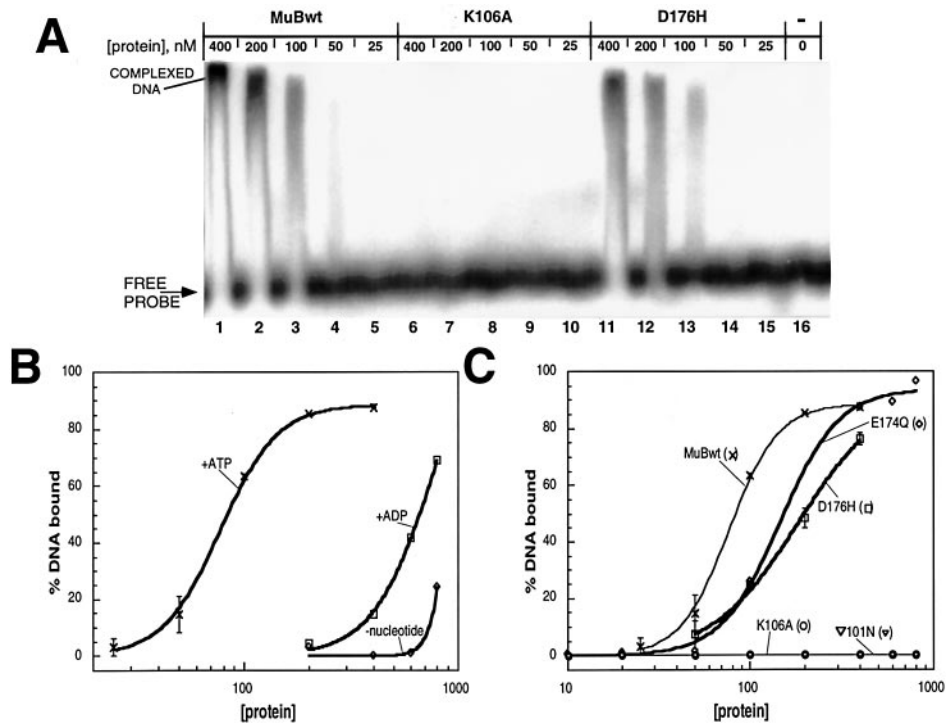
whereas E174Q and D176H altered the B box (Walker *et al.*, 1982; Pause and Sonenberg, 1992). Based on known structures of ATP- and GTP-binding proteins, the A and B boxes form a single ATP-binding pocket; A box residues contact the  $\alpha$  and  $\beta$  phosphates of ATP, and B box residues coordinate a magnesium ion in contact with the  $\beta$  and  $\gamma$  phosphates (la Cour *et al.*, 1985; Jurnak, 1985; Pai *et al.*, 1990; Müller and Schulz, 1992; Story and Steitz, 1992).

The mutant MuB proteins were purified and their ability to hydrolyze ATP determined (Figure 1B). At ATP concentrations above 300  $\mu$ M, wild-type MuB hydrolyzed ATP (~1 ATP/MuB•min) whereas the MuB mutants had little or no ATPase activity. These data therefore indicate that the proteins are in fact ATPase mutants.

### The two classes of MuB mutant proteins have different DNA-binding properties

Since MuB is an ATP-dependent DNA-binding protein, substitutions affecting nucleotide-binding motifs might also modulate DNA binding. We measured DNA binding by the MuB mutants using affinity co-electrophoresis (ACE, see Materials and methods; Lim *et al.*, 1991). Briefly, ACE involves forming protein-DNA complexes within an agarose gel during electrophoresis (Figure 2A). To analyze DNA binding by MuB, a  $^{32}$ P-labeled 30 bp fragment was used and the electrophoresis buffer was similar to that used for transposition. ATP was present at 0.5 mM during electrophoresis, unless otherwise noted. In the absence of nucleotide or when ADP was substituted in place of ATP, DNA binding was weakened dramatically (Figure 2B, Table I).

DNA-binding curves were obtained from ACE data for



**Fig. 2.** Proteins defective in ATP hydrolysis have altered DNA-binding affinities. (A) Affinity co-electrophoresis (ACE) gels were used to determine DNA-binding affinities for wild-type MuB and mutants (see Materials and methods). Briefly, wild-type MuB, K106A or D176H was embedded in the lanes of the gel at the concentrations indicated above the lanes whereas a  $^{32}\text{P}$ -labeled 30 bp dsDNA oligonucleotide was electrophoresed through the gel. Retardation of the radiolabeled oligonucleotide occurs as protein-DNA complexes are formed in the gel during electrophoresis. (B) DNA-binding dependence of MuB on nucleotide was determined using ACE. Data is shown from gels and buffer containing 0.5 mM ATP, ADP or no nucleotide as indicated. Data used to plot DNA binding in the presence of ATP was the average from two data sets, whereas data in the presence of ADP or in the absence of nucleotide were from one data set. Points were fitted to the following equations: +ATP,  $\theta = \theta_{\max} / \{1 + (1.6 \times 10^6 / [\text{protein}]^{3.3})\}$ ; +ADP,  $\theta = \theta_{\max} / \{1 + (5.0 \times 10^8 / [\text{protein}]^{3.0})\}$ ; and -nucleotide,  $\theta = \theta_{\max} / \{1 + (2.5 \times 10^{29} / [\text{protein}]^{9.6})\}$ . (C) Quantification of ACE gels for wild-type MuB, K106A and D176H. The amount of DNA retained in the top half of the gel (see Materials and methods) was quantified using ImageQuant software from Molecular Dynamics. Data for MuBwt, D176H and K106A are from two experiments and data for E174Q and  $\nabla 101\text{N}$  are from one. Data were fitted to the following equations: MuBwt,  $\theta = \theta_{\max} / \{1 + (1.6 \times 10^8 / [\text{protein}]^{3.3})\}$ ; E174Q,  $\theta = \theta_{\max} / \{1 + (8.2 \times 10^5 / [\text{protein}]^{2.7})\}$ ; and D176H,  $\theta = \theta_{\max} / \{1 + (1.8 \times 10^4 / [\text{protein}]^{1.9})\}$ . Hill coefficients ( $n$ ) were obtained from Hill plots of these data and are listed in Table I.

