The molecular chaperone ClpX of *Escherichia coli* plays two distinct functions for bacteriophage Mu DNA replication by transposition. As specificity component of a chaperone-linked protease, it recognizes the Mu immunity repressor for degradation by the peptidase component ClpP, thus derepressing Mu transposition functions. After strand exchange has been promoted by MuA transposase, ClpX alone can alter the conformation of the transpososome (the complex of MuA with Mu ends), and the remodeled MuA promotes transition to replisome assembly. Although ClpXP can degrade MuA, the presence of both ClpP and ClpX in the reconstituted transposition system did not destroy MuA essential for initiation of DNA replication by specific host replication enzymes. Levels of ClpXP needed to overcome inhibition by the repressor did not prevent MuA from promoting strand transfer, and ClpP stimulated alteration of the transpososome by ClpX. Apparently intact MuA was still present in the resulting transpososome, promoting initiation of Mu DNA replication by specific replication enzymes. The results indicate that ClpXP can discriminate repressor and MuA in the transpososome as substrates of the protease or the molecular chaperone alone, degrading repressor while remodeling MuA for its next critical function.

Chaperone-linked proteases employ the action of a protease component and a regulatory subunit with ATPase activity. In *Escherichia coli*, ClpA and ClpX proteins serve as regulatory subunits for the protease subunit ClpP (1–3). These regulatory subunits can by themselves act as molecular chaperones, breaking up aggregates or changing quaternary interactions of specific protein substrates (4, 5). The substrates of the molecular chaperone can become the targets of the chaperone-linked protease when the corresponding protease subunit is present (6). Thus, the molecular chaperones endow the protease with substrate specificity, most likely recognizing the substrate and presenting it to the protease subunit.

ClpP is synthesized as a protein of 207 amino acids (7), of which 14 amino acids at the N terminus are autocatalytically removed to yield the mature enzyme (8). The active protein consists of a tetradecamer, composed of two stacked heptameric rings (9, 10). The structure of ClpP is analogous to the 20 S proteasomes of eukaryotes and Archaeabacteria, with multiple active sites residing in the interior of the multimeric rings (10). Presumably, the molecular chaperones unfold the substrate and feed it into the proteolytic chamber of the ClpP tetradecamer, leading to the apparently processive degradation of the substrate (11).

ClpX is known to be involved in two distinct stages of the Mu life cycle. As part of the ClpXP protease, it can promote entry of a lysogen into lytic development by degrading the Mu immunity repressor (12, 13), which serves to shut down Mu transposition functions for the establishment and maintenance of lysogeny. ClpX also promotes initiation of Mu DNA replication, a process that does not require ClpP (14). It activates MuA’s function of promoting transition to DNA synthesis after MuA’s role in recombination has been completed (15).

During transposition, the MuA transposase binds to the Mu ends (16) and assembles into a tetramer (17), which catalyzes transfer of each Mu end to target DNA (18–20). This forms a branched DNA intermediate that contains a potential replication fork at each Mu end. Replication of Mu DNA by specific host enzymes completes transposition to form the cointegrate product (21–25), and MuA plays a critical role in this process. Upon completing Mu strand transfer, MuA remains very tightly bound to the Mu ends, still holding the Mu ends together in a nucleoprotein complex known as the type II transpososome (or the strand transfer complex 1, STC1) (19, 26). Removal of MuA by phenol extraction to create a deproteinized strand transfer product (STP) eliminates the requirement *in vitro* for specific host replication proteins implicated in Mu replication *in vivo* (25, 27), and if MuA in STC1 is damaged by partial proteolysis, replication cannot be initiated (25).

ClpX remodels MuA in STC1 to form STC2, destabilizing MuA’s tight grip on DNA without removing it from the nucleoprotein complex and activating MuA’s potential to promote transition to a replisome (15). Yet unidentified factors (Mu replication factor (MRF) α2) displace MuA in STC2 to form a new nucleoprotein complex STC3 (15), which permits initiation of DNA replication only by specific replication proteins including the DNA polymerase (pol) III holoenzyme and constituents of the multiprotein priming apparatus known as the φX174-type primosome (27).

