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# ClpX protein of *Escherichia coli* activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis

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**During transposition bacteriophage Mu transposase (MuA) catalyzes the transfer of a DNA strand at each Mu end to target DNA and then remains tightly bound to the Mu ends. Initiation of Mu DNA replication on the resulting strand transfer complex (STC1) requires specific host replication proteins and host factors from two partially purified enzyme fractions designated Mu replication factors α and β (MRFα and β). *Escherichia coli* ClpX protein, a molecular chaperone, is a component required for MRFα activity, which removes MuA from DNA for the establishment of a Mu replication fork. ClpX protein alters the conformation of DNA-bound MuA and converts STC1 to a less stable form (STC2). One or more additional components of MRFα (MRF$\alpha_2$) displace MuA from STC2 to form a nucleoprotein complex (STC3), that requires the specific replication proteins and MRFβ for Mu DNA synthesis. MuA present in STC2 is essential for its conversion to STC3. If MuA is removed from STC2, Mu DNA synthesis no longer requires MRF$\alpha_2$, MRFβ and the specific replication proteins. These results indicate that ClpX protein activates MuA in STC1 so that it can recruit crucial host factors needed to initiate Mu DNA synthesis by specific replication enzymes.**

*Keywords*: heat shock protein/*in vitro* DNA replication/ molecular chaperone/phage Mu/transposition

## Introduction

Bacteriophage Mu replicates its DNA by transposition, which is directed by the phage-encoded transposase MuA. MuA catalyzes the transfer of Mu ends to target DNA, forming a branched intermediate that is the template for Mu DNA replication. Upon catalyzing this recombination reaction MuA plays a role in promoting Mu DNA replication by specific host replication enzymes.

During transposition MuA is assembled into a higher order nucleoprotein complex to catalyze the recombination reaction, a complex that is subsequently disassembled for initiation of Mu DNA synthesis. MuA binds as a monomer (Kuo *et al.*, 1991) to three sites at each Mu end (Craigie *et al.*, 1984) and to the internal transpositional enhancer (Mizuuchi and Mizuuchi, 1989). On a supercoiled donor substrate, in which a mini-Mu element is carried on a plasmid vector, MuA is assembled into a tetramer in the presence of host HU protein (Lavoie *et al.*, 1991). This tetramer, which holds the two Mu ends together in a synaptic complex (see Figure 1), creates a nick at each 3′-end of Mu and transfers each end to target DNA (Craigie and Mizuuchi, 1987; Surette *et al.*, 1987; Mizuuchi *et al.*, 1992). A second Mu transposition protein (MuB) functions in the capture of target DNA, promoting intermolecular strand transfer into DNA to which it is bound (Adzuma and Mizuuchi, 1988). MuA remains very tightly bound to this strand transfer DNA product, maintaining the Mu ends in a synaptic complex (Surette *et al.*, 1987; Lavoie and Chaconas, 1990). The strand transfer product with bound transposition proteins, called the type II transpososome or strand transfer complex (STC), serves as the template for Mu DNA replication, which leads to formation of cointegrates. Alternatively, the strand transfer product can be processed without Mu DNA replication into a simple insert, which is a single mini-Mu element inserted into the target DNA (Craigie and Mizuuchi, 1985).

The very tightly bound MuA in the STC poses an impediment to the establishment of a Mu replication fork with host replication enzymes (Kruklitis and Nakai, 1994). A system of eight host replication proteins [DnaB helicase, DnaC protein, DnaG primase, DNA polymerase (pol) III holoenzyme, single strand binding protein (SSB), DNA gyrase, DNA pol I and DNA ligase; the eight-protein system] can convert the STC to a cointegrate provided that additional host factors from two partially purified enzyme fractions, designated MRFα or MRFβ, are present (Nakai and Kruklitis, 1995). MRFα and MRFβ are not required for Mu DNA synthesis if MuA is first removed from the STC by phenol extraction. However, while conversion of the STC to a cointegrate strictly requires host replication enzymes such as DnaB, DnaC and DNA pol III holoenzyme, which are host factors implicated in Mu DNA replication *in vivo* (Toussaint and Faelen, 1974; Toussaint and Résibois, 1983; Résibois *et al.*, 1984; Ross *et al.*, 1986), host protein requirements for replication of the deproteinized strand transfer product are relaxed (Kruklitis and Nakai, 1994). At relatively high concentrations of SSB or DNA pol I, the deproteinized strand transfer product can be converted to a cointegrate in the absence of the DnaB–DnaC complex or DNA pol III holoenzyme, respectively.

Thus after catalyzing strand transfer MuA in the STC plays a critical role in preparing the template for assembly of host replication enzymes. MRFα alters the STC, promoting removal of MuA from the DNA and forming a modified STC that requires MRFβ and the specific host replication proteins for conversion to a cointegrate (Nakai and Kruklitis, 1995). Such a modified STC could not be formed if the deproteinized strand transfer product was treated with MRFα. Thus MuA in the STC appears to play a critical function in promoting transition to a

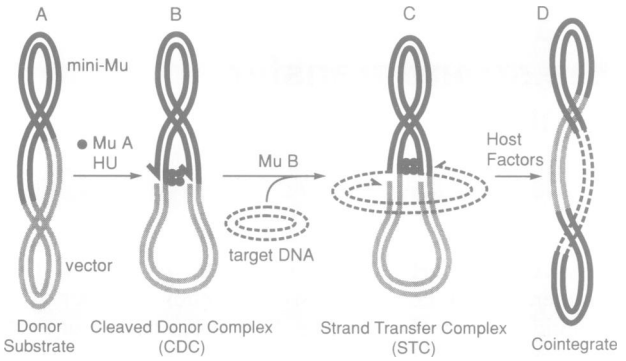


**Fig. 1.** Mu DNA strand transfer and replication. The donor substrate (**A**) in the *in vitro* Mu replication system is a negatively supercoiled plasmid which bears a mini-Mu element. In the presence of HU protein MuA binds to the Mu ends and brings them together in a synaptic complex. MuA, assembled into a tetramer, introduces a nick at each end to form the cleaved donor complex (**B**). In a process which requires MuB, the Mu 3′-ends are joined to target DNA; the MuA tetramer remains tightly bound to the Mu ends in the resulting strand transfer complex (**C**). Replication of Mu DNA initiating at one or both 3′-ends of the strand transfer complex produces the cointegrate (**D**), a fusion between the donor and target substrates.

