Disassembly of the Mu transposase tetramer by the ClpX chaperone

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Mu transposition is promoted by an extremely stable complex containing a tetramer of the transposase (MuA) bound to the recombining DNA. Here we purify the *Escherichia coli* ClpX protein, a member of a family of multimeric ATPases present in prokaryotes and eukaryotes (the Clp family), on the basis of its ability to remove the MuA from the DNA after recombination. Previously, ClpX has been shown to function with the ClpP peptidase in protein turnover. However, neither ClpP nor any other protease is required for disassembly of the transposase. The released MuA is not modified extensively, degraded, or irreversibly denatured, and is able to perform another round of disassembly in vitro. We conclude that ClpX catalyzes the ATP-dependent release of MuA by promoting a transient conformational change in the protein and, therefore, can be considered a molecular chaperone. ClpX is important at the transition between the recombination and DNA replication steps of transposition in vitro; this function probably corresponds to the essential contribution of ClpX for Mu growth. Deletion analysis reveals that the sequence at the carboxyl terminus of MuA is important for disassembly by ClpX and can target MuA for degradation by ClpXP in vitro. These data contribute to the emerging picture that members of the Clp family are chaperones specifically suited for disaggregating proteins and are able to function with or without a collaborating protease.

[Key Words: Clp; Hsp104; transposition; phage Mu; replication]

Received July 17, 1995; revised version accepted August 22, 1995.

Higher order protein–DNA complexes are often critical intermediates in initiation of transcription, recombination, and replication. Timely assembly and disassembly of these complexes is likely to be essential for the proper function and regulation of these processes. The protein–DNA complexes involved in site-specific recombination and transposition are among the best understood and, therefore, useful for dissecting the general principles governing the assembly, organization, and disassembly of such complexes.

Biochemical analysis of transposition by elements as diverse as phage Mu, Tn10, Tn7, and human immunodeficiency virus [HIV] indicate that the transposase and integrase proteins act by a similar mechanism (for review, see Mizuuchi 1992) and function in stable multimeric complexes (Surette et al. 1987; Haniford et al. 1991; Bainton et al. 1993; Ellison and Brown 1994). Although Mu transposase and HIV integrase have only a modest degree of amino acid sequence similarity [Baker and Luo 1994], recent determination of the structures of the core domains of these proteins reveal remarkable similarities surrounding the active sites [Dyda et al. 1994; Rice and Mizuuchi 1995].

Mu transposase [MuA] catalyzes the DNA cleavage and joining reactions central to recombination. MuA is monomeric in solution but forms a stable tetramer upon binding to specific sequences at each end of the phage genome. This tetramer pairs the two ends of the Mu DNA, cleaves these ends, and joins the cleaved ends to a new DNA site in a reaction called strand transfer (Craigie and Mizuuchi 1987; Surette et al. 1987, 1991; Lavoie et al. 1991; Mizuuchi et al. 1992). A second Mu-encoded transposition protein, MuB, participates in strand transfer by activating MuA and delivering an intermolecular target site to the transposase complex [Adzuma and Mizuuchi 1988; Baker et al. 1991; Surette and Chaconas 1991].

Transposition is used for two steps in the phage Mu life cycle: [1] integration of the Mu genome into that of the host cell during infection and [2] replicative amplification of the DNA during lytic growth. Although it is well established that phage Mu uses transposition to replicate its genome, how replication is initiated after the strand transfer reaction is not well understood. Mu replication in vivo requires several essential *Escherichia coli* genes that encode components of the host replication machinery, indicating that Mu replication forks are similar to those used for chromosomal replication [Tousaint and Faelen 1974; Toussaint and Resibois 1983; Resibois et al. 1984; Ross et al. 1986]. Strand transfer complexes [STC] can be replicated efficiently in *E. coli* extracts made from uninfected cells indicating that no Mu proteins other than MuA and MuB are required [Mizuuchi 1983], although the Mu arm functions stimulate...
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replication in vivo (Waggoner et al. 1981). Recently, in vitro replication with a more purified system confirmed roles for eight of the E. coli replication proteins and indicated that additional host factors are required (Kruklitis and Nakai 1994).

