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Complex Regulation of the DnaJ Homolog CbpA by the Global Regulators σ^S and Lrp, by the Specific Inhibitor CbpM, and by the Proteolytic Degradation of CbpM▽†

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CbpA is a DnaJ homolog that functions as a DnaK cochaperone. Several cellular processes, including growth at low and high temperatures and septum formation during cell division, require either CbpA or DnaJ. CbpA is encoded in an operon with the gene for CbpM, which is a specific in vivo and in vitro inhibitor of CbpA. Here, we have cooverexpressed CbpA with CbpM in a Δ*cbpAM* Δ*dnaJ* strain and examined the resulting phenotypes. Under these conditions, sufficient free CbpA activity was present to support growth at low temperatures, but not at high temperatures. Defects in cell division and in λ replication were also partially complemented by CbpA when cooverexpressed with CbpM. Utilizing reporter fusions, we demonstrated that the *cbpAM* operon was maximally transcribed at the transition from exponential growth to stationary phase. Transcription was controlled by the σ^S and Lrp global regulators, and both leucine availability and growth temperature influenced transcription. CbpA and CbpM accumulated to similar levels in stationary phase, ~2,300 monomers per cell. When not bound to CbpA, CbpM was unstable and was degraded by the Lon and ClpAP proteases. These data demonstrate that CbpA activity is controlled at multiple levels.

Chaperones assist in numerous cellular functions during both normal growth and times of stress. The DnaK/Hsp70 system is a universally conserved chaperone machine and is composed of DnaK and two cochaperones, DnaJ and GrpE, in *Escherichia coli* (8). DnaK is an energy-dependent chaperone that promotes remodeling of substrate polypeptides. DnaJ assists DnaK by facilitating substrate delivery and by stimulating the intrinsically weak ATPase activity of DnaK. GrpE stimulates nucleotide exchange, thereby assisting substrate release.

In addition to *dnaJ*, the *E. coli* chromosome contains genes for five other DnaJ homologs: *cbpA*, *djlA*, *hscB*, *ybeS*, and *ybeV*. CbpA (*c*urved DNA *b*inding *p*rotein *A*) functions not only as a multicopy suppressor for *dnaJ* mutations in vivo, but also as a cochaperone for the DnaK system in vitro (9, 27, 28). Unlike DnaJ, CbpA binds DNA efficiently and is associated with the nucleoid of stationary-phase cells (1, 27). CbpA is encoded in an operon that codes for a second protein, CbpM, which interacts specifically with CbpA to inhibit both the cochaperone and DNA binding activities of CbpA (9). CbpM homologs exist in a number of diverse bacteria, including the intracellular human pathogen *Coxiella burnetii* and the iron-reducing environmental organism *Geobacter sulfurreducens*. As in *E. coli*, CbpM homologs in other organisms are typically encoded in an operon that also codes for a CbpA-like protein (9). The common genetic organization suggests a conserved interaction between the CbpA and CbpM homologs. The fact that these homologs exist in diverse organisms with widely varying lifestyles implies that CbpA and CbpM fulfill basic physiological roles.

Previously, we demonstrated that CbpM binds to CbpA in vivo, resulting in an inactive complex (10). Overexpression of CbpM generates a Δ*cbpA* phenocopy for all known Δ*cbpA* phenotypes, which are evident only in Δ*dnaJ* strains due to functional overlap between CbpA and DnaJ (10). However, CbpM was present at much higher levels than CbpA in the experiments that detailed this inhibition. Since CbpA and CbpM are encoded by the same operon (9), we wanted to determine if CbpM inhibits CbpA in vivo when the two proteins are coexpressed. Also, we examined the regulation of the operon and the levels of CbpA and CbpM within the cell. CbpM partially inhibited CbpA when the two genes were coexpressed, and some phenotypes were more affected than others. The *cbpAM* operon was controlled at the level of transcription by σ^S and Lrp, and the two proteins accumulated to similar levels. Finally, we found that CbpM was degraded in a *lon*- and *clpP*-dependent manner in vivo and by Lon and ClpP in vitro when it was not bound to CbpA.

MATERIALS AND METHODS

Strains and culture conditions. Strains, plasmids, and phages used in this study are listed in Table 1. All strains used were derivatives of BW27784, a derivative of MG1655 (15). Transductions with P1vir were performed as described previously (19). The *lon*::Tn*10*, *clpP*::*kan*, and *rpoS*::*kan* alleles were acquired from Brill et al. (6), Sledjeski et al. (24), and Bohannon et al. (4), respectively. Unless noted otherwise, all strains were grown in LB and incubated at 30°C.