However, MuA is also a substrate for the ClpXP protease (28) just as other known substrates of ClpX and ClpA as molecular chaperones are substrates of the corresponding chaperone-linked protease (reviewed in Gottesman et al. (29)). Therefore,
can ClpX in the presence of ClpP remodel MuA in STC1 without degrading it? Whether a protein substrate is degraded or remodelled may simply be determined by the availability of ClpP when the substrate is bound by the molecular chaperone. Such a model raises the question of how ClpX could perform the two distinct functions for Mu lytic development, first promoting degradation of repressor by ClpP and then remodeling MuA in STC1 for transition to replisome assembly. The first function requires ClpP but the second function could potentially be disrupted by the engagement of the ClpP proteolytic activity. ClpX would not be expected to act in isolation from ClpP in vivo to perform the second function, raising the question of what effect ClpP has on the DNA replication phase of Mu transposition. Here we report that ClpP does not interfere with MuA-mediated transition to DNA synthesis even when present in saturating amounts and in fact stimulates ClpX-promoted conversion of STC1 to STC2.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains, Plasmids, and Proteins—E. coli BL21(DE3) (30) and plasmid pET-19b were purchased from Novagen. Plasmid pWC9 (8) including the coding sequences for ClpP and ClpX was obtained from Susan Gottesman (National Institutes of Health). Plasmid pGQ215 (19) bearing a mini-Mu element was used for Mu transposition in vitro. Phage f1 RFI DNA and dX174 RFI DNA were used as target substrates. Purified preparations of MuA, MuB, Muc (Rep) (Rep), Muc (Taqi), Mut (Taqm), polyethylenimine, polyacrylamide, Ni-NTA resin, was purchased from Qiagen. ATP, ADP, MgCl2, ATP-sulfurylase, was purchased from Sigma. Bovine serum albumin was from Worthington. Ni-NTA (BA83, 0.5-μm pore size) was from Biocon, Inc. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemiluminescence reagent was purchased from BioGlow. The enhanced chemi...
agiarose gel (TAE electrophoresis buffer containing 0.5 μg/ml EtBr). DNA was stained with SYBR® Green I for detection by the Molecular Dynamics Storm 840 system, and bands corresponding to STC1 and the disrupted complex (STC2) were quantitated using ImageQuant software.

Other Methods—Glutaraldehyde fixation of nucleoprotein complexes, Western blot analysis, and isolation of nucleoprotein complexes by gel filtration were performed as described previously (15). Densestometric analysis of Western blots detected by enhanced chemiluminescence was performed using Scan Analysis™ software from Biosoft. ClpP protein concentrations were determined by the method of Bradford (36). N-terminal sequencing of purified ClpP protein was performed by Dr. A. Fowler (UCLA Protein Microsequencing Facility).

RESULTS

Overproduction of Histidine-tagged ClpP and Rapid Purification of the Mature Protein—In plasmid pCPX01 the clpP and clpX genes are under the control of the phage T7 ϕ10 promoter (Fig. 1A). ClpP protein is expressed with a polyhistidine tag fused to the N terminus (h-ClpP) for rapid purification on Ni-NTA agarose columns (see “Experimental Procedures” and Table I). Induction of BL21(DE3) harboring pCPX01 resulted in the overproduction of proteins of molecular mass of 45, 23, and 21 kDa, apparently corresponding to ClpX, h-ClpP, and the mature form of ClpP (Fig. 1B, lane 2). The overproduction of mature ClpP suggested that h-ClpP could be processed to the mature form just as the wild-type precursor ClpP (pre-ClpP) is autokatically processed by cleavage of 14 amino acids at the N terminus (8).