In addition to the replication enzymes, the E. coli clpX gene product is required for lytic growth of phage Mu (Mhammedi-Alaoui et al. 1994). The kinetics of the block in phage growth after induction of a lysogen indicate that ClpX functions after the first strand transfer reaction but before onset of extensive replication (Mhammedi-Alaoui et al. 1994). ClpX is a member of a conserved family of ATPases (the Clp family) present in prokaryotes and eukaryotes (Gottesman et al. 1990, 1993). Many organisms, including E. coli, have multiple family members, many of which are heat shock proteins. The best studied Clp protein, E. coli ClpA, forms a complex with the ClpP protein, a small serine protease (unrelated in sequence to the other Clp proteins) to promote ATP-dependent degradation of specific proteins (Hwang et al. 1988; Katayama et al. 1988). ClpX was first purified based on its ability to degrade, with the ClpP protease, the replication initiator protein of phage λ, λO protein (Wojtkowiak et al. 1993). However, although Mu growth requires ClpX and some forms of the Mu repressor appear to be degraded by ClpXP (Geuskens et al. 1992), Mu propagates relatively normally in clpP-deficient cells (Mhammedi-Alaoui et al. 1994), indicating that the essential role of ClpX in Mu growth does not involve protein degradation by ClpXP.

In this study, we purify a factor from E. coli cell extracts on the basis of its ability to displace the MuA tetramer from the DNA after strand transfer. This factor is the ClpX protein. ClpX catalyzes the ATP-dependent disassembly of the MuA tetramer into monomers without detectable degradation and the released protein is active in another round of recombination. The importance of ClpX for Mu replication in vitro is also demonstrated. Implications of these data for the pathway of Mu replication and the mechanism of action of the Clp protein family are discussed.

Results

Purification of ClpX protein as a factor able to release MuA from the STC

Upon completion of the cleavage and strand transfer reactions in vitro, MuA remains bound to the recombined DNA in a protein–DNA complex called the STC or type II transposome. Although noncovalent, this complex is stable for days and resists treatment with 6 M urea and heating to 65°C (Surette et al. 1987). Factors able to remove MuA from the STC were detected in crude E. coli extracts active in Mu DNA replication (data not shown). One assay used to detect removal of MuA from the DNA depended on the difference in mobility between the STC and strand transfer products on a native agarose gel. The STC migrates as a single broad band that disappears with removal of MuA to give topoisomers of the DNA joined by strand transfer. The releasing activity in the extract was fractionated by chromatography, one activity behaved as a single component and was purified extensively (see Materials and methods). The peak of releasing activity correlated with a 46-kD protein during several chromatography steps; the activity of fractions from the penultimate column (Mono Q) is shown (Fig. 1a,b). After Superose 6 chromatography, this 46-kD protein was >90% pure (Fig. 1a).

Several lines of evidence established that the 46-kD protein was the ClpX protein. The protein (1) reacted efficiently with anti-ClpX antibody on a Western blot (data not shown); (2) supported degradation of a known substrate of the ClpXP protease in the presence of ClpP and ATP (see below); and (3) was more abundant in extracts made from cells overproducing ClpX than from nonoverproducing cells. Active fractions of ClpX purified from overproducing cells were >95% pure as judged by densitometry of a Coomassie Blue-stained SDS gel (Fig. 1c). Release of MuA from the STC by purified ClpX was relatively efficient; in a reaction containing 1.3 pmoles of MuA, 1.7 to 3.3 pmoles of ClpX gave rise to protein-free product DNA within 5 min (data not shown).

The ATP requirement for release of MuA from the STC by ClpX was investigated by modifying the reaction

\[ \text{ClpX} \to \text{MuA} \]

(a) SDS-PAGE of ClpX fractions after Mono Q (left) and Superose 6 (right) chromatography. Arrow indicates ClpX protein. Pre-stained protein markers are myosin (h-chain) 214 kD, phosphorylase B 111 kD, BSA 74 kD, ovalbumin 45 kD, and carbonic anhydrase 29 kD. (b) MuA releasing activity of Mono Q fractions of ClpX (5 μl of each fraction [0.2–1.0 μg of total protein] was added) assayed by agarose gel electrophoresis, arrow indicates STC, bracket indicates topoisomers of free strand transfer DNA. The protein–DNA complexes and free DNA products were visualized by staining of the gel with ethidium bromide (EtBr); a photograph of the negative is shown. (c) SDS-PAGE of ClpX purified from overexpressing cells.