Strain and plasmid construction. To construct plasmid p*cbpAM*+, *cbpAM* was amplified by PCR using primers listed in Table 2 and digested with EcoRI and HindIII. The inserts were ligated into pBAD24 (14). The ligated constructs were electroporated into DH5α, purified, sequenced, and electroporated into the BW27784 derivatives.

lacZ reporter constructs were made using plasmids pRS1551 and pRS1553, which encode the LacZ α-peptide and are derivatives of vectors pRS551 and pRS552 (23). Transcriptional (*cbpA1*::*lacZ*) and translational (*cbpA2*::*lacZ*) *cbpA* fusions were created with PCR products of *cbpA* that included 200 bp upstream

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TABLE 1. Strains utilized in this study

Strain, plasmid, or phage	(Parental strain) description	Reference or source
Strains		
BW27784	(BW25113) DE(*araFGH*) ϕ(Δ*araEp* P_{CP18}-*araE*)	15
DY330	(W3110) Δ*lacU169 gal490* λ*cI857* Δ(*cro-bioA*)	31
MC105	(BW27784) Δ*cbpA3*::*cat*	9
MC108	(BW27784) Δ*cbpM3*::*cat*	9
MC125	(MC105) *lon*::Tn*10*	This study
MC127	(MC105) Δ*clpP*::*kan*	This study
MC143	(BW27784) Δ*cbpAM3*::*cat*	9
MC144	(MC143) Δ*dnaJ*::*kan*	9
MC150	(BW27784) Δ*dnaJ*::*kan*	9
MC153	(BW27784) λMRC133 (*cbpA1*::*lacZ*)	This study
MC155	(BW27784) λMRC135 (*cbpAM1*::*lacZ*)	This study
MC158	(BW27784) λMRC138 (*cbpAM2*::*lacZ*)	This study
MC160	(BW27784) λMRC140 (*cbpA2*::*lacZ*)	This study
MC163	(MC153) Δ*rpoS*::*tet*	This study
MC247	(BW27784) λMRC141 (*cbpM1*::*lacZ*)	This study
MC342	(BW27784) Δ*lrp3*::*cat*	This study
MC351	(MC153) Δ*lrp3*::*cat*	This study
MC357	(MC163) Δ*lrp3*::*cat*	This study
MC367	(BW27784) λDDS314 (*dsrB1*::*lacZ*)	This study
MC371	(MC367) Δ*lrp3*::*cat*	This study
Plasmids		
pBAD24		14
p*cbpA*+	pBAD24 with *cbpA* insert	9
p*cbpM*+	pBAD24 with *cbpM* insert	9
p*cbpAM*+	pBAD24 with *cbpAM* insert	This study
pRS1551	Plasmid for construction of translational fusions	23
LacZ		
pRS1553	Plasmid for construction of transcriptional fusions	23
lacZ		
pMRC03	pRS1553 with *cbpA1*::*lacZ*	This study
pMRC05	pRS1553 with *cbpAM1*::*lacZ*	This study
pMRC08	pRS1551 with *cbpAM2*::*lacZ*	This study
pMRC10	pRS1551 with *cbpA2*::*lacZ*	This study
pMRC11	pRS1553 with *cbpM1*::*lacZ*	This study
Phage		
λRS468	Phage containing ω fragment of *lacZ*	23
λDDS314	imm^{21} *dsrB1*::*lacZ*	24
λMRC133	imm^{21} *cbpA1*::*lacZ*	λRS468 × pMRC03
λMRC135	imm^{21} *cbpAM1*::*lacZ*	λRS468 × pMRC05
λMRC138	imm^{21} *cbpAM2*::*lacZ*	λRS468 × pMRC08
λMRC140	imm^{21} *cbpA2*::*lacZ*	λRS468 × pMRC10
λMRC141	imm^{21} *cbpM1*::*lacZ*	λRS468 × pMRC11