**Table I.** DNA binding properties of MuB and mutants

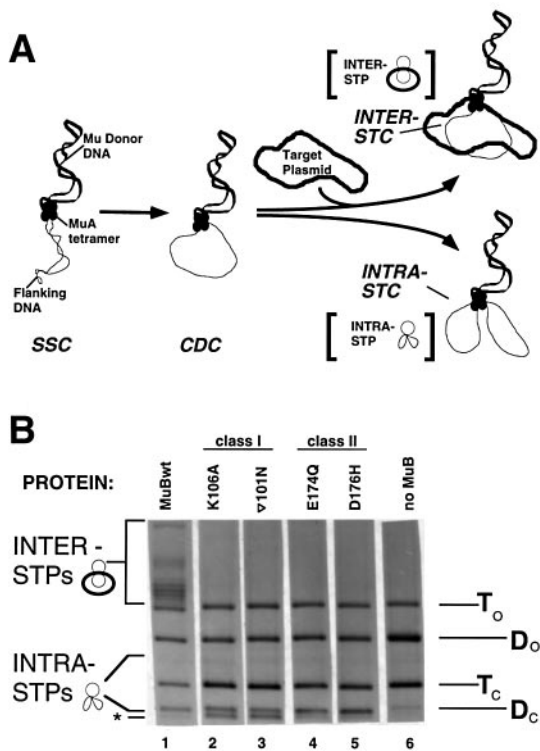
Protein	No nucleotide		ADP		ATP	
	K (nM)	$n$	K (nM)	$n$	K (nM)	$n$
MuBwt	1200	10	790	4.1	80	4.8
K106A	>800	n.a.	>800	n.a.	>800	n.a.
ins101N	n.a.	n.a.	n.a.	n.a.	800	n.a.
E174Q	n.a.	n.a.	n.a.	n.a.	150	2.3
D176H	580	n.a.	575	n.a.	180	2.5

wild-type MuB, K106A,  $\nabla 101\text{N}$ , E174Q and D176H (Figure 2C, Table I). The protein concentration required for half-maximal DNA binding was calculated from the concentration required to retain half of the DNA in the top half of the gel (see Materials and methods). Half-maximal binding occurred at 80 nM for wild-type MuB. Both E174Q and D176H bound DNA appreciably, with half-maximal DNA binding occurring at 150 nM and 180 nM, respectively. K106A and  $\nabla 101\text{N}$  showed no detectable binding even at concentrations as high as 800 nM. For the proteins that bound DNA, the ACE data also revealed differences in cooperativity (Table I). A Hill coefficient of 4.8 was calculated for wild-type MuB, suggesting that positive cooperativity strengthens DNA binding. The class II proteins exhibited some loss of

cooperativity, having Hill coefficients of 2.3 for E174Q and 2.5 for D176H (Table I). Thus, based on their respective abilities to bind DNA, the MuB mutants fell into two classes. Class I proteins are at least 10-fold defective in DNA binding, whereas class II proteins bind DNA with only a 2-fold defect.

#### **Loss of ATPase activity inhibits intermolecular recombination, but not MuB-dependent stimulation of transposase**

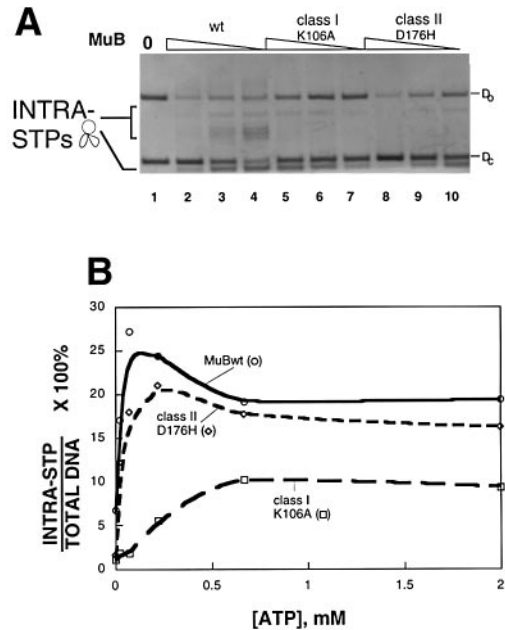
The impact of ATPase-defective MuB mutants on transposition was investigated. As MuB is involved in target site selection, the distribution of products generated in an *in vitro* transposition reaction is a sensitive diagnostic for MuB function. Depending on conditions, two classes of recombination products accumulate (Figure 3A). Intermolecular (bimolecular) strand transfer products (INTER-STPs), which result from recombination between a donor plasmid and a separate target DNA plasmid, are the predominant product in the presence of wild-type MuB and ATP. In contrast, intramolecular (unimolecular) strand transfer products (INTRA-STPs), resulting from recombination between the Mu end sequences and a second site on the same plasmid, are the exclusive product when MuB or ATP is omitted. Intramolecular strand transfer is also stimulated by MuB and ATP under some circumstances (for example when no other target DNA is available), and