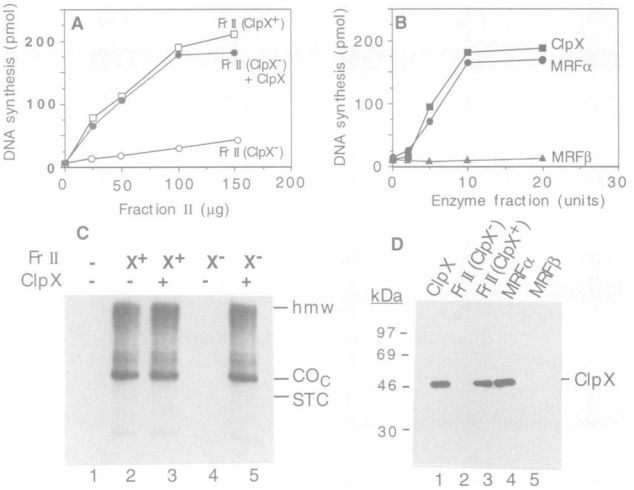


**Fig. 2.** ClpX protein, present in MRFα, is necessary for high levels of Mu DNA synthesis *in vitro*. (**A**) Mu DNA synthesis was catalyzed on STC1 (equivalent to 0.25 µg donor DNA in a 50 µl reaction mixture) using the indicated amount of fraction II extract prepared from either $ClpX^+$ (open squares) or $ClpX^-$ (*clpX*::*Kn*, open circles) WM433 strains. Where indicated fraction II ($ClpX^-$) extract was assayed in the presence of 100 ng ClpX protein (solid circles). (**B**) Mu DNA synthesis was catalyzed with 50 µg fraction II ($ClpX^-$) supplemented with ClpX protein (solid squares), MRFα (fraction IV, solid circles) or MRFβ (fraction IV, solid triangles). (**C**) Mu DNA synthesis was catalyzed with 50 µg fraction II of a $ClpX^-$ ($X^-$) or $ClpX^+$ ($X^+$) cell extract supplemented as indicated with 60 ng (10 U) ClpX protein. Products, labeled by incorporation of radioactive nucleotides, were resolved by electrophoresis on an alkaline agarose gel and visualized by autoradiography. More than 90% of the STC were converted to cointegrates. The total deoxynucleotide incorporation was as follows: lane 2, 174 pmol; lane 3, 172 pmol; lane 4, 11 pmol; lane 5, 168 pmol. $CO_C$, covalently closed circular cointegrate; hmw, high molecular weight product; STC, intermolecular strand transfer product. (**D**) Enzyme fractions were resolved by SDS–PAGE (10% gel) and the Western blot was probed with anti-ClpX antiserum. Lane 1, 100 ng ClpX protein; lane 2, 50 µg fraction II ($ClpX^-$); lane 3, 50 µg fraction II ($ClpX^+$); lane 4, 10 U MRFα (fraction IV); lane 5, 10 U MRFβ (fraction IV).

nucleoprotein complex that will permit only specific replication enzymes to assemble at the Mu fork.

An interesting finding in the light of the obstacle posed by the MuA tetramer on the STC is the requirement for *Escherichia coli clpX* function for Mu DNA replication *in vivo*. In a $ClpX^-$ strain Mu undergoes strand transfer, but the strand transfer product is not readily replicated (Mhammedi-Alaoui *et al.*, 1994). ClpX heat shock protein is one of the ATPase regulatory subunits of the Clp protease, conferring substrate specificity to the ClpP peptidase (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). ClpXP protease promotes rapid degradation of bacteriophage λ O protein (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993) and Mu *vir* protein (Geuskens *et al.*, 1992; Mhammedi-Alaoui *et al.*, 1994), a mutant form of the Mu immunity repressor conferring a *trans*-dominant virulent phenotype. ClpX protein can also act as a molecular chaperone independently of ClpP, illustrated by its ability to catalyze ATP-dependent disaggregation of λ O protein (Wawrzynow *et al.*, 1995).

While Mu DNA replication requires ClpX, Mu can replicate in a $ClpP^-$ host (Mhammedi-Alaoui *et al.*, 1994). Moreover, during Mu DNA replication *in vitro* MuA is removed from the STC by MRFα without any apparent degradation (Nakai and Kruklitis, 1995). All these results indicate that ClpX is involved in Mu DNA replication independently of ClpP and suggest the possibility that ClpX may participate in removing the MuA replication block from the STC. In this work we examine the participation of ClpX protein in Mu DNA replication *in vitro*. We characterize the function that ClpX plays in the process that promotes Mu DNA synthesis by specific replication enzymes.

## Results

### *ClpX protein is necessary for high levels of Mu DNA synthesis in vitro*

Because Mu DNA replication *in vivo* requires *clpX* function (Mhammedi-Alaoui *et al.*, 1994) we investigated whether a crude extract of strain WM433 *clpX*::*Kn* could support Mu DNA replication *in vitro*. For this reaction we used pGG215 donor substrate (Surette *et al.*, 1987), which is a plasmid bearing a 4 kb mini-Mu element. The type II transpososome or STC (hereafter referred to as STC1) was assembled in a strand transfer reaction using pGG215, a circular target DNA substrate, MuA, MuB and HU protein. STC1 was then introduced to the host enzyme fraction for the replication stage of the reaction.

Mu DNA replication on the STC1 was catalyzed at >8-fold higher levels with the $ClpX^+$ extract than with the corresponding $ClpX^-$ extract (Figure 2A). High levels of Mu DNA replication could be restored in the $ClpX^-$ extract by addition of MRFα (fraction IV) or purified ClpX protein, but not with MRFβ (fraction IV) (Figure 2A and B). Resolution of Mu replication products by alkaline agarose gel electrophoresis indicated that formation of cointegrates and high molecular weight Mu DNA, the product of rolling circle Mu DNA replication (Nakai, 1993), could be increased up to 50-fold by addition of ClpX to the $ClpX^-$ extract (Figure 2C, cf. lane 5 with 4). In the absence of added ClpX, low levels of cointegrate formation (2–5% of the level in the $ClpX^+$ extract; visible on long exposure of the autoradiogram shown in Figure

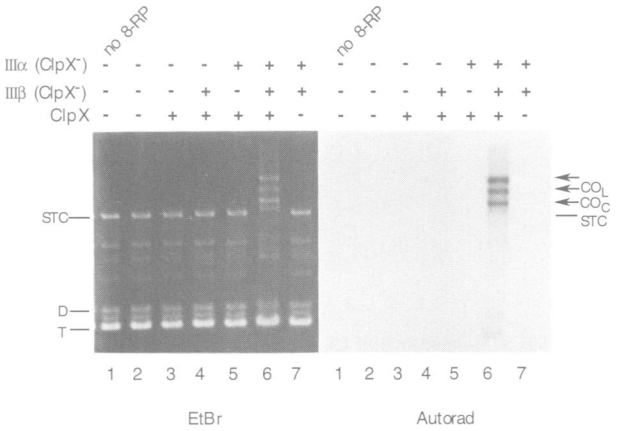


**Fig. 3.** The eight-protein system must be supplemented with ClpX protein, MRF$\alpha_2$ and MRFβ to support conversion of STC1 to a cointegrate. Mu DNA replication was catalyzed on STC1 (containing the equivalent of 125 ng donor DNA) using the eight-protein system supplemented with ClpX protein (60 ng), fraction IIIα (ClpX$^-$, 6 U) and fraction IIIβ (ClpX$^-$, 6 U) as indicated. Total deoxynucleotide incorporation in each reaction was as follows: lane 2, 8 pmol; lane 3, 7 pmol; lane 4, 15 pmol; lane 5, 5 pmol; lane 6, 121 pmol; lane 7, 12 pmol. Reaction products were resolved by alkaline agarose electrophoresis and dried for autoradiography. The three arrows indicate the positions of the cointegrate products: $CO_C$, covalently closed circular form of the cointegrate; $CO_L$, linear form of the cointegrate. The slowest migrating cointegrate band most likely corresponds to the single-stranded circular form, arising from denaturation of the open circular cointegrate on gel electrophoresis. The relative amounts of covalently closed circular and open circular cointegrates varied from experiment to experiment. EtBr, the gel stained with ethidium bromide; Autorad, the autoradiogram; D, donor substrate; T, target substrate; STC, the intermolecular strand transfer complex; 8-RP, the eight-protein system.

2C) could nevertheless be detected. Addition of ClpX protein to the ClpX$^+$ extract, on the other hand, promoted no significant increase in the level of the cointegrate and the high molecular weight Mu DNA (Figure 2C, cf. lane 3 with 2).

Consistent with these results, the presence of ClpX protein could be clearly detected on an immunoblot in the MRFα fraction, but not the MRFβ fraction (Figure 2D, lanes 4 and 5). Enzyme fractions from the *clpX*::*Kn* strain contained no detectable amount of this protein (lane 2). The results indicate that the presence of ClpX protein in the *in vitro* reaction system is necessary for high levels of Mu DNA replication.

### *In addition to ClpX protein, MRFα includes other host factors (MRF$\alpha_2$) required to promote Mu DNA replication*

The above results suggested that ClpX protein is essential for MRFα activity. Previously we determined that a crude enzyme fraction (fraction II) which promotes Mu DNA synthesis in the eight-protein system could be resolved into MRFα and MRFβ components by heparin–agarose chromatography (Nakai and Kruklitis, 1995). MRFα activity was present in the flow-through fractions and MRFβ activity in the 0.6 M NaCl eluate.