Both h-ClpP and mature ClpP were retained on the Ni-NTA column (Fig. 1C, lane 1). The removal of the precursor peptide is not required for ClpP oligomerization (37), and thus mature ClpP associated with h-ClpP should also be retained on Ni-NTA. A third polypeptide retained by the column corresponded to the molecular weight of pre-ClpP. The bound protein fraction from the Ni-NTA column was incubated at 37 °C for 3 h to promote further processing of the h-ClpP and then passed through the column a second time. Most of the protein passed through the column or was eluted at low (50 mM) imidazole concentrations. These protein fractions were pooled and were determined whether the presence of ClpXP protease allows the reconstituted Mu transposition system to overcome inhibition by the repressor.

Although the wild-type repressor (Rep) is a substrate for the ClpXP protease, it is degraded with a relatively high Km and assumes a ClpXP-resistant state in the presence of DNA (13). A mutant form of the repressor (Vir, repressor encoded by the virulent strain Muvir3060), which has an alteration at the C terminus and is rapidly degraded in vivo by the ClpXP protease (12, 14), is degraded by ClpXP with a 20-fold lower Km and is locked into the high affinity state even when bound to DNA (13). Both Rep and Vir are known to bind operator DNA with nearly equal affinity, Vir having a slightly higher dissociation constant (40). They both inhibited Mu strand transfer in the reconstituted system (Fig. 2, cf. lanes 6 and 10 with lane 2), but only inhibition by Vir could be eliminated by ClpXP (cf. lane 13 with 9). And even though MuA is a ClpXP substrate (28), amounts of ClpXP sufficient to remove Vir inhibition did not destroy MuA needed to catalyze strand transfer (Fig. 2, lanes 5 and 13). MuA is degraded slowly compared with Vir (13), permitting MuA to carry out its transposition function at levels of ClpXP that effectively eliminate the repressor.

ClpP Stimulates Action of ClpX on MuA Present in STC1—One method for examining the conversion of STC1 to STC2 is to examine disassembly of the transpososome by agarose gel electrophoresis (15). Treatment of STC1 with ClpX destabilizes tight binding of MuA to the DNA such that it readily disassembles during challenge with heparin and resolution by electrophoresis, and disruption of the synaptic complex of Mu ends results in a change in mobility of the strand transfer product. We used this disassembly assay to compare the kinetics with which ClpX acts on STC1 in the presence or absence of ClpP.

The presence of ClpP increased by 2–3-fold the amount of STC1 disassembled at 30 min by limiting concentrations of ClpX (Fig. 3A). The level of ClpP used was saturating; the
presence of 2-fold higher levels of ClpP did not result in any additional stimulation (data not shown). Time course experiments (Fig. 3, B and C) revealed that the extent of STC1 disassembly was approximately proportional to the amount of added ClpX and that ClpP increased the extent of STC1 disassembly at each ClpX concentration. Even in the presence of ClpP, however, an excess of ClpX monomers over STC1 was required for 100% disassembly, with at least 2.5 pmol of ClpX being required to disassemble 0.05 pmol of STC1 (formed from 250 ng of donor substrate in a reaction mixture containing 55 ng of MuA). If sufficient ClpX was present, ClpP was not essential for complete disassembly of STC1 (e.g. see Fig. 5, lane 7) as shown previously (15, 28).

**ClpXP Promotes Transition from STC1 to STC2, Leaving Activated MuA Molecules Bound to the Mu Ends**—The disassembly assay used to detect ClpX action above does not necessarily measure conversion of STC1 to STC2. STC2 includes activated MuA that remains bound to DNA and plays a critical role in the transition to DNA synthesis. The removal of that MuA from the DNA by artificial means such as phenol extraction eliminates the specific requirement *in vitro* for replication enzymes that are needed *in vivo*. For example, on the deproteinized STP, potential primers for leading strand synthesis at the Mu left and right ends can be extended by DNA pol I or Klenow fragment, whereas they cannot be extended by pol I in STC1, STC2, or STC3, even when all components of the Mu replication system except pol III holoenzyme are present (15, 27). We determined whether ClpX in the presence of ClpP removes MuA from STC1, leaving the ends of Mu accessible to DNA pol I, or converts STC1 to STC2 in which MuA remains bound to the ends.