**Fig. 3.** MuB mutants are defective in stimulating intermolecular strand transfer *in vitro*. (A) Diagram depicting intermolecular versus intramolecular strand transfer. The donor DNA is a plasmid containing the ends of the Mu genome that divide the plasmid into Mu sequences and non-Mu flanking sequences as shown. A tetramer of MuA binds the Mu end sequences to form the stable synaptic complex (SSC). Cleavage of the ends results in the cleaved donor complex (CDC). At this stage, the 3' hydroxyls exposed during cleavage go on to attack a DNA target site on a different molecule, resulting in an intermolecular strand transfer complex (INTER-STC), or they can attack sequences within the donor DNA plasmid, resulting in an intramolecular strand transfer complex (INTRA-STC). Deproteinization of complexes by SDS treatment leads to the final DNA products [called intermolecular strand transfer products (INTER-STP) and intramolecular strand transfer products (INTRA-STP)] discussed in the text. (B) *In vitro* transposition reactions were treated with SDS and electrophoresed. The reaction contains a target DNA molecule, which migrates as supercoiled ( $T_c$ ) or relaxed ( $T_o$ ), and a donor DNA plasmid, which migrates as supercoiled ( $D_c$ ) or relaxed ( $D_o$ ). Reactions contained either wild-type MuB (330 nM) or a MuB mutant (330 nM) as indicated above the lanes. INTER-STPs migrate as a series of topoisomers just above  $T_o$  as indicated. INTRA-STPs migrate as a series of topoisomers between  $T_c$  and  $D_o$  and as a band just below  $D_c$  as indicated by \*. Although multiple products are possible (see text) the \*-marked product is the predominant form generated when MuB is not bound to DNA.

under the conditions used in this study, little or no INTRA-STPs are observed when MuB is omitted (Figure 3B, lane 6). Thus, accumulation of INTER-STPs is a measure of MuB's ability to deliver target DNA and stimulate transposase, whereas accumulation of INTRA-STPs provides an assay for stimulation of MuA by MuB without requiring MuB's target DNA delivery functions (see below).

None of the four mutant MuB proteins supported intermolecular strand transfer (Figure 3B, lanes 2–5, INTER-STPs). They all, however, allowed intramolecular recombination. Intramolecular strand transfer with the mutant proteins was characterized further in the absence of the target DNA plasmid (Figure 4). Data are shown for



**Fig. 4.** MuB mutants stimulate MuA. (A) Transposition reactions carried out in the absence of target DNA were treated with SDS and electrophoresed. Lanes contained either no MuB, or 50 nM, 150 nM or 450 nM wild-type MuB, K106A or D176H as indicated below the lanes. (B) Graphical representation of the ATP concentration dependence of wild-type MuB and mutants during transposition. Data were obtained from quantitation of *in vitro* transposition products by autoradiography of Southern blots probed with  $^{32}P$ -labeled donor DNA as described in Materials and methods.

wild-type MuB; the class I protein, K106A; and the class II protein, D176H; the other two representatives of these classes performed similarly (data not shown). Based on the MuB concentration required to stimulate strand transfer, the mutant proteins fell into the same two classes as was observed for DNA binding. Class I proteins (K106A and ∇101N), which failed to bind DNA, stimulated strand transfer maximally at concentrations between 1000 and 2000 nM (Figure 3A, lanes 5–7; and data not shown). Class II proteins (E174Q and D176H), bound DNA appreciably, and, like wild-type MuB, maximally stimulated strand transfer when present between 200 nM and 400 nM (Figure 4A, lanes 8–10; and data not shown), with higher concentrations being inhibitory.

Both class I and class II MuB mutants required ATP to stimulate intramolecular recombination. Class II proteins (D176H is shown; E174Q behaves similarly.) required an ATP concentration similar to that needed by wild-type MuB whereas class I proteins (K106A is shown, ∇101N behaves similarly) required higher levels of ATP to stimulate MuA. These data suggest that the class I proteins have a defect in ATP binding not exhibited by class II proteins. This conclusion was further supported by 8- $N_3$ - $\alpha$ - $^{32}P$ -ATP cross-linking experiments, which revealed an ATP-binding defect for the class I proteins relative to the class II proteins and wild-type MuB (data not shown). Although defects in ATP binding by MuB appear to be associated with alterations to the A box, this does not appear to be a general rule based on mutational analysis of other proteins (Blum *et al.*, 1992; Pause and Sonenberg, 1992).

The class II proteins stimulated strand transfer to a

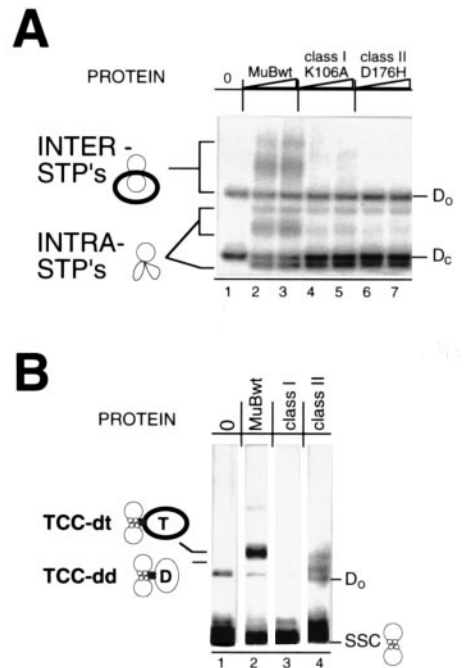
similar extent as wild-type MuB (Figure 4B). The class I proteins exhibited an ~2-fold defect in their ability to stimulate MuA at high ATP concentrations, possibly due to the fact that the reactions were performed at a protein concentration slightly below the optimum for class I proteins. However, the distribution of intramolecular products generated in the presence of the mutant proteins differed from those formed with wild-type MuB (Figure 4A). Four classes of intramolecular products (different topoisomers) can form during transposition depending on whether the target site is within the Mu portion of the donor plasmid or in the flanking sequences, and which strands are joined to each other (Maxwell *et al.*, 1987; Baker *et al.*, 1991). Both classes of altered MuB proteins preferentially formed the product that results from strand transfer of the cleaved Mu DNA ends into the same strands of the flanking sequence on the donor plasmid (see legend to Figure 3). This product migrates faster than the supercoiled donor DNA during agarose gel electrophoresis (Maxwell *et al.*, 1987; Baker *et al.*, 1991). It has been shown previously that this is the predominant product formed when MuB stimulates intramolecular strand transfer while not bound to the DNA (Baker *et al.*, 1991; Surette and Chaconas, 1991). Formation of this specific INTRA-STP depended on MuB–MuA interactions as it was not generated when MuB was absent (lane 1) or when MuA was replaced by a deletion derivative known to be defective in interacting with MuB (MuA1-615; Baker *et al.*, 1991; Leung and Harshey, 1991; data not shown).