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conditions. Strand transfer normally involves MuB protein as well as MuA. MuB is an ATP-dependent nonspecific DNA-binding protein that targets strand transfer to intermolecular sites [Adzuma and Mizuuchi 1988; Baker et al. 1991]. When MuA promotes strand transfer without MuB and ATP, recombination occurs using target sites within the donor plasmid (intramolecular strand transfer). ClpX disassembled efficiently the intramolecular STC formed in the absence of MuB [Fig. 2]. Removal of MuB from the reaction renders strand transfer insensitive to ATP allowing the role of ATP in ClpX-mediated disassembly to be analyzed. ATP was necessary for detectable disassembly of the STC by ClpX; ATP[γS] did not support the reaction, indicating that ATP hydrolysis is probably essential [Fig. 2].

**ClpX disassembles the MuA tetramer without degradation**

ClpX was purified previously for its ability direct the ClpP protease to degrade λO protein [Wojtkowiak et al. 1993]. In contrast, disassembly of MuA from the STC required ClpX but not ClpP. After gel filtration on Superose 6, ClpX fractions active in MuA release were free from ClpP by Western blot analysis [data not shown]. Furthermore, these fractions were unable to degrade λO protein without addition of exogenous ClpP, confirming the absence of an enzymatic contamination [Fig. 3]. Thus, degradation of MuA by ClpXP does not appear to be the mechanism of disassembly of the STC.

To investigate the physical state of the released MuA, STCs were purified away from monomeric MuA by gel filtration, treated with ClpX, and the products of the releasing assay were sedimented in a glycerol gradient [Fig. 4a]. MuA sedimented with the DNA [fractions 4—9] when either ClpX or ATP was omitted from the releasing reaction. However, when ClpX and ATP were present, MuA remained at the top of the gradient in the position of the monomeric MuA marker [fractions 1,2]. These fractions contained MuA that appeared to be full length [75 kD] by SDS gel electrophoresis [Fig. 4a]. Quantitative Western blot analysis confirmed that the MuA released was not degraded even after prolonged incubation with ClpX [data not shown]. Disassembly was also monitored by protein cross-linking, which revealed that monomeric MuA was the form released from the STC [data not shown].

**MuA released by ClpX is active**

To investigate further the mechanism of ClpX action, the ability of the released MuA to catalyze another recombination reaction was tested. The released MuA [fraction 1 from a glycerol gradient analogous to that in Fig. 4a] was incubated with 32P-labeled oligonucleotides carrying the Mu right end sequence [which served as the donor DNA] and an unlabeled target DNA [pX174 replicative form 1 (RFI)] [Fig. 4b]. The released MuA produced labeled strand transfer products efficiently; the specific activity of the protein was comparable to that of transposase that had never been reacted with ClpX [Fig. 4b]. This strand transfer reaction demands that MuA is able to bind specifically to DNA, form the tetramer, catalyze strand transfer, and interact with MuB protein. Clearly the MuA released by ClpX must be folded properly. Therefore, these data argue that MuA is not extensively modified, degraded, or irreversibly denatured as a result of being disassembled by ClpX. Thus, ClpX appears to release MuA from the STC by promoting a transient conformational change in the protein.

**ClpX is important for Mu replication in vitro**

Mu replicates by transposition; DNA synthesis on the Mu portion of the DNA covalently joined by strand transfer generates a cointegrate containing the new and old target sequences and two copies of the phage DNA. Mu DNA replicated poorly in an extract made from ClpX-deficient cells [Fig. 5a]. This extract replicated a plasmid carrying the E. coli chromosomal origin [oriC] as
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**Figure 4.** ClpX releases active MuA protein. (a) Analysis of released MuA by glycerol gradient sedimentation. (Top) Distribution of MuA in gradient fractions when STC was incubated without ClpX; (middle) distribution of MuA in gradient fractions after incubation of the STC with ClpX and ATP; (bottom) migration of DNA from the top [■] and middle [△] panels and free MuA protein. ([b]) Agarose gel of strand transfer products made in reactions with different levels of released MuA. The leftmost set of reactions contained released MuA, fraction 1 from glycerol gradient analogous to that shown in a; the concentration of MuA in this fraction was estimated to be 1 ng/μl. The control for the released MuA (middle set of lanes) used the same volume of material from glycerol gradient fractions in which the STC had not been treated with ClpX (as in a top). The rightmost set of reactions contain MuA that had not been used in a previous reaction. Arrows indicate donor DNA, and the strand transfer products that result from joining of one [single-end strand transfer] or two [pair-wise strand transfer] of the donor oligonucleotides into the target DNA.