Upon treatment of STC1 with ClpXP, the leading strand primers at the Mu left and right ends could not be appreciably extended by the Klenow fragment (Fig. 4B, lanes 5–7). This was true even after incubation for 60 min, twice the time required for 100% of STC1 to be disassembled by the gel electrophoresis assay (Fig. 3B). Identical results were obtained with STC1 treated with ClpX alone (Fig. 4B, lanes 2–4). Although a low level of nucleotide incorporation was detected after prolonged incubation of ClpXP-treated STC with Klenow, this was not associated with any measurable extension of the leading strand primers. Thus, only a limited amount of leading strand synthesis if any could have been catalyzed, and most of the incorporation was due to nonspecific DNA synthesis such as the alternating action of 3′ to 5′ exonuclease and polymerase activities of Klenow at 3′-hydroxyl ends. In contrast, leading strand primers on the deproteinized STP could be readily extended by the Klenow fragment (Fig. 4A, lanes 2–5), and the presence of ClpXP could not inhibit this reaction (data not shown).

The synaptic complex in STC treated with ClpXP could be preserved with glutaraldehyde (Fig. 5, lane 10), just as it is when STC1 is treated with ClpX alone (lane 12). This indicates that MuA maintains the synaptic complex in STC even after treatment with ClpXP. Cross-linking MuA with glutaraldehyde stabilizes the synaptic complex in STC2 such that it is pre-

**FIG. 2.** ClpXP relieves inhibition of MuA transposase by Vir *in vitro*. Reaction mixtures (25 μl) including DNA transposition substrates (0.25 μg of donor DNA and 0.5 μg of ϕ1 RFI target DNA) and Rep (275 ng) or Vir (510 ng) proteins were incubated on ice for 10 min in the presence of all reaction components for strand transfer except MuA. MuA (70 ng), ClpX (114 ng), and ClpP (440 ng) proteins were then added as indicated, and reactions were incubated at 37 °C for 30 min. Reaction products were then digested with PstI, treated with SDS (1%), and incubated at 65 °C for 5 min. Products were separated on a 0.6% agarose gel (TAE electrophoresis buffer containing 0.5 μg/ml EtBr). T, target DNA; M, donor DNA containing mini-Mu.

**FIG. 3.** ClpP stimulates action of ClpX on STC1. The action of ClpX on STC1 in the absence (open symbols) or presence of ClpP (440 ng; closed symbols) was determined using the gel disruption assay described under "Experimental Procedures." Values are the average of two independent trials with absolute variance given by error bars. A, reaction mixtures contained 0–190 ng ClpX; incubation time was 30 min. B, reaction mixtures contained 114 ng of ClpX; incubation time was 2–30 min. C, reaction mixtures contained 57 ng of ClpX; incubation time was 2–30 min.
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FIG. 4. The ends of STC1 treated with ClpXP cannot be extended by DNA pol I. Replication was conducted in the six-protein system, which lacks DNA ligase and intact DNA pol I, supplemented with high levels of DNA pol I Klenow fragment (5 units) as indicated (A and B, lane 1, six-protein system alone). After various incubation times (15–60 min), DNA products were extracted and digested with BamHI, which cuts asymmetrically in the vector of donor DNA. This allows leading strand primers extended by pol I at the Mu left and right ends to be distinguished by size on an alkaline agarose gel. Total deoxynucleotide incorporation (picomoles) was determined from one-tenth of each reaction. A, deproteinized STP (A/X74 RFI target) treated with ClpX (760 ng) or ClpXP (228 and 880 ng, respectively) as indicated. B, position of primers at either end of Mu prior to the initiation of replication (visualized by SYBR® Green I staining); Ex, products resulting from primer extension by DNA pol I Klenow fragment; lane 5 reaction volume, 0.25 μg of donor substrate, f1 RFI target). We confirmed that this concentration of ClpX and ClpP (25-μl reaction volume) at the concentrations indicated at 37 °C for 30 min. Reactions were stopped with TLCK (10 mM) and EDTA (20 mM) and subjected to SDS-PAGE on a 12% gel. The gel was blotted and probed with polyclonal antibody specific for MuA.