When fraction II from WM433 *clpX*::*Kn* was resolved by heparin–agarose chromatography no significant level of MRFα activity was present in the flow-through fractions (fraction IIIα; <4% of the activity present in the corresponding fraction of WM433 ClpX$^+$), whereas the level of MRFβ activity in the 0.6 M NaCl eluate (fraction IIIβ) was comparable with that found in the ClpX$^+$ strain (data not shown). However, ClpX protein was not sufficient for MRFα activity (Figure 3, lane 4). Fraction IIIα (ClpX$^-$) and ClpX protein were both required to convert STC1 to a cointegrate (lane 6).

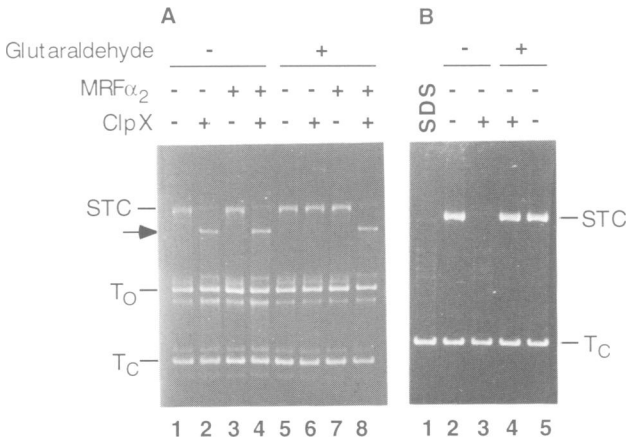


**Fig. 4.** ClpX protein can destabilize the MuA nucleoprotein complex of STC1 so that it can be disassembled by MRF$\alpha_2$. STC1 was treated as indicated with ClpX and MRF$\alpha_2$. Nucleoprotein complexes were fixed with glutaraldehyde as indicated. The DNA was digested with *Pst*I and resolved by agarose gel electrophoresis. (**A**) STC1 formed with f1 DNA as target substrate. (**B**) STC1 formed with pXP10 as target substrate. The arrow indicates the position of the DNA after treatment of STC1 (f1 DNA target) with SDS. $T_C$, covalently closed circular target substrate; $T_O$, open circular target substrate; STC, STC1 or STC2 which retain the synaptic complex of Mu ends.

Thus Mu DNA replication requires at least two distinct components from MRFα, ClpX protein (MRF$\alpha_1$) and an additional, as yet undefined, factor(s) (MRF$\alpha_2$) present in fraction IIIα. By supplementing fraction IIIα (ClpX$^-$) with ClpX protein MRFα activity could be reconstituted to the same level found in ClpX$^+$ cells (data not shown).

### *ClpX protein can convert STC1 to STC2, a MuA nucleoprotein complex that can be disassembled by MRF$\alpha_2$*

We previously determined that MRFα directs removal of MuA from STC1, forming a modified STC that requires MRFβ and the specific replication proteins for Mu DNA synthesis (Nakai and Kruklitis, 1995). We determined whether ClpX protein could alter STC1. Upon digestion with *Pst*I, which cleaves once within the mini-Mu element and once within the donor vector, STC1 (formed with a f1 DNA target which contains no *Pst*I site) resolves as a tight band on an agarose gel. Disassembly of the synaptic complex of Mu ends causes the DNA to migrate faster on the agarose gel. Treatment of STC1 with ClpX protein or with both ClpX and MRF$\alpha_2$ formed the faster migrating DNA species (Figure 4A, lanes 2 and 4). The mobility of STC1 was unaffected by incubation with MRF$\alpha_2$ (lane 3). An immunoblot of this gel verified that bound MuA had been removed from the faster migrating DNA species (data not shown). Levchenko *et al.* (1995) have also recently characterized release of MuA from STC1 catalyzed by the ClpX protein.

If, on the other hand, nucleoprotein complexes were fixed with glutaraldehyde before resolution by gel electro-

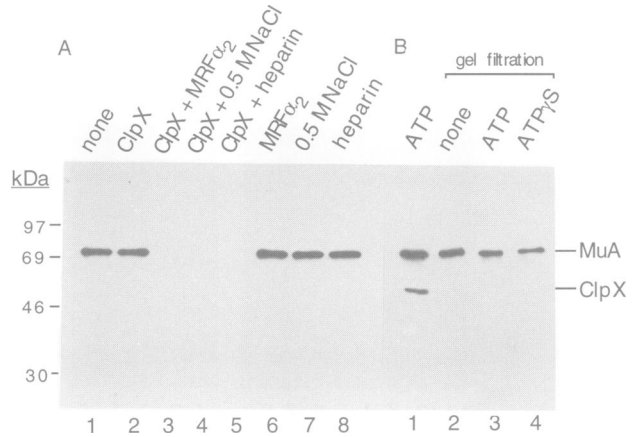


**Fig. 5.** MuA and ClpX protein content of STC2 and STC3 isolated by gel filtration. (**A**) Isolated STC1 was treated with ClpX protein and $MRF\alpha_2$ as indicated. After incubation of STC1 with or without ClpX protein some reaction mixtures were adjusted to contain 500 mM NaCl or 2 mg/ml heparin and incubated for an additional 15 min at 37°C (lanes 4, 5, 7 and 8). Proteins present in the resulting nucleoprotein complexes, isolated by gel filtration, were resolved on a SDS–polyacrylamide gel (10%). Western blots were probed with anti-MuA antibody. (**B**) Isolated STC1 was treated with ClpX protein in the absence of nucleotides (lane 2), in the presence of 2 mM ATP (lanes 1 and 3) or in the presence of 2 mM ATPγS (lane 4). The protein content of resulting nucleoprotein complexes, isolated by gel filtration (lanes 2–4), was determined on Western blots probed with both anti-MuA and anti-ClpX antibodies. As a control the protein content of a reaction mixture not subjected to gel filtration chromatography was determined (lane 1).

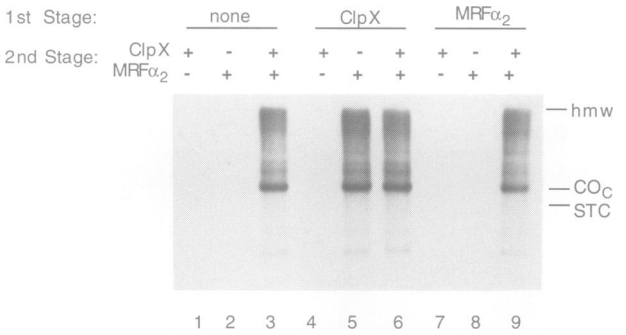


**Fig. 6.** STC2, isolated free of ClpX protein, can be converted to cointegrates by the eight-protein system supplemented with $MRF\alpha_2$ and MRFβ. Isolated STC1 was incubated with ClpX protein or $MRF\alpha_2$ as indicated (first stage). The resulting nucleoprotein complex (equivalent to 0.25 μg donor DNA), isolated by gel filtration, was introduced to the eight-protein system supplemented with $MRF\alpha_2$ (fraction IV, 10 U), MRFβ (fraction IV, 10 U) and ClpX protein (60 ng) as indicated (100 μl reaction mixture; second stage). The total deoxynucleotide incorporation (pmol) in each reaction was as follows: lane 1, 5 pmol; lane 2, 5 pmol; lane 3, 156 pmol; lane 4, 16 pmol; lane 5, 201 pmol; lane 6, 181 pmol; lane 7, 9 pmol; lane 8, 6 pmol; lane 9, 203 pmol. Labeled products of DNA synthesis were resolved by alkaline agarose electrophoresis and visualized by autoradiography. STC, position of the intermolecular strand transfer product; $CO_C$, covalently closed circular cointegrate; hmw, high molecular weight product.

phoresis, contrasting results were obtained. The mobility of STC1 on the agarose gel was unchanged after treatment with ClpX protein alone (Figure 4A, lane 6). The faster migrating form was produced only after treatment with both ClpX protein and $MRF\alpha_2$ (lane 8). To verify that the synaptic complex of Mu ends was retained after treatment with ClpX protein, STC1 was formed with target DNA that contains a single *Pst*I site (pXP10; Figure 4B). Digestion of this STC1 with *Pst*I separates the two Mu ends into two DNA molecules, which are held together by the MuA nucleoprotein complex. This complex resolves as a tight band so long as the synaptic complex remains intact (Figure 4B, lane 2). Since the two DNA molecules of the complex are not of uniform size, they do not form discrete bands upon disrupting the synaptic complex with SDS (lane 1). After treatment of STC1 with ClpX protein the synaptic complex could be detected, but only if the nucleoprotein complex was fixed with glutaraldehyde (cf. lanes 3 and 4).