of the ATG start codon and the first 900 bp of the 921-bp *cbpA* gene. The primers used for amplification were MC.026 and MC.028 (Table 2). Transcriptional (*cbpAM1*::*lacZ*) and translational (*cbpAM2*::*lacZ*) *cbpM* fusions were created with PCR products of *cbpAM* that included 200 bp upstream of the ATG start codon, the entire *cbpA* gene, and the first 300 bp of the 306-bp *cbpM* gene. The primers utilized were MC.026 and MC.030 (Table 2). Additionally, a *cbpM* transcriptional fusion lacking the *cbpAM* promoter (*cbpM1*::*lacZ*) was created with a PCR product that included bases 211 to 921 of *cbpA* and the first 300 bp of *cbpM*. The primers utilized were MC.046 and MC.030 (Table 2). The amplified products were cloned into the EcoRI and BamHI sites of pRS1551 and pRS1553 for translational and transcriptional fusions, respectively (Table 1). The resulting plasmids were crossed with λRS468, which contains the ω fragment of *lacZ* (23), to obtain the corresponding transducing phage. As such, the transducing phage carries either *cbpA* or *cbpAM* fragments fused to a complete *lacZ* gene. The recombinant phage were used to lysogenize BW27784 (Table 1). Monolysogens were identified as described previously (20).

The *lrp* deletion/insertion strain was created as previously described (31). Chloramphenicol cassettes were amplified using the oligonucleotide primer pair Δ*lrp3*for and Δ*lrp3*rev (Table 2). The resulting Δ*lrp3* allele removed all sequence between the *lrp* start and stop codons and was confirmed by sequencing.

Lambda replication. Plaquing efficiency (2) and burst size (16) were determined as described previously. A multiplicity of infection of ~3 was used to determine burst size in the single-step infections.

Microscopy. Cell morphology was examined by phase-contrast as previously described (10).

Western blotting. Cultures were adjusted to an optical density at 595 nm (OD_{595}) of 0.4, precipitated with 10% trichloroacetic acid (vol/vol), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. The protein of interest was detected by probing with specific rabbit serum using a Western immunodetection kit (Western-Breeze; Invitrogen, Carlsbad, CA).

RNA isolation and Northern blotting. RNA was isolated by hot-phenol extraction, and DNA was removed (17). Northern blotting was performed as described previously (17). The biotinylated probes used for detection of *cbpA*,

TABLE 2. Oligonucleotides used for plasmid and strain construction and for Northern blotting

Oligonucleotide	Sequence (5′–3′)
Construction of plasmids	
*cbpAM*for	CGCGGAATTCACCATGGAATTAAAGGATTATTACGCCATCATG
*cbpAM*rev	CGCCTGCAGTCACGGATGAGCTACAAACCGGGAAAGCCG
Chromosomal mutations	
Δ*lrp3*for	CAGACAGGAGTAGGGAAGGAATACAGAGAGACAATAATATT ACGCCCCGCCCTGCCACTC
Δ*lrp3*rev	TTAGCGCGTCTTAATAACCAGACGATTACTCTGCTTGACTTAC GCCCCGCCCTGCCACTC
Construction of *lacZ* fusions	
MC.026	CGCGAATTCTCAACTATCAAAAATCGCTCACCC
MC.028	CGCGGATCCCGACGTGGATCAAAAGACGACTGGGCG
MC.030	CGCGGATCCCGATGAGCTACAAACCGGGAAAGCCG
MC.046	CGCGAATTCCATCGCAACGATCCGCAATTTAACCGT
Biotinylated probes for Northern blots	
cbpA probe	CCACGGGCTAACCGGCACCACAATTTCCAGATCCTGGCCG
cbpM probe	CCAGTTCATGACGCAGGCGTACCGCGCGTTGCACCACAAT
crl probe	CGTTGACGCATACAGCCAGACAATCGAAAAAGAATCGATT
dps probe	TGTTCCAGTGCGCTTGTTTGGTAATCAAAGAAAGATCAAT

cbpM, *crl*, and *dps* are listed in Table 2. Detection was performed with a BrightStar Biodetect kit (Ambion, Austin, TX) according to the manufacturer's instructions.

β-Galactosidase assays. Samples from strains containing *lacZ* fusions were lysed in Fastbreak lysis buffer (Promega, Madison, WI), and the levels of β-galactosidase activity were determined by utilizing a β-galactosidase enzyme assay system (Promega). Activity was determined as Miller units (22).