**Figure 5.** ClpX rescues the replication defect of ClpX-deficient extracts. (a) Replication in vitro of the a mini-Mu plasmid and a plasmid containing the E. coli chromosomal origin [oriC] in extracts prepared from C600 [clpX+] and pSG22101 cells [clpX−] cells. (b) Replication of mini-Mu DNA in ClpX− extract; the STCs in the samples on the right were treated with purified ClpX before initiation of the replication reaction, whereas those on the left were not treated with ClpX. Replication was for the times as indicated. DNA products were cleaved with Hpal before electrophoresis. Arrows mark positions of the mini-Mu plasmid [donor] and the products of replicative transposition [cointegrate].

well as the control [clpX+] extract, indicating that the defect was specific for a factor required for Mu replication. Mu replication in the extract was restored by treatment of the STCs with purified ClpX [Fig. 5b]. Thus, ClpX is important for Mu-specific DNA replication in vitro. A defect in Mu DNA replication in fractions from clpX− cells has been reported by Nakai and Kruklitis (1995); they also find that efficient replication is restored by addition of ClpX protein to the in vitro reaction [H. Nakai, pers. comm.].

The carboxyl terminus of MuA is required for disassembly by ClpX and degradation by ClpXP

The carboxy-terminal domain of MuA has been shown previously to be required for MuA–MuB interactions in vitro (Baker et al. 1991; Wu and Chaconas 1994). However, tetramers that contain a mixture of wild-type MuA and a deletion derivative lacking 47 amino acids from
the carboxyl terminus retain the ability to interact with MuB protein but are not efficient substrates for initiation of Mu DNA replication in vitro (Nakai and Kruklitis 1995). Therefore, we investigated the ability of derivatives of MuA carrying deletions of the carboxyl-terminus to be disassembled by ClpX.

ClpX was unable to disassemble STCs containing a MuA protein missing the last 48 amino acids (Fig. 6a) [MuA1-615; described in Baker et al. 1991]. In contrast, the STCs made with a MuA derivative lacking 76 amino acids from the amino terminus were disassembled efficiently by ClpX (data not shown). To define more precisely the region of the carboxyl terminus required for disassembly by ClpX, smaller carboxy-terminal deletions were constructed by moving the truncations made directly by ClpX. The carboxy-terminal domain of MuA is important for disassembly and may be recognized directly by ClpX. The carboxy-terminal domain of MuA is required for replicative transposition in vivo, but is not essential for the nonreplicative transposition that occurs during infection (Betermier et al. 1989; Desmet et al. 1989); whether the requirement for this domain during replicative transposition reflects the need for MuA–MuB interactions or MuA–ClpX interactions, or both, is not yet clear.

Figure 6. Carboxy-terminal sequence of MuA is required for disassembly by ClpX and for degradation by ClpXP. (a) Strand transfer complexes (STC) were made with wild-type MuA and with carboxy-terminal deletion variants lacking 4 (Δ4), 8 (Δ8), and 48 (Δ48) amino acids. The STC, CDC and free product DNAs are indicated by arrows. (b) Western blot analysis of wild-type MuA and Δ4, Δ8, and Δ48 MuA variants incubated with ClpXP for 0, 30, and 60 min at 30 °C.

The last 8 amino acids at the carboxyl terminus of MuA are QNRRKKAI (Harshey et al. 1995). This sequence appeared similar to the carboxy-terminal amino acid sequence of λ protein (Sanger et al. 1982), P1 Phd protein (Lehnherr et al. 1993; Lehnherr and Yarmolinsky 1995), and the virulent derivatives of the Mu repressor (Geuskens et al. 1992; Laachouch et al. 1995); these proteins are all known or suspected substrates of the ClpXP protease. Therefore, the ability of ClpXP to degrade MuA and the carboxy-terminal deletion derivatives was investigated. Purified ClpP protein was added to reactions containing monomeric MuA, ClpX, and ATP. Under these conditions, the full-length MuA monomer was degraded by ClpXP, the variants lacking 4 or 8 amino acids were not degraded efficiently, and the protein with the 48-amino-acid deletion was refractory to degradation [Fig. 6b]. These data suggest that the carboxy-terminal sequence of MuA is recognized by ClpX during disassembly and this recognition can result in ClpX-dependent degradation by ClpP in vitro.