FIG. 5. The synaptic complex of the Mu ends is maintained following treatment with ClpXP. STC1 isolated by gel filtration (equivalent of 0.25 μg of donor substrate, f1 RFI target) was incubated with ClpX and ClpP (25-μl reaction volume) at the indicated concentrations at 37 °C for 30 min. Products were then treated with glutaraldehyde (0.1% w/v) as indicated and digested with PstI. Products were separated on a 0.6% agarose gel (TAE electrophoresis buffer containing 10 μg/ml EtBr). STC, STC with synaptic complex intact; D, strand transfer product after disruption of the synaptic complex; T, target DNA; M, donor DNA containing mini-Mu.

FIG. 6. MuA is present in STC following treatment with ClpXP. STC1 isolated by gel filtration (equivalent of 0.25 μg of donor substrate, f1 RFI target) was incubated with ClpX and ClpP (25-μl reaction volume) at the concentrations indicated at 37 °C for 30 min. Reactions were stopped with TLCK (10 mM) and EDTA (20 mM) and subjected to SDS-PAGE on a 12% gel. The gel was blotted and probed with polyclonal antibody specific for MuA.

Table: Table 1

DISCUSSION

ClpP plays at least two critical functions in the phage Mu life cycle. Together with protease component ClpP, it can catalyze degradation of the Mu immunity repressor to induce lytic de-
DNA replication, can be disaggregated by ClpX alone (5), and aggregates of the phage can also be degraded when ClpP is also present. For example, can be remodeled by molecular chaperone ClpA or ClpX alone (28, 41). ClpP stimulates ClpX without degrading crucial MuA (14), activating MuA in the transpososome so that MuA can simultaneously function as a molecular chaperone as well as a specificity component for the protease. ClpP interacts with ClpX to stimulate chaperone activity is not consistent with the finding that ClpP is not required for Mu DNA replication in vivo (14). The stimulation of ClpX activity suggests that ClpP is interacting with ClpX; however, how ClpP interacts with ClpX to stimulate chaperone activity is not yet clear. Our results indicate that a molar excess of ClpX monomers over STC1 was required for complete conversion of STC1 to STC2 and that the extent of STC2 formation was proportional to the ClpX concentration. The molar excess of ClpX used in the STC1 disassembly assay is typical of amounts used in previous work (15, 28, 41), and it is possible that ClpX is not turning over catalytically in this reaction. Through its interaction with ClpX, ClpP may be increasing the effective concentration of active ClpX in the reaction mixture. ClpP could allosterically activate the ClpX molecular chaperone or stabilize an active multimeric configuration of ClpX.

Alternatively, the ClpP proteolytic function could facilitate transition to STC2. STC2 formed in the presence of ClpXP was functionally identical to that formed with ClpX alone, but we consistently noticed some decrease in total MuA upon treatment of STC1 with ClpXP. Transpososomes are made up of a core tetramer of MuA plus additional, more loosely bound MuA protomers that play an auxiliary function (45). It is possible that these auxiliary MuA protomers are degraded during ClpXP treatment, thereby facilitating STC1 to STC2 transition. The oligomeric structure of MuA must nevertheless be retained in the resulting STC2 for Mu ends to be held together in a synaptic complex. When ClpX promotes transition of STC1 to STC2, the oligomeric structure of MuA is preserved (15).
The versatility of ClpXP to act either as molecular chaperone or protease may play a key role in determining the course of biochemical reactions such as MuA-catalyzed transposition. While the decision not to degrade MuA in STC1 and to promote transition to STC2 would assure Mu DNA replication, degradation of MuA under certain cellular conditions could permit other pathways for processing the strand transfer intermediate to yield the final recombination product. Thus, the choice of acting as protease or chaperone could influence key decision points in biochemical pathways.

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