Quantitative Western blots. Stationary-phase culture samples were precipitated with trichloroacetic acid, and multiple dilutions of the experimental sample were loaded onto the gel with known concentrations of the purified protein (1 to 15 ng). After SDS-PAGE separation and Western immunodetection, the film was scanned and analyzed with ImageJ software (http://rsb.info.nih.gov/ij/). A standard curve was constructed using the purified proteins, and the amount of CbpA or CbpM per sample was calculated in the linear range of the standard curve. These data were used in conjunction with plate counts to determine the number of monomers of each protein per cell.

Protein purification. CbpA (27), CbpM (9), ClpP (18), ClpA (18), and ClpX (18) were isolated as described previously. Lon was a gift from Michael Maurizi (National Institutes of Health, Bethesda, MD).

RESULTS

Cooverexpression of CbpM with CbpA in a Δ*dnaJ* Δ*cbpAM* strain inhibits growth at high but not low temperatures. When overexpressed from a plasmid, CbpM inhibits CbpA's ability to support growth at high and low temperatures in a Δ*dnaJ* strain (10). Since *cbpA* and *cbpM* are located in the same operon, we wanted to know whether CbpM exerts its inhibitory effect when cooverexpressed with CbpA. To address this question, the plating efficiencies of Δ*dnaJ* Δ*cbpAM* triple-deletion strains constitutively expressing CbpA or both CbpA and CbpM from a plasmid were examined over a range of temperatures. The Δ*dnaJ* Δ*cbpAM* strain grew at 25 and 30°C, although growth was reduced by ~100-fold compared to the wild type (10) (Fig. 1A, columns 1 and 2). Growth of the triple-deletion strain was reduced ~10,000-fold at 16 and 37°C compared to the wild type, and there was no detectable growth at 42°C. When CbpA was reintroduced to the Δ*dnaJ* Δ*cbpAM* strain via a multicopy plasmid, growth at 16, 25, 30, and 37°C was restored to wild-type levels (Fig. 1A, column 3). At 42°C, CbpA overexpression was only sufficient to allow low-level growth, ~10,000-fold lower than the wild type (Fig. 1A, column 3). Overexpression

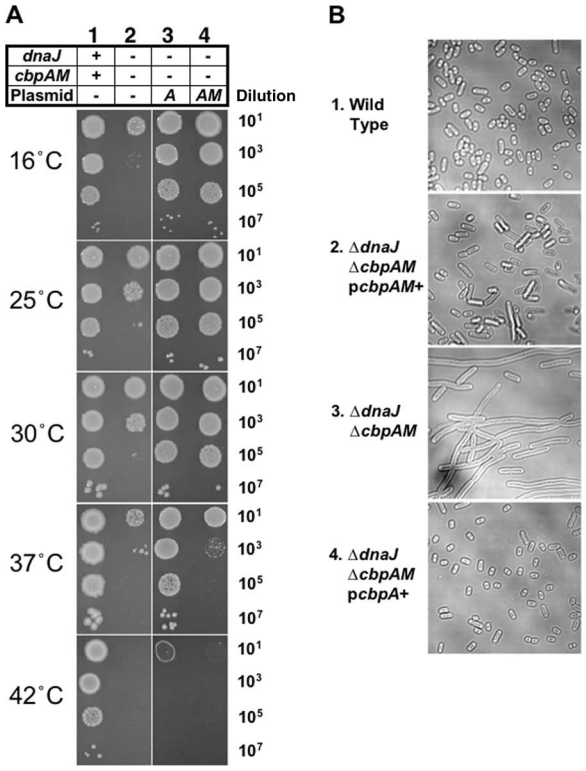


FIG. 1. In vivo effects of cooverexpression of CbpA and CbpM. (A) *E. coli* strains were grown in LB for 24 h at 25°C and serially diluted as indicated, and 10 μl of each dilution was plated on LB agar. The plates were incubated at the indicated temperatures. Ampicillin (50 μg/ml) and arabinose (0.02%) were added to the plates for strains containing plasmids. The relevant genotypes are shown at the top. Column 1, wild type; column 2, Δ*dnaJ* Δ*cbpAM*; column 3, Δ*dnaJ* Δ*cbpAM* p*cbpA*+; column 4, Δ*dnaJ* Δ*cbpAM* p*cbpAM*+. (B) *E. coli* strains were grown in LB overnight (16 h) at 25°C, and the cell morphologies were examined by phase-contrast microscopy. Ampicillin (50 μg/ml) and arabinose (0.02%) were added to cultures of cells containing plasmids.