Discussion

ClpX functions as a molecular chaperone to disassemble the MuA tetramer.

The E. coli ClpX protein catalyzes disassembly of the MuA tetramer–DNA complex, the active form of MuA responsible for transpositional recombination. Disassembly (1) depends on ATP, and probably on ATP hydrolysis; (2) does not require the ClpP protease; (3) occurs without degradation; and (4) releases active, monomeric MuA. Clearly, ClpX must recognize MuA and promote a transient conformational change in the protein to mediate disassembly. Therefore, we conclude that ClpX functions as a molecular chaperone during Mu transposition in vitro.

Extracts made from ClpX-defective cells replicate Mu DNA poorly but maintain the ability to replicate a plasmid carrying the E. coli chromosomal origin. Therefore, these extracts must contain the essential E. coli replication fork proteins that are responsible for Mu DNA replication as well. Treatment of STCs with purified ClpX before their introduction into the defective replication extract restores Mu replication. Thus, ClpX is important for Mu replication in vitro. In vivo, Mu replication is blocked by mutations in the clpX gene but proceeds normally in clpP-deficient cells, leading to the suggestion that ClpX might act alone during Mu replication as a chaperone or that ClpX may function with a protein other than ClpP [Mhammedi-Alaoui et al. 1994]. These data, taken together with the results presented here that ClpX recognizes MuA directly and disassembles the STC, strongly support the hypothesis that the chaperone activity promoted by ClpX is the essential function of this protein during phage replication.

It has been reported previously that MuA prevents initiation of Mu replication by purified host replication proteins in the absence of additional unidentified host factors [Kruklitis and Nakai 1994; Nakai and Kruklitis 1995]; ClpX is very likely one of these additional factors.
The details of when ClpX functions with respect to other events necessary for replication initiation are not yet clear; however, both in vivo and in vitro analyses now place its role at the transition between recombination and assembly of the replication forks at the newly formed Mu DNA–host DNA junctions. The role of MuB protein in Mu replication also needs to be clarified. The requirement of MuB for Mu replication in vivo and in vitro has been reported (Mizuuchi 1983; Chaconas et al. 1985), but in a reconstituted replication reaction MuB was not found to be required (Kruklitis and Nakai 1994). It is now apparent that ClpX and MuB recognize overlapping regions at the carboxyl terminus of MuA, suggesting that competition between MuB and ClpX for this region of MuA may be important during the transposition pathway. Preliminary results indicate that, in vitro, high levels of MuB protein antagonize the MuA-releasing activity of ClpX (data not shown).

The Clp protein family as chaperones and mediators of proteolysis.

The mechanism by which ClpX disassembles the MuA tetramer and the way it targets proteins for degradation by ClpP are probably closely related. Although we purified ClpX on the basis of assays that require only the chaperone activity, and the chaperone activity appears to be its essential function, MuA is also a substrate for degradation by ClpXP in vitro. Both chaperone activity and degradation require ATP and are sensitive to the specific amino acid sequence at the carboxyl terminus of MuA. As disassembly must involve recognition of MuA by ClpX and an ATP hydrolysis-dependent conformational change in MuA to destabilize the stable tetramer, it is reasonable to conclude that during proteolysis by ClpXP, ClpX recognizes the substrate and promotes a conformational change in the target protein to render it susceptible to peptide bond hydrolysis by the active sites in ClpP. In this regard, it is interesting that the MuA released from the STC is active, and therefore, must be folded properly. As MuA is a large, multidomain protein that does not refold spontaneously and efficiently in vitro (data not shown), these data suggest either that ClpX does not “globally” unfold MuA during disassembly or that after disassembly, ClpX promotes the accurate refolding of MuA.

Although ClpXP can degrade MuA in vitro, ClpP is not essential for Mu growth and replication. It is difficult to tell if MuA is normally degraded by ClpXP. In vivo, both physical and functional assays demonstrate that MuA is unstable (Pato and Reich 1982, 1984; Gama et al. 1990). Mutations in hflA and hflB stabilize MuA (Gama et al. 1990), whereas a clpX mutation does not have a substantial effect (Mhammedi-Alaoui et al. 1994), indicating that the Hfl proteases, rather than ClpXP, are principally responsible for its degradation. However, it is possible that the pool of MuA in the STCs is usually degraded by ClpXP, this would explain why MuA is still functionally unstable in hfl defective host cells (Gama et al. 1990).