Collectively, these data suggest that although the mutant proteins fail to promote intermolecular strand transfer, they retain the ability to bind ATP, contact MuA and stimulate catalysis of strand transfer. Both classes of altered MuB proteins stimulate strand transfer by MuA in response to the presence of ATP, but appear to do so only when not bound to DNA. In this context, MuA is responsible for choosing the target site and MuB stimulates catalysis by forming a protein–protein complex with MuA. Thus, the mutant MuB proteins appear specifically defective in some aspect of target DNA delivery required for intermolecular transposition.

#### **Class II MuB ATPase mutants form target capture complexes with MuA**

A defect in supporting intermolecular strand transfer is not surprising for the class I proteins given their defect in DNA binding. This binding defect is seen even at 2 mM ATP, conditions where these proteins stimulate intramolecular strand transfer (data not shown). Without the ability to bind DNA, they could not deliver the target DNA to transposase. However, the class II proteins do bind DNA in an ATP-dependent manner like wild-type MuB, and interact with MuA to stimulate recombination. Since these proteins are defective in ATP hydrolysis, these data suggest that ATP hydrolysis by MuB is specifically required for intermolecular transposition.

ATP hydrolysis by MuB is known to be important for target immunity (Adzuma and Mizuuchi, 1988). Therefore, the class II MuB mutants are probably unable to discriminate between the non-Mu target plasmids and donor plasmids. Indeed, one explanation for their intermolecular strand transfer defect could be the disruption of target



**Fig 5.** Class II proteins exhibit a defect after target capture, but before stimulation of strand transfer. (A) *In vitro* transposition with pre-incubated donor and target DNAs in high concentration. ATP and non-Mu target DNA were pre-incubated for 10 min at 30°C with no protein (lane 1), wild-type MuB (0.33 μM, lane 2; 1.0 μM, lane 3), K106A (0.33 μM, lane 4; 1.0 μM, lane 5) or D176H (0.33 μM, lane 6; 1.0 μM, lane 7) prior to addition to MuA, HU and donor DNA (also pre-incubated for 10 min at 30°C). Products are as indicated. (B) Target capture complexes (TCC) formed *in vitro*. Wild-type MuB (1.0 μM, lane 2); the class I protein, K106A (2.7 μM, lane 3); the class II protein, D176H (2.1 μM, lane 4); or no protein (lane 1) were incubated with pre-formed SSCs and cross-linked with DSP. Products were electrophoresed through a 0.95% agarose gel and transferred to nitrocellulose. TCCs containing both a donor (pMK586) and target DNA molecule (φX174) are marked TCC-dt. TCCs with two donor DNA molecules are labeled TCC-dd.

immunity, since in the absence of immunity, intramolecular target sites are preferred due to their high local concentration with respect to the Mu DNA ends. Therefore, the ability of a class II MuB mutant, D176H, to support intermolecular strand transfer was assayed under conditions that optimize target DNA binding. Wild-type or mutant MuB, ATP and the target plasmid were pre-incubated for 10 min under conditions where MuB and the mutant protein bind target DNA ([MuB or mutant] = 0.33 or 1 μM). After pre-incubation, the MuB–target DNA complexes were mixed with pre-assembled MuA–donor DNA complexes to allow for strand transfer. No detectable intermolecular strand transfer products were observed either by ethidium bromide staining of the agarose gel or on an overexposed Southern blot (Figure 5A). Thus, even when allowed to pre-bind to the target DNA, the class II MuB proteins are unable to activate transposase to use this DNA during transposition.

To investigate further when during the transposition pathway the class II MuB mutants fail at intermolecular recombination, the ability of a class II representative to physically bring target DNA to the MuA–donor DNA complex was addressed. Adding target DNA pre-bound by MuB to MuA–donor DNA complexes allows formation of target capture complexes (TCCs; Naigamwalla and

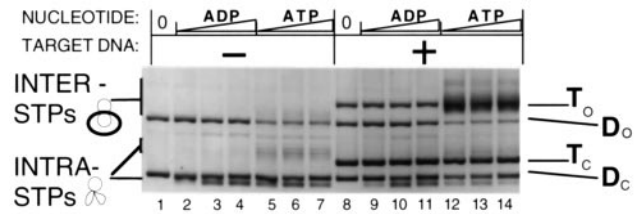
Chaconas, 1997). Within these TCCs, the donor and target DNA plasmids are held together by protein–protein interaction between MuA and MuB. These complexes can be chased further into intermolecular strand transfer products (Naigamwalla and Chaconas, 1997). To ask whether the class II MuB mutant, D176H, supports TCC formation even though it is defective in intermolecular strand transfer, the MuA–donor DNA complexes (SSCs) were prevented from catalyzing cleavage or strand transfer owing to an active site mutation in MuA (MuAE392Q; Figure 5B), or the presence of  $\text{Ca}^{2+}$  with wild-type MuA (data not shown). TCCs formed in this way and stabilized by cross-linking with the lysine–lysine cross-linker DSP, are detectable after native agarose gel electrophoresis.