TABLE 3. Lambda plaquing efficiencies and burst sizes

Parameter	Value			
	WT	Δ*dnaJ*	Δ*dnaJ* p*cbpA*+	Δ*dnaJ* p*cbpAM*+
PFU[a]	$1.2 \pm 0.2 \times 10^{10}$	$<10^{3}$	$7.3 \pm 2.8 \times 10^{9}$	$6.0 \pm 1.4 \times 10^{9}$
Efficiency (%)[b]	100	$<10^{-5}$	62	51
Burst size[c]	170 ± 70	1.4 ± 0.4	147 ± 64	26 ± 4.5

[a] PFU is the average ± standard deviation of three independent experiments.
[b] The efficiency of the wild type (WT) was set to 100%, and those of the other strains are relative to it.
[c] Burst size is the average ± standard deviation of three independent experiments.

of CbpA and CbpM together from the p*cbpAM*+ multicopy plasmid in the Δ*dnaJ* Δ*cbpAM* strain yielded unexpected results. At 16, 25, and 30°C, this strain exhibited wild-type growth, indicating that there was sufficient CbpA activity to allow growth at low temperatures (Fig. 1A, column 4). However, at 37 and 42°C, cooverexpression of CbpA and CbpM in the Δ*dnaJ* Δ*cbpAM* strain resulted in severe growth defects, similar to those evident in the Δ*dnaJ* Δ*cbpAM* triple mutant (Fig. 1A, column 4). Taken together, these results indicate that when CbpM is present with CbpA, there is sufficient CbpA activity to support growth at low temperature, but not at high temperature.

Cooverexpression of CbpA and CbpM partially suppresses the cell division defect of Δ*dnaJ* Δ*cbpAM* cells. Our previous work showed that (i) a Δ*dnaJ* Δ*cbpAM* strain exhibits filamentous cell morphology and overexpression of CbpA restores the wild-type rod-shaped morphology and (ii) overexpression of CbpM in a Δ*dnaJ* strain results in a filamentous morphology like that of a Δ*dnaJ* Δ*cbpA* or a Δ*dnaJ* Δ*cbpAM* strain (10). We wanted to determine if CbpA could repair the cell division defect in Δ*dnaJ* Δ*cbpAM* cells when it was cooverexpressed with CbpM at 25°C. Interestingly, this resulted in slightly elongated cell morphology compared to the wild type (Fig. 1B, images 1 and 2). Δ*dnaJ* Δ*cbpAM* cells bearing the p*cbpAM*+ plasmid did not form the filaments typical of Δ*dnaJ* Δ*cbpAM* cells (Fig. 1B, image 3) or short rods typical of a Δ*dnaJ* Δ*cbpAM* strain overexpressing CbpA alone (Fig. 1B, image 4). Thus, cooverexpression of CbpM with CbpA in a Δ*dnaJ* Δ*cbpAM* strain at 25°C results in sufficient CbpA activity to correct the defect in cell division to nearly the wild-type state.

Overexpression of CbpA with CbpM only partially suppresses the growth defect of bacteriophage λ on Δ*dnaJ* strains. Because cooverexpression of CbpM with CbpA prevented CbpA from participating in cell growth under some conditions but not others, we were interested to know how λ replication would be affected in a Δ*dnaJ* background when both *cbpA* and *cbpM* were coexpressed on a multicopy plasmid. Previous work showed that λ is unable to grow on *dnaJ* mutant strains (25) (Table 3) due to a defect in DNA replication (21, 30) and that multicopy *cbpA* complements the defect (27, 28) (Table 3). Interestingly, when we cooverexpressed CbpM with CbpA in a Δ*dnaJ* strain, the plaquing efficiency of λ was not significantly reduced (Table 3). However, the plaques formed on the Δ*dnaJ* p*cbpAM*+ strain were noticeably smaller than those produced on either the wild-type or Δ*dnaJ* p*cbpA*+ strain.

To investigate the difference in plaque size, the burst sizes were measured. The Δ*dnaJ* cells overexpressing CbpA produced nearly wild-type levels of phage, but Δ*dnaJ* cells overexpressing both CbpA and CbpM produced about sixfold-lower levels of phage (Table 3). Therefore, in the absence of DnaJ, CbpM limits λ replication when CbpA and CbpM are coexpressed from a plasmid, indicating partial inhibition of CbpA activity.