The possibility that the Clp ATPases activate proteolysis by ClpP by catalyzing a chaperone-like function was proposed by Squires and Squires (1992) (see also Gottesman and Maurizi 1992; Craig et al. 1994) and this conclusion was strongly supported by the discovery that ClpA can function as a chaperone in vitro (Wickner et al. 1994). ClpA, like ClpX, was purified for its ability to promote degradation in concert with ClpP (Hwang et al. 1988; Katayama et al. 1988). The chaperone activity was uncovered subsequently by the observation that ClpA, acting alone, could substitute in vitro for the Hsp70 chaperone, DnaK, and its co-chaperone DnaJ, in activating the replication initiator protein of phage P1 by converting inactive dimers into active monomers (Wickner et al. 1994). While this study on ClpX was nearing completion, a publication appeared demonstrating that ClpX disaggregates heat-denatured λO protein in vitro and exhibits λO protein-stimulated ATPase activity (Wawrynow et al. 1995). In agreement with our characterization of the mechanism by which ClpX disassembles the MuA tetramer, this study concluded that ClpX has the activities normally attributed to a molecular chaperone (Wawrynow et al. 1995). Thus, both of the Clp ATPases known to function with the ClpP protease have intrinsic chaperone activity in vitro, ClpX appears to function as a chaperone in vivo as well [see discussion above].

Discovery that ClpA and ClpX are chaperones underscores that the unifying activity of the Clp ATPase family is their chaperone activity rather than their ability to promote proteolysis. There are several members of the Clp family for which a direct role in proteolysis has not been indicated. A Clp homolog from Saccharomyces cerevisiae, Hsp104, appears to take apart protein aggregates that arise during exposure of cells to high temperature, but does not appear to promote their degradation (Parsell et al. 1991). The closest E. coli homolog of Hsp104 is ClpB; ClpB, like Hsp104, is a heat shock protein essential for cell growth at high temperature (Kitagawa et al. 1991; Squires et al. 1991). ClpB has protein-stimulated ATPase activity, but has not been shown to function as an activator of degradation by ClpP (Woo et al. 1992). Thus, the Clp family members appear to be chaperones specialized for protein disassembly. Whether the end result of this chaperone activity is a disassembled or degraded version of the substrate protein probably depends on several factors, including the substrate protein, the Clp family member catalyzing the reaction, and the availability of a collaborating protease.

Electron microscopy reveals a striking similarity between the ClpAP protease and proteasome complex of eukaryotes and archaeabacteria (Kessel et al. 1995; for a review of proteasome, see Peters 1994; Lowe et al. 1995). Both protein complexes form a multimeric ring-shaped structure. Like ClpAP, the 26S proteasome complex consists of a proteolytic core particle (20S proteasome) and a ATPase-containing complex (19S cap complex). Degradation of ubiquitinated proteins by 20S proteasome is probably coupled to a chaperone-like activity of the 19S complex. Thus, the coupling of chaperone-catalyzed protein unfolding and proteolytic degradation appears reca-
ClpX disassembles the Mu transposase

MuA release reactions
Mu-releasing reactions were performed as follows. Mu strand transfer reactions were supplemented by 8 mM ATP, 40 mM creatine phosphate, and 0.1 mg/ml creatine kinase; 2.5–5.0 pmoles of ClpX protein (assuming a native molecular weight of a tetramer) was added to start reaction. Creatine phosphate and creatine kinase were omitted when reactions contained ATP[$\gamma$S] (5 mM). After incubation at 30°C for 60 min portions of the samples were analyzed by gel electrophoresis as described (Baker et al. 1993), except the gel and electrophoresis buffer contained 10 μg/ml of heparin and 80 μg/ml of BSA.

Materials and methods

DNA
ClpX gene was amplified by PCR from *E. coli* (strain C600, New England Biolabs) chromosomal DNA, using primers designed based on the published sequence of (Gottesman et al. 1993): TB189: GGAAATCTGATCAGACATAAACGGCAAG; TB190: GCCGCCGATTCGCGCCGATTTCAACAGATGCTCTGTCCCTTC.