These results suggested that ClpX protein converts STC1 to a less stable form (STC2) which falls apart during gel electrophoresis. To test this hypothesis we subjected STC1 to ClpX treatment and isolated the resulting complex by gel filtration to remove any unbound proteins. Proteins eluting together with DNA were resolved by SDS–PAGE and the presence of MuA was examined on an immunoblot. MuA remained bound to DNA after treatment with ClpX (Figure 5A, lane 2) and it was displaced from DNA after treatment with both ClpX and $MRF\alpha_2$ (lane 3). As observed in Figure 4, no MuA was removed from STC1 by $MRF\alpha_2$ alone (Figure 5A, lane 6). In these experiments we isolated nucleoprotein complexes under the ionic conditions (200 mM potassium glutamate) used for catalyzing Mu DNA replication. Alternatively, other procedures for examining STC1 after treatment with MRFα (Nakai and Kruklitis, 1995; the gel electrophoresis analysis) or ClpX protein (Levchenko *et al.*, 1995) involve resolution of the resulting product in the presence of heparin or under conditions of moderately high ionic strength (≥300 mM NaCl). We therefore examined whether heparin or moderately high NaCl concentrations could cause MuA to dissociate from STC2. After ClpX treatment MuA readily dissociated from the strand transfer product upon challenge with 500 mM NaCl or heparin, whereas MuA from untreated STC1 could not be removed under these conditions (cf. lanes 4 and 5 with 7 and 8). Previously we determined that MRFβ does not remove MuA from STC1 (Nakai and Kruklitis, 1995) and we have also determined that MRFβ cannot remove MuA even in the presence of ClpX protein (data not shown).

These results indicated that STC2, formed by treatment of STC1 with ClpX, has characteristics that distinguish it from STC1, even though MuA remains bound to DNA and the synaptic complex of Mu ends is maintained. No detectable ClpX protein remained bound to STC2 (Figure 5B, lane 3). Although we were able to detect as little as 10 fmol ClpX monomers, no measurable amount of ClpX protein eluted together with 500 fmol STC2 on a gel filtration column (data not shown). Under our reaction conditions for Mu DNA synthesis, 1 fmol ClpX could not promote detectable levels of Mu DNA synthesis on 50 fmol STC2 (data not shown). Nevertheless, the isolated STC2 could be converted to a cointegrate in the absence of ClpX (Figure 6, lane 5), and STC2 could not be replicated if $MRF\alpha_2$, MRFβ or the specific replication proteins were omitted (Table I). In contrast, upon treatment of STC1 with $MRF\alpha_2$, no altered STC was formed that could be isolated and replicated in the absence of $MRF\alpha_2$ (Figure 6, lane 7).

**Table I.** MuA protein maintains the requirement for MRF$\alpha_2$, MRFβ, DnaB, DnaC and pol III holoenzyme for replication of STC2

| Component omitted | DNA synthesis on STC2 after treatment | | | | | |
|---|---|---|---|---|---|---|
| | No treatment | | High NaCl | | Heparin | |
| | pmol[a] | % CO[b] | pmol | % CO | pmol | % CO |
| None | 134 | 100 | 141 | 100 | 124 | 100 |
| MRF$\alpha_2$ | 5 | <1 | 121 | 95 | 128 | 96 |
| MRFβ | 7 | <1 | 149 | 100 | 118 | 82 |
| pol III | 4 | <1 | 54 | 49 | 45 | 43 |
| DnaB | 4 | <1 | 69 | 70 | 59 | 61 |
| DnaC | 2 | <1 | 56 | 68 | 48 | 57 |

STC2 was treated with 500 mM NaCl or 2 mg/ml heparin as indicated and then isolated by gel filtration. A portion of the isolated STC2 (equivalent to ~0.25 μg donor DNA) was introduced to the eight-protein system (50 μl reaction mixture with 9 μg/ml SSB and 4 U/ml DNA pol I) supplemented with MRF$\alpha_2$ (fraction IV) and MRFβ (fraction IV). Host factors in the reaction system were omitted as indicated.
[a]Total deoxynucleotide incorporation (pmol) was determined.
[b]DNA products were resolved by alkaline agarose electrophoresis and dried for autoradiography to measure cointegrate (CO) formation. For each template >75% of the donor substrates were converted to cointegrates. This level of cointegrates for each template was arbitrarily set at 100%.

All of these results suggest that some characteristic of the MuA nucleoprotein complex is altered by ClpX to form STC2, which is an intermediate in the transition from Mu strand transfer to DNA replication. It is unlikely that MuB and HU protein play any role in the biochemical properties of STC2. We have previously demonstrated that MuB and HU do not play any role in the replication block imposed by STC1 and that no MuB is required for initiation of Mu DNA synthesis in the presence of the MRF components (Kruklitis and Nakai, 1994). Moreover, isolated STC1 stripped free of MuB and HU by challenge with 0.5 M NaCl and heparin can still be converted by ClpX protein to STC2, which requires the specific host factors for conversion to cointegrates (data not shown).

The conversion of STC1 to STC2 required ATP (data not shown). Destabilization of the MuA nucleoprotein complex in STC1 was not catalyzed by ClpX if ATP was not present or if it was replaced by the non-hydrolyzable analog ATPγS or AMP-PNP. Moreover, after incubation of STC1 with ClpX in the absence of ATP, the isolated complex could not be replicated in the eight-protein system supplemented with MRF$\alpha_2$ and MRFβ (data not shown).

### *MuA in STC2 plays a critical function in conversion to STC3, which requires specific host factors for initiation of DNA synthesis*

We wished to determine whether MuA present in STC2 plays a critical function in the transition to Mu DNA replication with specific replication proteins. Conversion of STC2 to a cointegrate required MRF$\alpha_2$, MRFβ, DnaB, DnaC and DNA pol III holoenzyme (Table I). Upon removal of MuA from STC2 by phenol extraction the strand transfer product could be replicated even when one of these host factors was omitted (data not shown). As demonstrated in Figure 5A, MuA was removed from STC2 by challenge with heparin or 0.5 M NaCl. The resulting strand transfer product could also be converted to a cointegrate even if one of the essential host factors was omitted (Table I). Thus, as is the case with STC1, removal of MuA from STC2 results in a relaxed host factor requirement for cointegrate formation.

Removal of MuA from STC1 with ClpX and MRF$\alpha_2$ formed a modified STC (STC3) identical to that formed after treatment with MRFα (Nakai and Kruklitis, 1995).