CbpA and CbpM are coexpressed during the growth cycle. As it is perplexing that the gene for an inhibitor of CbpA appears in the same operon with *cbpA*, we wanted to know if there are some situations in vivo when CbpA is in excess of CbpM.

To determine if *cbpA* and *cbpM* may be differentially regulated, the expression of these two genes was examined by Northern blot analysis using oligonucleotides specific for *cbpA* and *cbpM* (see Fig. S1 in the supplemental material). Both genes were transcribed on a 1.4-kb mRNA, indicating that they are likely cotranscribed (Fig. 2A). The expression of *cbpA* and *cbpM* reached a maximum during the transition from exponential-phase growth to stationary phase, and then transcription of both genes rapidly decreased to almost undetectable levels (Fig. 2A). This pattern of transcription was also displayed by *dps*, a σ^S-dependent stationary-phase gene, and by *crl*, a σ^S-independent stationary-phase gene (see Fig. S2 in the supplemental material), suggesting that *cbpA* and *cbpM* transcription is typical of stationary-phase genes. Importantly, the pattern of expression of *cbpA* was indistinguishable from that of *cbpM*.

To further monitor the expression of *cbpA* and *cbpM*, transcriptional *lacZ* gene fusions were made to *cbpA* and to *cbpAM* and inserted into the chromosome in single copies. Using these fusions, we found that the transcriptional patterns of *cbpA* and *cbpAM* were similar (Fig. 2B). With both reporter fusions, transcription was low during exponential growth, increased sharply as cells transitioned to stationary phase, and leveled off in stationary phase (Fig. 2B), similar to the previously reported *cbpA* transcriptional profile (29). Approximately 40% more β-galactosidase was produced by the *cbpAM1*::*lacZ* fusion than by the *cbpA1*::*lacZ* fusion, possibly due to differences in mRNA stability. However, the ratio of β-galactosidase produced by the *cbpAM* fusion compared to that produced by the *cbpA* fusion was nearly constant at 1.4 ± 0.1 during the growth period. Additionally, a *cbpM* transcriptional fusion (*cbpM1*::*lacZ*) lacking the promoter upstream of *cbpA* failed to produce β-galactosidase activity, indicating that *cbpM* probably does not have a separate promoter (see Fig. S3 in the supplemental material). These observations, together with the Northern blot analysis, show that *cbpA* and *cbpM* follow similar transcription profiles throughout growth.

The roles of σ^S and Lrp in *cbpAM* transcription. Previously, transcription of *cbpA* was shown to be induced by σ^S during stationary phase (29) and repressed by Lrp during exponential growth (26). We wanted to further study the effects of these global regulators on the *cbpAM* operon to determine if there was interplay between the two regulatory systems. After 24 h of growth (OD_{595}, ~2.5), a Δ*rpoS* strain produced ~80% less β-galactosidase activity from the *cbpA1*::*lacZ* transcriptional fusion than from the wild-type strain (Fig. 2C). Introduction of the Δ*lrp3* allele to the wild type resulted in a ~40% decrease in *cbpA* promoter activity (Fig. 2C). Interestingly, introduction of the Δ*lrp3* allele into a strain already bearing the *rpoS* deletion had no further effect upon *cbpA* transcription (Fig. 2C). The