The clpX gene was cloned using the NdeI and BamHI sites of the expression vector PET-3a (Novagen) [plasmid PET-3a/ClpX]. MuA deletion proteins lacking 4 (Δ4) and 8 (Δ8) amino acids from the carboxyl terminus were PCR-amplified from plasmids pN85 and pN81 (Wu and Chaconas 1994), using primers: TB256: GGAGGCTCATGTCAGAGG and TB258: CCCGGCCGCGGCTGAATATCGCCGCCCGCGA-AAAACAC.

The resulting PCR fragments were cloned into pWZ170 (Wu and Chaconas 1994) a derivative of pMK591 (Baker et al. 1993). The Mu donor DNA used in all experiments was pSG1 (Baker and Luo 1994). ΔX174 RFI was [target DNA] purchased from GIBCO-BRL.

Proteins
MuA protein, Δ4, Δ8, and Δ48 [MuA1–615] were purified as described in Baker et al. [1993]. MuB and Hu proteins were the same fractions described previously (Baker et al. 1994). ClpP protein was purified from cells containing the overexpressing plasmid pAED4/ClpP as described (Thompson and Maurizi 1994). The ΔO protein was purified from cells containing the overexpressing plasmid pRLM73 as described (Roberts and McMacken 1983).

ClpX was purified from 325 grams of C600 cells based initially on the activity in a Mu-releasing reaction and later by following AO degradation [with the addition of ClpP protein] as well. Briefly, the method was as follows: fraction II, active in mini-Mu plasmid replication in vitro was chromatographed on a phosphocellulose P11 column (Whatman), equilibrated with buffer A [50 mM Tris HCl (pH 7.2), 10% glycerol, 2 mM DTT, 0.1 mM EDTA] with 50 mM KCl. Flowthrough fraction active in Mu-releasing reaction was loaded directly onto the DEAE cellulose column equilibrated with buffer used for phosphocellulose. Bound proteins were eluted directly with gradient of KCl from 50 to 400 mM. Fractions eluted at 250–300 mM KCl, active in Mu-releasing reaction, were directly loaded onto hydroxyapatite column (fraction I), equilibrated with buffer A. Mu-releasing activity was eluted by gradient of potassium phosphate [pH 7.2] from 0 to 300 mM and was purified further by chromatography on Affi-Gel Blue (eluted by KCl gradient from 100 to 800 mM). Heparin-Sepharose [eluted by KCl gradient from 0 to 250 mM], hydroxyapatite [fraction II] [gradient from 10 to 250 mM potassium phosphate [pH 7.0]] Mono Q and Superose 6 (at conditions described below). After Superose 6 ClpX was >90% pure as determined by SDS-PAGE stained with Coomassie Blue.

ClpX protein was also purified from 20 grams of BL21 (DE3)pLysS cells containing overexpressing plasmid PET-3a/ClpX. Cell lysis was as described (Baker et al. 1993). Lysate centrifuged at 20,000 rpm for 40 min (SS34 rotor, Sorvall). Clear lysate was dialysed overnight against buffer B [25 mM Tris HCl (pH 7.5), 1% glycerol, 2 mM DTT, 0.1 mM EDTA], containing 50 mM KCl. Dialysed material was loaded on Q-Sepharose Fast Flow column [Pharmacia], equilibrated with buffer B and 50 mM of KCl. Proteins were eluted by gradient of KCl from 50 to 400 mM. Fractions, containing ClpX as determined by SDS-PAGE, were pooled and dialysed overnight against buffer B with 10 mM MgCl2. Dialysed material was loaded onto a heparin-Sepharose [Pharmacia] equilibrated with the same buffer. Proteins were eluted by KCl gradient from 0 to 250 mM. Fractions containing ClpX were pooled. ClpX was purified further by chromatography on Mono Q (PC 1.6/5) (elution by 50–400 mM KCl gradient) and gel filtration on Superose 6 (PC 3.2/30) [using buffer B with 150 mM KCl]. After Superose 6 ClpX was >95% pure as determined by scanning of Coomassie Blue-stained SDS-PAGE.