**Table II.** Host factor requirement for Mu DNA replication on STC3

| Component omitted | DNA synthesis | | | |
|---|---|---|---|---|
| | Isolated STC3 | | Deproteinized DNA | |
| | pmol | % CO[a] | pmol | % CO |
| None | 113 | 100 | 109 | 100 |
| MRFβ | 2 | <1 | 130 | 98 |
| pol III | 7 | <1 | 71 | 58 |
| DnaB | 4 | <1 | 101 | 87 |
| DnaC | 4 | <1 | 121 | 74 |

STC3 was isolated by gel filtration and then deproteinized by phenol extraction as indicated. The strand transfer product (equivalent to ~0.25 μg donor DNA) was introduced to the eight-protein system (50 μl reaction mixture with 9 μg/ml SSB and 4 U/ml DNA pol I) supplemented with MRFβ (fraction IV). Host factors were omitted from the reaction system as indicated.
[a]More than 70% of STC3 and the deproteinized strand transfer product were converted to cointegrates in the complete system, and this level of cointegrates for each template was arbitrarily set at 100%

STC3, isolated by gel filtration, required MRFβ, DnaB, DnaC and DNA pol III holoenzyme for cointegrate formation, but after phenol extraction the specific requirements for these host factors were lost (Table II). In addition, polyclonal antibodies against MuA inhibited replication of STC1 and STC2 to form a cointegrate, but not replication of STC3 (Table III). We previously demonstrated that STC3 could not be formed by incubating the deproteinized strand transfer product with MRFα (Nakai and Kruklitis, 1995). These results indicate that MuA in STC2 plays an important function in the transition to STC3. Furthermore, anti-ClpX antibodies inhibited replication of STC1, but not replication of STC2 or STC3 (Table III), confirming that the function of ClpX protein has been completed upon formation of STC2.

ClpX protein was unable to destabilize a synaptic complex of STC1 formed with a mixed tetramer of MuA and mutant MuA211 (data not shown), which is truncated by 47 amino acids at the C-terminus (Harshey and Cuneo, 1986). We previously determined that STC1 formed with this mixed tetramer cannot be disassembled by MRFα and cannot readily be converted to a cointegrate (Nakai and Kruklitis, 1995). Levchenko *et al.* (1995) have also recently demonstrated that MuA variants truncated at the

**Table III.** Effect of anti-MuA and anti-ClpX antibodies on conversion of STC1, STC2 and STC3 to cointegrates

| STC | DNA synthesis in the presence of antibody | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No antibody | | Non-immune | | Anti-MuA | | Anti-ClpX | |
| | pmol | % CO[a] | pmol | % CO | pmol | % CO | pmol | % CO |
| STC1 | 126 | 100 | 140 | 97 | 1 | <1 | 12 | <1 |
| STC2 | 172 | 100 | 115 | 90 | 7 | 5 | 160 | 87 |
| STC3 | 119 | 100 | 103 | 72 | 129 | 95 | 129 | 84 |

STC1 (equivalent to 0.25 μg donor DNA in a 25 μl reaction mixture) was treated at 37°C for 30 min with ClpX (60 ng) or ClpX (60 ng) and MRF$\alpha_2$ (fraction IV, 10 U) to form STC2 and STC3 respectively. Anti-MuA (15 μg total protein), anti-ClpX (20 μg total protein) or non-immune polyclonal antibodies (15 μg total protein) were then introduced to the reaction mixture, which was allowed to incubate on ice for 20 min. Mu DNA replication was catalyzed in the eight-protein system supplemented with MRFα (fraction IIIα, 10 U) and MRFβ (fraction IIIβ, 10 U).
[a]For each template >80% of the strand transfer products were converted to cointegrates in the absence of antibody and this level of cointegrates was arbitrarily set at 100%.

C-terminus are poor substrates for ClpX protein. The present results indicate that STC1 containing MuA truncated at the C-terminus cannot be converted to STC2 and is unable to promote the transition to DNA replication. Overall, the results indicate that the function of ClpX protein is to alter MuA in STC1, forming STC2, which can interact with MRF$\alpha_2$ to prepare the template for Mu DNA replication.

### *Tetrameric MuA in STC1 is not disassembled back to monomers during conversion to STC2 and STC3*

Since ClpX protein has been determined to be a molecular chaperone that can catalyze disaggregation of λ O protein (Wawrzynow *et al.*, 1995), we wished to determine whether ClpX protein and MRF$\alpha_2$ could promote disassembly of tetrameric MuA back to monomers. The assembly of MuA monomers to tetramers for formation of STC1 has been examined by chemical cross-linking (Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1992). We examined the quaternary structure of MuA as STC1 is converted to STC2 and STC3 by cross-linking with disuccinimidyl suberate (DSS). This agent allowed us to cross-link MuA under reaction conditions that are compatible with Mu DNA replication. The oligomeric structure of MuA in STC1 could be detected as a ladder of dimers, trimers and tetramers (Figure 7A, lane 4). A low level of MuA of higher molecular weight could also be detected under our reaction conditions. MuA that did not assemble into tetramers was detected as monomers after DSS treatment. This is evident in strand transfer reaction mixtures which contained no HU protein (lane 2) or which contained linearized donor DNA (lane 3). Treatment of STC1 with ClpX protein caused no apparent change in the oligomeric structure of MuA (lane 5), indicating that it was not converted back to monomers. The MuA tetramer holds the synaptic structure together in STC1 (Lavoie *et al.*, 1991) and the MuA tetrameric structure is most likely retained in STC2 to maintain its synaptic complex. As expected, treatment of STC1 with MRF$\alpha_2$ alone promoted no apparent change in MuA quaternary structure (data not shown).

Upon treatment of STC1 with both ClpX and MRF$\alpha_2$ no MuA eluted together with DNA on a gel filtration column (Figure 7A, lane 6). To examine released MuA we treated STC2, isolated free of ClpX protein and unbound MuA, with MRF$\alpha_2$. MRF$\alpha_2$ removed MuA from isolated STC2 in an ATP-dependent process (data not shown). After incubation of STC2 with MRF$\alpha_2$, the mixture was passed through a gel filtration column to separate DNA from released MuA, which was then cross-linked with DSS. The free MuA eluting in the included fractions clearly retained its oligomeric structure (Figure 7B, lanes 4 and 5). In a control experiment we tested whether any substance in the MRF$\alpha_2$ fraction could cause MuA to oligomerize. MuA monomers treated with DSS in the presence of MRF$\alpha_2$ were not cross-linked (Figure 7A, lane 7). Identical results were obtained in analogous control experiments in which MuA monomers were treated with DSS in the presence of ClpX protein or ClpX protein and MRF$\alpha_2$. While the cross-linking studies cannot definitively establish that MuA dissociates as a tetramer, the results clearly indicate that MuA is not converted back to its original monomeric form during conversion of STC2 to STC3. All these results suggest that ClpX alters the overall conformation of MuA in STC1 while retaining the tetrameric structure of MuA.

## Discussion

### *Function of ClpX protein in the initiation of Mu DNA synthesis*

The MuA tetramer present in the type II transpososome (STC1), which is formed during strand transfer, plays a critical role in the transition to DNA replication. For Mu DNA replication *in vitro* it imposes a requirement for specific host factors that have been implicated in Mu DNA replication *in vivo*. The deproteinized strand transfer product can be replicated in the absence of DNA pol III holoenzyme or DnaB helicase, which are required for replication of STC1 (Kruklitis and Nakai, 1994). Access of forked junctions of the strand transfer product to inappropriate enzymes *in vivo* could slow down or interfere with replication of the 37 kb genome of Mu. A system that directs assembly of a specific replisome on the strand transfer product would assure efficient replication of Mu.