The class II MuB protein (D176H) supported TCC formation, although the efficiency was reduced compared with wild-type MuB (Figure 5B). Furthermore, with the mutant protein, roughly equal amounts of TCCs containing two donor plasmids (TCC-dd) and those containing one donor and one target plasmid (TCC-dt) were observed, whereas only donor–target TCCs were formed by wild-type MuB. This pattern of complex formation reflects the loss of preference for non-Mu target DNA normally conferred by MuB as a result of target immunity. The reduced number of TCCs formed by class II proteins could additionally be explained by a third type of TCC, a unimolecular complex; this complex is also expected to be present due to the loss of target immunity, but would not be detected because its mobility would be similar to that of the SSC.

#### ADP–MuB supports strand transfer but not target delivery

Experiments presented above reveal that the class II MuB mutants can bring an intermolecular target DNA to MuA, although at a reduced efficiency compared with wild-type MuB. Our ability to detect intermolecular TCCs with MuAE392Q (Figure 5B) or wild-type MuA (data not shown), taken together with the inability to detect intermolecular strand transfer products (Figure 5A) indicates that the class II proteins are defective during a recombination step that occurs after formation of a MuB–ATP–DNA complex with MuA, but prior to MuB's stimulation of strand transfer. Because the mutant proteins are defective in hydrolyzing ATP, these data suggest that during intermolecular transposition, stimulation of strand transfer may normally occur after MuB hydrolyzes ATP. If this is true, the ADP-bound form of MuB also might be expected to stimulate strand transfer. Therefore, the ability of ADP to support MuB-stimulated transposition was investigated.

Transposition was carried out in the presence of ADP or ATP under conditions where strand transfer is dependent on MuB and nucleotide (Figure 6). ADP clearly supported MuB-stimulated strand transfer; at high ADP concentrations (e.g. 0.4 mM, lane 4) the efficiency of strand transfer was similar to that seen with ATP (although, as described above, the distribution of products suggested that MuB was not bound to DNA when it stimulates MuA, Figure 3). Only intramolecular strand transfer products were observed, regardless of whether target DNA was present (Figure 6, lanes 2–4 and 9–11). The lack of intermolecular strand transfer with ADP was attributed to weak DNA binding by MuB–ADP (Figure 2B; Table I). Thus, these



**Fig. 6.** MuB stimulates strand transfer in the presence of ATP or ADP. Transposition products were formed in the presence of varying levels of ATP (lanes 5–7 and 12–14), ADP (lanes 2–4 and 9–11) or no nucleotide (lanes 1 and 8). Target DNA was omitted (lanes 1–7) to visualize the effect of nucleotide on intramolecular transposition, whereas it was included (lanes 8–14) to analyze effects on intermolecular transposition. Nucleotide concentrations were 0.04 mM, 0.4 mM or 4 mM as indicated. DNA species are as described in the legend to Figure 3.

data indicate that ADP–MuB, although it binds only weakly to DNA, forms a productive complex with MuA that stimulates its recombinase functions. Stimulation of MuA by ADP–MuB supports the notion that, during the normal process of intermolecular transposition, MuB can stimulate strand transfer after it has hydrolyzed ATP.

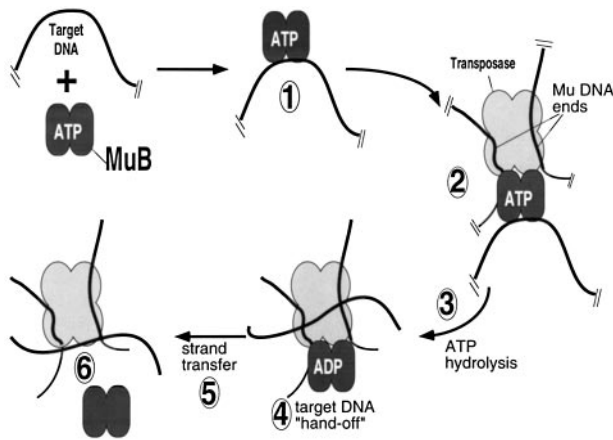
## Discussion

### Two classes of MuB mutants suggest an ATP–ADP switch during target delivery

MuB protein participates in two independent binding interactions critical during transposition: MuB binds DNA and forms a complex with the MuA transposase. Both of these interactions are modulated by adenine nucleotide binding to MuB. We have isolated and characterized MuB mutants with altered ATP interactions in order to investigate the roles of ATP binding and hydrolysis during transposition. These mutant proteins fall into two functional classes and aid in defining a mechanism for target DNA delivery during transposition.

The class I MuB mutants are defective in ATP-dependent DNA binding. They also appear to have an ATP-binding defect that can be rescued by high concentrations of ATP. In the presence of high levels of ATP, these proteins interact with MuA to stimulate intramolecular transposition and to protect MuA complexes from disassembly by ClpX (data not shown). The observation that tight DNA binding is not rescued by increasing the ATP concentration suggests that the nucleotide-dependent changes in MuB needed for the MuA–MuB interaction differ from those required for high-affinity DNA binding. The class I mutants appear specifically defective in reaching this high-affinity DNA-binding state. As a result of this defect, the class I proteins function analogously to previously characterized DNA-binding-defective versions of MuB (Baker *et al.*, 1991; Surette and Chaconas, 1991; Millner and Chaconas, 1998). These proteins, which like the class I mutants, are modified or mutated at cysteine 99, carry alterations very near the A box of the nucleotide-binding motif. The proximity of these alterations to a sequence shown here to be important for ATP binding and hydrolysis, suggests that this region of MuB functions to communicate the ATP-bound state to the protein determinants responsible for high-affinity DNA binding.