Transposition reactions
Strand transfer reactions were performed as described previously (Baker et al. 1993). Reaction mixtures (25 μl) contained 25 mM HEPES/KOH [pH 7.8], 156 mM NaCl, 10 mM MgCl2, 2 mM ATP, 15% glycerol, 10 μg/ml of mini-Mu donor DNA [pSc1,] and 10 μg/ml of ΔX174 DNA. The protein levels were HU, 3 pmoles; MuB, 6.5 pmol; MuA [Δ4, Δ8, or Δ48 MuA] (0.0014 pmole) were performed as described (Savilaitå et al. 1995).
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Glycerol gradient sedimentation of released MuA

Intramolecular STCs were assembled as described above. Unre­acted MuA protein was removed as described (Baker et al. 1993) and the purified STCs were incubated with ClpX protein as described above. After completion of the reaction, NaCl was added to 300 mM and samples (50 |xL) were loaded onto 1.1-mL glycerol gradients [14%–20%, with an 80% shelf containing 25 mM HEPES/KOH [pH 7.8], 300 mM NaCl, 2 mM DTT, and 0.1 mM EDTA]. Gradients were run for 90 min at 55,000 rpm [4°C] using a TLS-55 rotor and fractionated by collecting 90- |xL samples from the top of the gradient. The distribution of DNA in the gradient fractions was determined by gel electrophoresis and quantitated using the gel print 2000i (Bio Photonics). The presence of MuA was determined by Western blot analysis. The concentration of released MuA was estimated by scanning of Western blots, developed with 125I-labeled protein A on Phosphorlmager 445 SI.

Protein degradation by ClpXP

The XO degradation conditions were as described (Wojtkowiak et al. 1993). Protein levels were as follows: 100 ng of XO protein, 0.5 mg of ClpX protein, and 1.0 mg of ClpP protein [fraction from DEAE—cellulose (Wojtkowiak et al. 1993)]. The reaction mixture (25 |xL) was incubated for 60 min at 30°C. Degradation of XO was monitored by Western blot analysis. MuA degrada­tion conditions were as follows: reactions (20 |xL) contained 25 mM of HEPES/KOH (pH 7.8), 156 mM NaCl, 10 mM MgCl2, 10 mM ATP, 15% glycerol, 200 ng of ClpX, 100 ng of ClpP, and 1 |xg of MuA. Reactions were incubated at 30°F. Samples were analyzed by Western blot, using anti MuA antibody and 125I-labeled protein A. Blots were scanned on Phosphorlmager 445 SI.

In vitro replication reactions

Replication extracts [fraction II] were prepared as described [Fuller et al. 1981] and in vitro replication of pBSoriC and the mini-Mu DNA was performed as described previously [Fuller et al. 1981, Mizuuchi et al. 1983; Krukli­ts and Nakai 1994]. Reaction mixture contained 25 mM of HEPES/KOH (pH 7.9), 40 mM of KCl, 10 mM MgCl2, 50 mg/ml of BSA, 4% polyethylene glycol 8000, 2 mM of ATP, 40 mM each of dATP, dTTP, dCTP, dGTP, 40 mM of creatine phosphate, 0.1 mg/ml of creatine kinase, 30 |xL of rifampicin, [3H]TTTP or [32P]dATP [for a final specific activity of 1000 cpm/|xg]) and 0.5 |xg of pBSoriC or STC. The reaction mixtures were incubated at 30°C for 60 min. The replication activity for oriC template was measured by scintilla­tion counting of TCA-perceptible DNA. One hundred percent of replication activity for pBSoriC plasmid is equal to 96.8 pmoles of dTTP incorporated under standard conditions. The replication activity for mini-Mu template was measured by scanning of dried agarose gels, containing 32P-labeled DNA replication products on Phosphorlmager 445 SI. For enzymatic manipulations 32P-labeled DNA replication products were purified as described (Krukli­ts and Nakai 1994). Samples were digested with HpaI and analyzed by gel electrophoresis. Dried gels were scanned on Phosphorlmager 445 SI.

Acknowledgments

We wish to thank Susan Gottesman for gifts of strains and ant­ibodies against ClpX and ClpP; Roger McMacken for antibody to XO protein; Ross Inman for a strain overproducing LXO; Arthur Horwich for the strain overproducing ClpP; Zhen­guo Wu and George Chaconas for plasmids encoding deletions of do­main III of MuA; and Hiroshi Nakai for communicating results

before publication. Steve Bell, Alan Grossman, Patrick Waller, Michael Maurizi and members of our laboratory provided help­ful comments on the manuscript. T.A.B. is a recipient of an Na­tional Science Foundation Young Investigator Award.

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*Genes Dev.* 1995, 9:
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