The ClpX protein apparently carries out the first step in the transition from Mu strand transfer to DNA replication (Figure 8). In the presence of ATP, ClpX converts STC1 to STC2, a form in which MuA is less tightly bound to DNA. STC2, which retains the synaptic complex of Mu ends, can interact with MRF$\alpha_2$ to form STC3, a nucleoprotein complex that contains no MuA (and no synaptic complex of Mu ends) and that requires MRFβ and specific replication proteins for initiation of Mu DNA synthesis. Proteins bound to STC3 can be detected (data

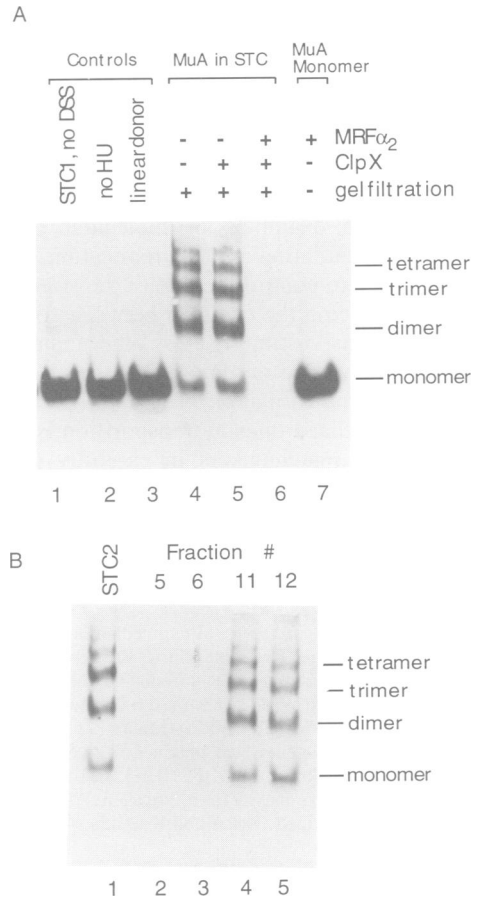


**Fig. 7.** MuA remains oligomeric after treatment of STC1 with ClpX protein and after displacement from DNA in the presence of MRF$\alpha_2$. Monomeric MuA and MuA in nucleoprotein complexes were treated with DSS and resolved by electrophoresis on a SDS–agarose–polyacrylamide gel. (**A**) Lane 1, STC1 (no DSS treatment); lane 2, HU protein was omitted from the reaction mixture for strand transfer; lane 3, donor substrate linearized with *Bam*H1 was present in the reaction mixture for strand transfer; lanes 4–6, STC1 was incubated with ClpX protein and MRF$\alpha_2$ as indicated and the resulting nucleoprotein complex isolated by gel filtration and treated with DSS; lane 7, MuA (150 ng) treated with DSS in the presence of 10 U MRF$\alpha_2$ (26 µl reaction mixture). (**B**) STC1 was incubated with ClpX protein and the resulting nucleoprotein complex isolated by gel filtration (isolated STC2 containing the equivalent of 1 µg donor substrate in 50 µl). Lane 1, STC2 (12.5 µl) isolated by gel filtration and then treated with DSS (lane 1); lanes 2–5, isolated STC2 (50 µl) incubated with MRF$\alpha_2$ (50 U) in the presence of 2 mM ATP (75 µl reaction mixture). The mixture was then filtered through a 1 ml Bio-Gel A-15m column at a flow rate of 1 ml/h. Fifteen fractions (75 µl each) were collected. Fractions 5 and 6 contained DNA (lanes 2 and 3) and fractions 11 and 12 contained MuA disassembled from STC2 (lanes 4 and 5). Aliquots of 25 µl of these fractions were subjected to treatment with DSS.

not shown), but we have not yet been able to determine which are the critical components for initiation of Mu DNA synthesis. If proteins in STC3 are removed by phenol extraction, the requirement for host factors MRFβ, DnaB, DnaC and DNA pol III holoenzyme is lost.

Therefore, the function of ClpX protein is to alter MuA in STC1 so that it can recruit specific host factors which will most likely participate in initiation of Mu DNA synthesis. MuA in STC1 is inert to the action of MRF$\alpha_2$, and ClpX apparently induces a stable conformational change in MuA to form STC2, which is now able to

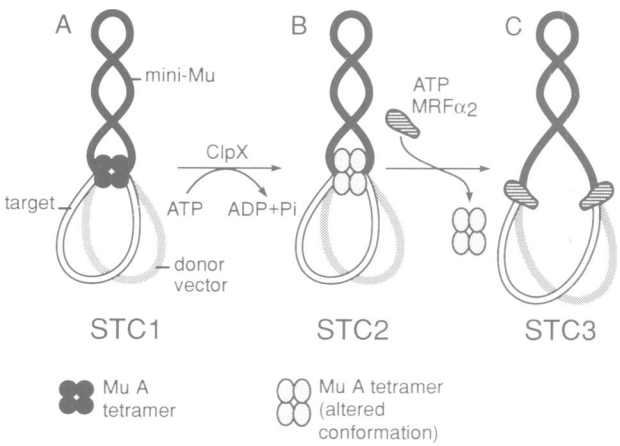


**Fig. 8.** Model for conversion of STC1 to STC2 and STC3. The type II transpososome or STC1 (**A**) consists of a strand transfer product with Mu ends tightly held together as a synaptic complex by the MuA tetramer. Upon treatment with ClpX protein, STC1 is converted to STC2 (**B**), in which the MuA tetramer is less tightly bound to DNA. This is the result of altering the conformation of the MuA tetramer. This nucleoprotein complex is now able to interact with MRF$\alpha_2$, hypothesized to be a protein that displaces MuA as a tetramer and binds to each Mu end, disrupting the synaptic complex and forming STC3 (**C**). The depicted protein content and organization of STC3 are purely speculative. STC3 allows initiation of Mu DNA synthesis by specific replication proteins in the presence of MRFβ.

interact with MRF$\alpha_2$. A mixed MuA–MuA211 tetramer does not appear to make the transition to STC2 and this is the likely reason why Mu DNA replication does not initiate efficiently on STC1 containing this tetramer (Nakai and Kruklitis, 1995).

MuA remains an oligomer upon ClpX treatment and upon removal from DNA by MRF$\alpha_2$ under reaction conditions for Mu DNA synthesis. One reason for postulating that ClpX might play a role in converting the MuA tetramer to the monomer had been the finding that ClpX can catalyze disaggregation of inactive λ O protein (Wawrzynow *et al.*, 1995). Moreover, the ClpA protein, another ATP-dependent specificity component of the Clp protease (Hwang *et al.*, 1987, 1988; Katayama-Fujimura *et al.*, 1987), can catalyze conversion of inactive RepA of plasmid P1, a protein that tends to dimerize, to the active monomeric form (Wickner *et al.*, 1994). In contrast, MuA in the STC need not be converted back to monomers by ClpX protein for initiation of Mu DNA replication. We have not further investigated the fate of oligomeric MuA that dissociates from the strand transfer product. Other *E.coli* or Mu factors may be involved in regenerating monomeric MuA, which is the form that initially binds to Mu DNA for assembly of the synaptic complex (Kuo *et al.*, 1991). On the other hand, free oligomeric MuA may be an inactive form that is marked for disposal. This latter mechanism would be consistent with the stoichiometric action of Mu transposase in promoting Mu DNA replication *in vivo* (Pato and Reich, 1982, 1984).

Levchenko *et al.* (1995) have recently characterized release of MuA from STC1 catalyzed by ClpX protein alone. Moreover, they have demonstrated that the released MuA can be re-isolated in the form of monomers, which are active in catalyzing strand transfer. Our present results do not contradict their findings. To isolate STC2 for our experiments we chose ionic conditions (200 mM potassium

glutamate or 60 mM KCl) that we use for Mu DNA replication reactions. On the other hand, Levchenko *et al.* (1995) isolated released MuA on glycerol gradients containing 300 mM NaCl. We have confirmed in our cross-linking experiments that MuA is released from STC2 in monomeric form when placed in 300 mM NaCl (data not shown). Our results also indicate that upon removal of MuA from STC2, specific host factors are no longer required for cointegrate formation (Table I); i.e. after ClpX treatment MuA must still perform an important function in promoting initiation of DNA synthesis before dissociating from the DNA template. The results of Levchenko *et al.* (1995) indicate that the action of ClpX protein destabilizes the MuA quaternary structure. Our results indicate that the destabilized MuA oligomer in STC2 is needed for the transition to STC3.