The class II MuB mutants bind DNA and ATP nearly



**Fig. 7.** Model for target DNA delivery to the MuA active site. MuB is bound to target DNA in the presence of ATP (step 1; although MuB is drawn as a dimer here, the quaternary structure of MuB involved in target delivery is unknown, and may be much larger). This is a non-Mu target (see Introduction) that does not have MuA binding sites. Through protein-protein interactions between MuB and MuA, the MuA-donor DNA complex is brought together with the MuB-ATP-target DNA complex (step 2). MuA stimulates MuB to hydrolyze its ATP (step 3), thus causing MuB to release the target DNA, all the while maintaining contacts with MuA. After the target DNA 'hand-off' has occurred (step 4), MuB-ADP stimulates MuA to catalyze strand transfer (step 5) and MuB leaves the complex (step 6) allowing for ClpX to remodel the STC prior to initiation of DNA replication.

as well as the wild-type protein but are defective in hydrolyzing ATP. These proteins therefore allowed us to specifically investigate the roles of ATP hydrolysis during transposition. Neither class II mutant supported the normal intermolecular recombination pathway, indicating that ATP hydrolysis by MuB has an important function. The proteins do, however, participate in intermolecular target capture complexes, demonstrating that they can interact with DNA and MuA simultaneously, and deliver a target DNA segment to the MuA-donor DNA complex. Based on these observations, we suggest that ATP hydrolysis by MuB is important for the transition between the target delivery step and the stage where MuB stimulates strand transfer by MuA. Consistent with this conclusion, we find that the ADP-bound form of MuB also stimulates strand transfer by MuA.

Thus, this study reveals the presence of two forms of MuB protein that probably function during target delivery: (i) the MuB-ATP-DNA complex, which binds tightly to DNA and interacts with MuA; and (ii) the MuB-ADP complex, which interacts with MuA but has a reduced affinity for DNA. A model for how these forms of MuB may function to control progression of the transposition pathway is presented below.

#### **Model for control of target delivery and strand transfer by MuB protein**

Figure 7 depicts a six step target DNA delivery model for events that occur during Mu transposition. This model focuses on how the MuB interactions change throughout the pathway; for simplicity we have not considered the multiple conformations of MuA that are certainly also involved. ATP binding is required for MuB to bind target DNA (step 1), and this MuB-ATP-target DNA complex associates with a MuA-donor DNA complex (step 2).

Since the combination of MuA and DNA stimulate ATP hydrolysis by MuB (Maxwell *et al.*, 1987; Adzuma and Mizuuchi, 1991), MuB probably hydrolyzes its bound ATP in the context of this complex (step 3). The target DNA 'hand-off' step, (step 4), where the target DNA segment is bound by MuA but no longer bound by MuB, may be facilitated by ATP hydrolysis, as suggested by the lower DNA-binding affinity of MuB-ADP compared with MuB-ATP (we do not, however, know how the ADP + Pi form of MuB interacts with DNA). Since both MuB-ADP and MuB-ATP interact with MuA to stimulate catalysis, we suggest that MuB-ADP generated by ATP hydrolysis stimulates strand transfer by MuA (step 5). Finally, after strand transfer, MuB may dissociate (step 6) from the MuA complex allowing for remodeling and the onset of replication. ADP appears to bind MuB less tightly than does ATP; therefore, ADP release, which in turn would destabilize the MuA-MuB interaction, may trigger dissociation of MuB.

Although many details in the pathway remain to be elucidated (and division into the steps outlined above is quite arbitrary), this type of model is attractive for several reasons. First, it suggests that at least two types of MuA-MuB complexes participate in the pathway and that MuB stimulates catalysis by MuA only in the later complexes. MuB can interact with the MuA-donor DNA complex at many different stages (Mizuuchi *et al.*, 1995; Naigamwalla and Chaconas, 1997), including the earliest known MuA assembly intermediate (Watson and Chaconas, 1996; Naigamwalla and Chaconas, 1997). In this early complex, MuA has not yet engaged the DNA cleavage sites (Watson and Chaconas, 1996), and probably has also not secured a target site. It would seem advantageous for the MuA-MuB interaction established early during transposition not to stimulate catalysis, but rather to assist in MuA complex assembly. This type of MuB complex, which interacts with but does not stimulate MuA, is observed when MuB-ATP-DNA is frozen as such by a class II mutation.

A second attractive feature of the target delivery model is that the proposed interactions between MuA and MuB closely parallel those described previously for establishing target immunity. The biased distribution of MuB on DNA that causes a DNA molecule carrying a MuA-binding site to be an immune target involves the following steps (Adzuma and Mizuuchi, 1988, 1989): MuB binds ATP and then DNA; MuA bound to DNA molecules with MuA-binding sites contacts the DNA-bound MuB; this MuA-interaction stimulates ATP hydrolysis by MuB and its dissociation from the DNA. Very similar interactions are recapitulated during the target delivery cycle outlined in Figure 7. However, although the mechanism by which MuB-MuA interactions lead to immunity versus transposition is not thoroughly understood, there are differences between the MuA-MuB interactions that cycle MuB off the DNA to establish immunity and those involved in target delivery. For example, monomers of MuA can stimulate ATP hydrolysis and DNA dissociation by MuB (Darzins *et al.*, 1988; Adzuma and Mizuuchi, 1989); in contrast, only MuA in a transpososome would be prepared to accept the target DNA from MuB during the delivery process. Establishing the balance between target immunity and target delivery is probably more complex than outlined above, with factors including the multimeric state of MuB,

the rate of assembly of transpososomes, and the relative activities of different forms of MuA at cycling MuB, influencing the process.

Finally, we have proposed previously that MuB needs to leave the transpososome after strand transfer in order to reveal the C-terminal peptide of MuA. This C-terminal region bound by MuB, is also recognized by the ClpX chaperone to initiate remodeling and Mu-specific DNA replication (Levchenko *et al.*, 1997). Having ATP-hydrolysis by MuB precede stimulation of strand transfer provides one means for the complexes at different stages in recombination to be distinguishable with respect to ClpX recognition. Complexes that have not completed strand transfer would be protected from the disassembly activities of ClpX by the bound MuB. This protection expires after ATP hydrolysis and dissociation of MuB from the complex.

### What is the active form of MuB?

Prior to this study, ATP hydrolysis by MuB protein was not thought to be important for intermolecular strand transfer because both ATP $\gamma$ s and AMPPNP support MuB-dependent intermolecular recombination (Adzuma and Mizuuchi, 1988; Teplow and Harshey, 1988; Baker *et al.*, 1991; Surette *et al.*, 1991; M.Yamauchi and T.A.Baker, unpublished data). Although we do not understand fully why ATP analogs and the ATP-hydrolysis defective MuB mutants do not exhibit the same effects on transposition, there are clear differences between reactions performed in the presence of the analogs compared with ATP. For example, ATP $\gamma$ s causes MuB to form large protein complexes not seen with ATP, as detected by gel filtration (Adzuma and Mizuuchi, 1991) and gel mobility shift assays (data not shown). Furthermore, transposition in the presence of these analogs is slow, stalled cleaved donor complexes accumulate, and little strand transfer product is formed (data not shown). In contrast, the class II MuB mutants stimulate (intramolecular) strand transfer with similar kinetics as wild-type MuB. Also, they form the same protein-DNA complexes as wild-type MuB in gel mobility shift experiments, in contrast to the large complexes seen with ATP $\gamma$ s (data not shown). Taken together, these data suggest that ATP analogs allow complexes to form, and reactions to occur, that do not occur with wild-type MuB in the presence of ATP or with the mutants. However, although we favor the idea that the class II MuB proteins form MuB-ATP-DNA complexes that closely mimic those formed by wild-type MuB prior to ATP hydrolysis, this is also probably not simply the case. Differences between the mutants and wild-type MuB are evident in their 2-fold lower apparent binding affinity for DNA and lower binding cooperativity. Thus, we do not rule out the possibility that some of the defects seen with the mutant proteins reflect changes in addition to their inability to hydrolyze ATP. Perhaps the mutant MuB proteins assemble only poorly into the most active form of MuB (which may be larger than a dimer), or the complexes formed are fragile and dissociate before MuA's catalytic functions are stimulated.

The level of detail in this study is not sufficient to allow high resolution examination of the active form of MuB during target delivery. This analysis has focused our interest on the step in target delivery we call 'DNA hand-

off', during which MuA chooses to use the DNA segment originally bound by MuB as the transposition target. It is not clear what part of the target DNA gets incorporated into the MuA active site (i.e. is it the same sequence that is bound by MuB or a neighboring sequence?), what the DNA becomes handed to (i.e. is it handed directly to the active site?), or how many steps are involved in the hand-off. Clarification of these points would illuminate further the process of target site selection during Mu transposition. This analysis will possibly also continue to provide insight into the general strategies that proteins (including other transposases) use to interact with specific regions of DNA due to the presence of bound proteins rather than the DNA nucleotide sequence.

## Materials and methods

### Bacterial strains and plasmids

The mini-Mu donor plasmid pMK586 (Mizuuchi *et al.*, 1992) was used for all *in vitro* assays.  $\phi$ X174 DNA from Gibco-BRL was used without further purification as the target DNA in all transposition reactions.

A 6 $\times$  histidine tagged MuB protein (hisMuBwt) was constructed by amplifying the *MuB* gene from the plasmid pMK209 (Craigie and Mizuuchi, 1985) via the polymerase chain reaction (PCR). Additional silent restriction sites were added via the method of Kunkel (1985) as follows: an *SpeI* site was created by a A $\rightarrow$ T top-strand substitution at nucleotide (nt) 252, and a *BstEII* site was created by a C $\rightarrow$ T change at nt 300 and a T $\rightarrow$ C change at nt 303. The amplified product was digested with *NdeI* and *BamHI* and cloned into pET14B (Novagen) between the *NdeI* and *BamHI* sites. MuB mutants were constructed using a two-step site-directed PCR mutagenesis protocol as described previously (Cormack, 1997). Changes were as follows (top strand altered sequence is shown; substitutions are lower case): K106A (nt 313-321), 5'-GGCgcAACC-3';  $\nabla$ 101N (nt 301-306), 5'-AATAatCCT-3'; E174Q (nt 517-525), 5'-GACcAgGCT-3'; D176H (nt 523-531), 5'-GCTcATCAT-3'. All substitutions were confirmed by DNA sequencing.

### Proteins

The bacterial strain HMS174(DE3)pLysS (Novagen) was transformed with hisMuBwt, hisK106A, his $\nabla$ 101N, hisE174Q or hisD176H. Cells were grown to an OD<sub>600</sub> of 0.5, induced with IPTG to a final concentration of 0.1 mM, and allowed to grow for an additional 1 h. Cells were harvested, centrifuged, resuspended and lysed as described previously (Baker *et al.*, 1991). Lysates were mixed in batch with Ni-NTA-agarose (Qiagen) and incubated at 4°C for 30 min. The lysate slurry was then poured into a column and the matrix was allowed to settle. The resin was washed with five column volumes of load buffer (50 mM NaPO<sub>4</sub>, pH 7.8, 300 mM NaCl) and 20 column volumes of wash buffer I (50 mM NaPO<sub>4</sub>, pH 6.0, 1 M NaCl, 10 mM imidazole, 10% glycerol). Protein was eluted with elution buffer (50 mM NaPO<sub>4</sub>, pH 6.0, 1 M NaCl, 250 mM imidazole, 10% glycerol) and the elution was dialyzed against MuB dilution buffer (25 mM HEPES-KOH pH 7.6, 1 M NaCl, 0.1 mM EDTA, 20% glycerol, 2 mM DTT). Proteins were analyzed by SDS-PAGE and found to be >90% pure with concentrations in the mg/ml range.