### *General strategy of employing molecular chaperones to coordinate macromolecular interactions*

The function of MuA and ClpX protein in Mu DNA replication is reminiscent of a class of proteins termed molecular matchmakers by Sancar and Hearst (1993). These are proteins that promote association between two macromolecules in an ATP-dependent process, the energy of ATP hydrolysis being utilized to induce a conformation change in one or both matched molecules and thus promote their interaction. The matchmaker, which is able to form a complex with each matched component, mediates association and then leaves the complex. Sancar and Hearst (1993) cite UvrA protein of *E.coli* as one example of a molecular matchmaker. It is involved in promoting binding of UvrB protein with high specificity to damaged DNA, the DNA-bound UvrB protein being subsequently involved in recruiting the necessary enzymes for nucleotide excision repair.

Similar types of mechanisms are employed by the Mu transposition system for the transition from strand transfer to DNA replication. MuA, whose conformation has been altered by ClpX protein to form STC2, plays a key role in binding essential host factors to the strand transfer product. The energy of ATP hydrolysis is utilized by ClpX to induce a conformational change in the MuA tetramer of STC1. As MRF$\alpha_2$ interacts with STC2, MuA leaves the complex in an ATP-dependent process and STC3 is formed. Whatever factors bind to the strand transfer product to form STC3, they do not bind to the deproteinized strand transfer product or to STC1.

Just as molecular matchmakers are employed to target proteins to specific sites on DNA such as damaged DNA, the MuA protein functions in targeting host factors to the strand transfer product. However, MuA has a dual function in replicative transposition. Upon assembly of the MuA tetramer on the donor substrate to form the stable synaptic complex (Lavoie *et al.*, 1991; Mizuuchi *et al.*, 1992) the first function of MuA is to catalyze all of the chemical steps involved in strand transfer. Upon completion of this process, its function turns to the ultimate targeting of specific replication enzymes to the strand transfer product. ClpX protein functions as a molecular chaperone that switches the MuA tetramer from the first function to the second.

This sequence of events, from assembly of the MuA tetramer to alteration of STC1 to STC2, may be a means of ensuring that proper macromolecular interactions take place in an orderly, timely fashion. *Escherichia coli* heat shock proteins DnaJ and DnaK, which participate in the orderly assembly and disassembly of nucleoprotein complexes at the bacteriophage λ origin (Alfano and McMacken, 1989a,b; Dodson *et al.*, 1989; Zylicz *et al.*, 1989), are classic examples of molecular chaperones functioning in the initiation of DNA replication. Molecular chaperones may also control the fate of the strand transfer product by influencing the decision between conservative and replicative transposition. Factors other than ClpX and MRF$\alpha_2$ may be involved in removing MuA or altering its conformation for the formation of simple inserts. Such a role of molecular chaperones in transposition is similar to that of molecular matchmakers in carefully controlling protein–protein and protein–DNA interactions.

## Materials and methods

### *Bacterial strains and plasmids*

*Escherichia coli* MC4100 *clpX::Kn* (Mhammedi-Alaoui *et al.*, 1994) was obtained from Dr Ariane Toussaint (Université Libre de Bruxelles). WM433 *clpX::Kn* was constructed by introducing the *clpX::Kn* allele from MC4100 *clpX::Kn* into WM433 (*dnaA204*) (Tippe-Schindler *et al.*, 1979) by P1 transduction. Plasmid pWPC9 (Maurizi *et al.*, 1990), which contains the *clpP* and *clpX* genes, was obtained from Susan Gottesman (National Institutes of Health). Strain BL21(DE3) (Studier and Moffatt, 1986) and pET19B were purchased from Novagen. All other plasmids have been previously described (Nakai and Kruklitis, 1995).

### *Chemicals, antibodies and purified proteins*

DSS was from Pierce and glutaraldehyde was from Electron Microscopy Sciences. Dimethylsulfoxide and polyethylenimine were from Sigma. Hydroxylapatite (Bio-Gel HTP) and Bio-Gel A-15m were from Bio-Rad. Rabbit antibody against ClpX protein was a gift from Susan Gottesman and Michael Maurizi (National Institutes of Health). Rabbit antibody against MuA protein was prepared by Biocon Inc. Antibodies partially purified by ammonium sulfate precipitation (50% saturation) were used for inhibition studies of Mu DNA replication. The precipitated immunoglobulins were resuspended in phosphate-buffered saline (10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 137 mM NaCl, 3 mM KCl, pH 7.4) and then dialyzed for 30 min against the same buffer. The enhanced chemiluminescence detection system from Amersham was used for all immunoblots. With the exception of ClpX protein, whose purification is described below, all purified proteins and all other reagents have been previously described (Nakai and Kruklitis, 1995).

### *Buffers*

Reaction buffer was 25 mM HEPES–KOH, pH 7.5, 12 mM magnesium acetate, 5 mM dithiothreitol (DTT), 200 mM potassium glutamate, 50 μg/ml bovine serum albumin (BSA). Buffer A was 50 mM Tris–HCl, pH 8.0, 20 mM DTT, 4 mM EDTA. Buffer B was 25 mM Tris–HCl, pH 7.8, 10 mM DTT, 25 mM NaCl. Buffer C was 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, 10% (v/v) glycerol. Buffer D was 50 mM Tris–HCl, pH 7.0, 10 mM DTT, 5 mM $MgCl_2$, 10% (v/v) glycerol. Buffer E was 50 mM Tris–HCl, pH 8.2, 10 mM DTT, 5 mM $MgCl_2$, 10% (v/v) glycerol. Buffer F was 25 mM HEPES–KOH, pH 7.5, 12 mM magnesium acetate, 60 mM KCl.

### *Bacteriophage Mu DNA replication in vitro*

Strand transfer to form STC1 and Mu DNA replication on STC1 (catalyzed by a crude cell extract or by the eight-protein system supplemented with MRFα and MRFβ) were carried out as previously described (Nakai and Kruklitis, 1995). The target substrate for strand transfer was pXP10 (Wolffe *et al.*, 1986), a plasmid chosen simply because it is readily prepared in high yields (where indicated phage f1 RFI DNA was used instead). All DNA synthesis reactions were carried out at 37°C for 30 min. When products of DNA replication were resolved by electrophoresis on an alkaline agarose gel (0.7%) total deoxynucleotide incorporation in 10% of the reaction mixture was measured as previously described (Kruklitis and Nakai, 1994).

### Preparation of host enzyme fractions

Fraction II, which provides all necessary host factors for Mu DNA replication, and protein fractions containing MRFα and MRFβ were prepared as previously described (Nakai and Kruklitis, 1995). One unit of MRFα or MRFβ activity is defined as the amount required to promote incorporation of 20 pmol deoxynucleotides in the eight-protein system (standard 25 μl reaction mixture containing 125 ng donor substrate), using 6 U MRFβ (fraction IV) or MRFα (fraction IV) as the source of complementing activity. The preparation of $MRF\alpha_2$ (fractions IIIα and IV) was identical to the preparation of MRFα fractions except for the following changes. $MRF\alpha_2$ was prepared from WM433 *clpX*::*Kn* and was assayed for its ability to complement the eight-protein system supplemented with ClpX protein and MRFβ (fraction IV) in the standard Mu replication assay (25 μl reaction mixture). One unit of $MRF\alpha_2$ activity is defined as the amount required to promote 20 pmol deoxynucleotide incorporation in the eight-protein system supplemented with 40 ng (7 U) ClpX protein and 6 U MRFβ (fraction IV). In the preparation of $MRF\alpha_2$ (fraction IV) concentration of fraction IIIα by ammonium sulfate precipitation for resolution on a Sepharose S-300 column was omitted.

### Purification of ClpX protein

ClpX protein was assayed by its ability to promote Mu DNA synthesis in the presence of 50 μg fraction II of WM433 *clpX*::*Kn* (standard 25 μl reaction system). One unit of ClpX activity is defined as the amount required to promote 20 pmol DNA synthesis in this system.