MuA and HU proteins were purified as described previously (Baker *et al.*, 1993, 1994).

### Mu transposition in vitro

*In vitro* reactions were carried out as described previously (Craigie *et al.*, 1985) except for the modification indicated in the text and legends. Briefly, reactions contained 30 mM HEPES-KOH pH 7.6, 10 mM MgCl<sub>2</sub>, 3.2% glycerol, 144 mM NaCl, 0.02 mM EDTA, 10  $\mu$ g/ml pMK586 plasmid DNA and 10  $\mu$ g/ml  $\phi$ X174 DNA. Unless otherwise noted, the ATP concentration was 2 mM. Protein levels were as follows: MuA monomer, 53.3 nM; HU, 120 nM. Unless otherwise noted, the standard concentration of MuB or MuB mutants was 330 nM. Reactions were incubated in a final volume of 25  $\mu$ l for 20 min at 30°C and then stopped by the addition of 6  $\mu$ l stop solution (0.1% bromophenol blue, 2.5% SDS, 50 mM EDTA, 25% glycerol). Products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining or by autoradiography of Southern blots probed with <sup>32</sup>P-labeled pMK586.



Figures with ethidium-bromide-stained gels are shown as a photograph of a negative of the gel upon UV illumination.

### Target capture complex (TCC) assay

Reactions were based on the system described by Naigamwalla and Chaconas (1997). Briefly, 5.3 pmol of the MuAE392Q protein was pre-incubated for 10 min at 30°C with 125 pmol HU and 750 ng pMK586 DNA (Gibco-BRL) in a final volume of 18 µl with 36.1 mM HEPES-KOH pH 7.6, 13.9 mM CaCl<sub>2</sub>, 16.7 mM NaCl, 0.1 mM EDTA and 21.7% glycerol. At the same time, 750 ng φX174 plasmid DNA and 10 mM ATP were pre-incubated with 26.5 pmol wild-type MuB, 75 pmol K106A, 75 pmol D176H, or no protein in a final volume of 7 µl with 430 mM NaCl, 43 mM EDTA and 11.4% glycerol. The pMK586 pre-incubation mix was then added to the φX174 mix producing a final volume of 25 µl and protein concentrations as follows: MuAE392Q, 212 nM; HU, 500 nM; MuB or mutant, 3 µM. This mix was incubated for 20 min at 30°C. DSP (Pierce) was then added to a final concentration of 250 µg/ml and the mixture was incubated at room temperature for 10 min. The cross-linking was quenched by the addition of 10 µl quench mix (300 mM Tris-HCl pH 7.5; 60 mM lysine). Cross-linked products were separated on a 0.95% agarose gel and transferred to a nitrocellulose membrane. The membrane was probed with internally <sup>32</sup>P-labeled pMK586 plasmid DNA. Radioactivity was visualized using a PhosphorImager 445SI (Molecular Dynamics).

### ATP hydrolysis

ATP hydrolysis was determined by analyzing the products of an *in vitro* reaction by chromatography on a polyethyleneimine (PEI) TLC plate in 0.4 M LiCl and 1 M acetic acid. Since the rate of ATP hydrolysis by MuB depends on the MuB concentration in a manner suggestive of positive cooperativity (Adzuma and Mizuuchi, 1991), conditions yielding maximal hydrolysis rates were determined. Consistent with a previous study (Adzuma and Mizuuchi, 1991), maximal rates of ATP hydrolysis occurred when the MuB concentration exceeded 0.1 µM (data not shown); the ATPase activity of wild-type MuB and the mutants were therefore assayed at 0.2 µM protein, over a range of ATP concentrations. The *in vitro* reactions also contained 28 mM HEPES-KOH pH 8.0, 10 mM MgCl<sub>2</sub>, 120 mM NaCl, 12 mM EDTA and 2.4% glycerol. Unlabeled ATP was added to 0.5 µl α-<sup>32</sup>P-ATP at >400 Ci/mmol (Amersham) to vary the final ATP concentration (0.5 µM, 20 µM, 80 µM, 320 µM, 1280 µM). Reaction products were visualized using a PhosphorImager 445 SI and quantitated using ImageQuant software (Molecular Dynamics).

### DNA binding

Affinity co-electrophoresis was performed essentially as described by Lim *et al.* (1991). Protein was embedded into 1% LMP-agarose and <sup>32</sup>P-end-labeled dsDNA oligonucleotide (5'-CTTTGCGTTTATCTGT-CATATGGAATCCC-3') was electrophoresed through the gel. The final protein concentrations in the gel varied from 1 µM to 10 nM as indicated in the text and figure legends. The gels and electrophoresis buffer contained 25 mM Tris-HCl pH 8.0, 0.1 mg/ml bovine serum albumin (BSA), 10 mM magnesium acetate and 50 mM potassium acetate. Additionally, gels and buffer were supplemented with 0.5 mM ATP, ADP or without nucleotide. Gels were run at 4°C with circulating buffer at 50 V for 3 h. The gels were then fixed for 15 min in 7% acetic acid, dried and scanned using a PhosphorImager 445 SI from Molecular Dynamics. The fraction of DNA bound was taken as the percent of radioactivity in the top half of each lane. Quantification was performed using ImageQuant software (Molecular Dynamics).

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