The *clpX* gene from pWPC9 was placed under the control of the T7 promoter in plasmid pET19B. Strain BL21(DE3), transformed with this plasmid, was grown in 10 l modified terrific broth (Sambrook *et al.*, 1989; half the amount of yeast extract) in a 14 l New Brunswick fermenter (37°C, to $OD_{595}$ = 2) and ClpX protein overproduction was induced by the addition of isopropyl β-D-thiogalactopyranoside (Boehringer-Mannheim) to 0.4 mM. After 1 h induction cells (130 g wet cells) were harvested, washed with 50 mM Tris–HCl, pH 8.0, 10% (w/v) sucrose and resuspended in the same buffer to $OD_{595}$ = 300. Cells were frozen in liquid nitrogen and stored at –80°C.

All steps in the purification of ClpX were carried out at 4°C unless noted otherwise. A portion of the cell suspension (18.5 ml, 10.5 g wet cells) was diluted with buffer A (11.5 ml) and lysozyme added to a concentration of 300 μg/ml. After 60 min on ice the suspension was heated for 5 min at 42°C, placed on ice for 10 min and then subjected to centrifugation in a Beckman Ti45 rotor at 130 000 *g* for 45 min. The cell pellet, which was found to contain the majority of the ClpX protein, was solubilized in 30 ml buffer B containing 7 M urea (Yoo *et al.*, 1994) by stirring for 6 h on ice. Upon removing any insoluble materials by centrifugation, ClpX protein was renatured by dialysis against 500 ml buffer C (four times for a total of 18 h). The solution was clarified by centrifugation, yielding fraction I (12 400 000 U, 278 mg, 26 ml). Only 5% of fraction I was carried though the entire purification protocol, described below. The indicated yields have been normalized to reflect fractionation of the entire preparation.

Fraction I was adjusted to 850 mM NaCl and polyethylenimine was added dropwise [0.06 vol. 10% (w/v) stock, pH 7.8] over 15 min with occasional mixing. Upon removing the precipitate by centrifugation the supernatant (fraction IA, 270 mg, 30 ml) was loaded onto a hydroxyapatite column (0.2 ml bed volume/mg fraction IA) equilibrated in buffer D containing 850 mM NaCl and the column was washed with 3 column vol of the same buffer. Bound proteins were eluted (2 column vol/h) with a linear gradient (10 column vol.) of 0–300 mM potassium phosphate, pH 7.0, in buffer D containing 850 mM NaCl. Active fractions, eluting at ~125 mM potassium phosphate, were pooled (fraction II, 11 400 000 U, 180 mg, 120 ml). Fraction II was then dialyzed twice, for 10 h each time, against buffer D (40 times the volume of fraction II); during this time a white precipitate formed. This precipitate, collected by centrifugation (Sorvall SS-34 rotor, 10 000 *g*, 30 min), was resuspended in buffer E containing 1 M NaCl (one third of the volume of fraction II) over a period of 30 min (fraction III, 7 500 000 U, 48 mg, 60 ml). Fraction III was then dialyzed against buffer E containing 150 mM NaCl until the conductivity was equivalent to this buffer and then clarified by centrifugation. In multiple runs fraction III (1.5 mg/cycle) was applied to a HR 5/5 MonoQ column (1 ml, flow rate 12 ml/h; Pharmacia) equilibrated in buffer E containing 150 mM NaCl. After washing with the same buffer (3 ml) a linear gradient (10 ml) of 150–500 mM NaCl in buffer E was applied. Active fractions (fraction IV, 6 000 000 U, 36 mg, 120 ml), which eluted at 350 mM NaCl, were pooled and judged to be >95% homogenous on a SDS–polyacrylamide gel (stained with Coomassie Blue R-250, Bio-Rad). Fraction IV was dialyzed extensively against buffer E containing 400 mM NaCl, frozen in liquid nitrogen and stored at –80°C. ClpX protein stored under these conditions had no detectable decline in specific activity after 3 months.

### Gel electrophoresis assay to detect disruption of the synaptic complex

STC1, formed from 0.25 μg donor substrate, was treated with ClpX protein (2.0 μg/ml) and $MRF\alpha_2$ (fraction IV, 335 U/ml) in reaction mixtures (30 μl) containing reaction buffer, 100 μg/ml creatine kinase, 20 mM creatine phosphate, 32 μg/ml rifampicin and 2 mM ATP. Incubation was at 37°C for 30 min. Where indicated glutaraldehyde was added to a final concentration of 0.1% (w/v) and the mixture was incubated for 15 min at room temperature. A one tenth volume of 1 M Tris–HCl, pH 8.0, and 1 M glycine was then added in order to quench unreacted glutaraldehyde, and the mixture was placed on ice for 5 min. *Pst*I (10 U) was then added and the mixture was incubated for 60 min at 37°C. Reactions were stopped by addition of EDTA to 15 mM and Triton X-100 to 1% (w/v). Nucleoprotein complexes were analyzed by electrophoresis on a neutral agarose gel (0.7%) and by immunoblotting as previously described (Nakai and Kruklitis, 1995).

### Isolation of nucleoprotein complexes by gel filtration

STC1 was formed in standard reaction mixtures (75 μl) in which pGG215 donor substrate, target substrate, MuA, MuB and HU were present at 2-fold higher concentrations. STC1 was challenged with heparin (2 mg/ml) and then purified from free proteins and nucleotides by filtration through a Bio-Gel A-15m column (1 ml) equilibrated in reaction buffer. DNA-containing fractions in the void volume (125 μl, equivalent to 1.5 μg donor DNA) were pooled. An aliquot of isolated STC1 (equivalent to 0.5 μg donor DNA) was treated with ClpX (2.4 μg/ml) or $MRF\alpha_2$ (400 U/ml) under the reaction conditions described above for disruption of the synaptic complex (50 μl reaction mixture). The resulting complexes were isolated by gel filtration (50 μl, equivalent to 0.25 μg donor DNA) and then assayed in the Mu DNA replication system. Alternatively, Western blot analysis was performed to determine the protein content of the isolated complex.

STC2 and STC3 were formed from STC1 (equivalent to 2.0 μg donor DNA) by treatment with 4.0 μg/ml ClpX or 4.0 μg/ml ClpX and 670 U/ml $MRF\alpha_2$ (fraction IV), respectively (120 μl reaction mixture, reaction conditions as described above for the disruption of the synaptic complex). STC2 and STC3 were then isolated by gel filtration (150 μl, equivalent to 1.5 μg donor DNA).

### Analysis of oligomeric MuA structure

STC1 was formed in reaction mixtures (25 μl) in which potassium glutamate was replaced with 60 mM KCl. In addition, pGG215, target DNA, MuA and HU were present at 5-fold higher concentrations and MuB was present at a 10-fold higher concentration. Where indicated STC1 was incubated with 7.5 μg/ml ClpX protein and 1250 U/ml $MRF\alpha_2$ in 40 μl reaction mixtures which contained buffer F, 5 mM DTT, 50 μg/ml BSA, 32 μg/ml rifampicin, 100 μg/ml creatine kinase, 20 mM creatine phosphate and 2 mM ATP. Incubation was at 37°C for 30 min. The resulting nucleoprotein complexes were isolated by filtration through a Bio-Gel A-15m column equilibrated in buffer F.

Proteins were treated for 30 min at room temperature in reaction mixtures (26 μl) containing buffer F and 200 μg/ml DSS (from 5 mg/ml stock solution in dimethylsulfoxide), using nucleoprotein complexes (equivalent to 0.25 μg donor DNA) isolated by gel filtration or 5 μl aliquots of the above strand transfer reaction mixture. Excess DSS was quenched by addition of Tris–HCl, pH 8.0, and glycine to 25 mM each. Proteins treated with DSS were denatured by heating in 2% (w/v) SDS (65°C for 15 min) and were resolved on a SDS–agarose–acrylamide composite gel (Kiehm and Ji, 1977) as described by Lavoie *et al.* (1991). MuA was detected on immunoblots and the positions of the MuA oligomers were assigned relative to catalase (232 kDa), myosin (210 kDa), phosphorylase b (97 kDa) and BSA (69 kDa).

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