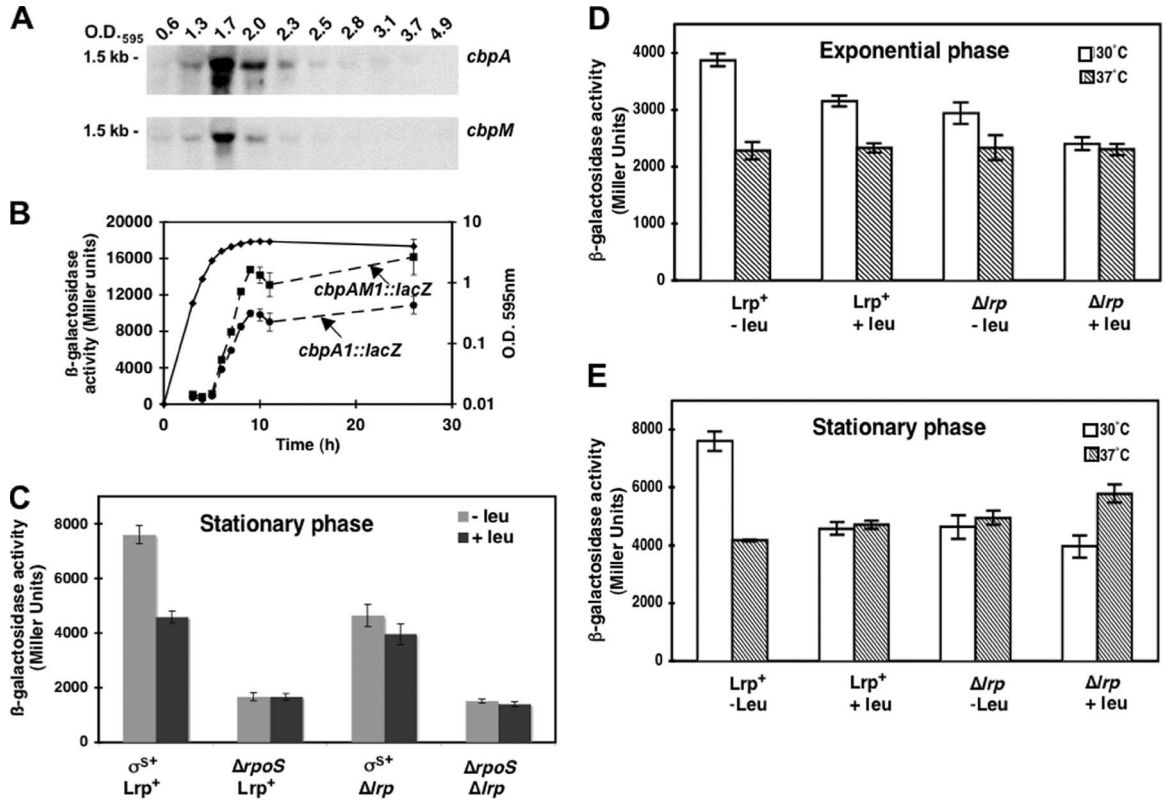


FIG. 2. Expression of *cbpA* and *cbpM* during growth and dependence upon global regulators. (A) Northern analysis of *cbpA* and *cbpM* transcription. Wild-type *E. coli* was grown in LB at 30°C, and RNA samples were collected at the indicated cellular densities during the growth cycle. Transcripts were detected by Northern blotting utilizing biotinylated oligonucleotides specific for *cbpA* (top) or *cbpM* (bottom). (B) Transcriptional analysis of *cbpA* and *cbpM* utilizing *lac* fusions. *E. coli* strains bearing *cbpA* or *cbpAM* transcriptional fusions were grown in LB at 30°C, and β-galactosidase activity from *cbpA1*::*lacZ* (circles) or *cbpAM1*::*lacZ* (squares) was determined throughout the growth cycle at the indicated time points. Each time point represents the average ± standard deviation (SD) of three independent assays. Diamonds, OD_{595} measurements of the culture. (C) Roles of σ^S, Lrp, and leucine in the transcription of *cbpA*. *E. coli* strains containing *cbpA1*::*lacZ* fusions were grown at 30°C in MOPS (morpholinepropanesulfonic acid) minimal medium supplemented with 0.2% glucose, 0.4 mM valine, and 0.4 mM isoleucine (26) to late stationary phase (OD_{595}, ~2.5; 24 h of growth) and assayed for β-galactosidase activity. Combinations of *rpoS* and *lrp* deletions were introduced as indicated. The light-gray bars indicate that no leucine was added to the medium (− leu), and the dark-gray bars indicate 0.4 mM leucine was added to the medium (+ leu). The values represent the average ± SD of at least three independent assays. (D) Role of growth temperature in the transcription of *cbpA* in exponential phase. Cultures were grown to mid-exponential phase (OD_{595}, ~0.3; 7 h of growth), and the transcriptional activity of the *cbpA* promoter was monitored as for panel C. Combinations of *lrp* deletion and leucine addition were introduced as indicated. (E) Role of growth temperature in the transcription of *cbpA* in stationary phase. Transcription of *cbpA* in late-stationary-phase cultures (OD_{595}, ~2.5; 24 h of growth) grown at either 30 or 37°C was determined as in panel D.

effects of these deletions upon *cbpM* transcription followed the same patterns seen for *cbpA* (data not shown). In a control experiment, the *lrp* deletion had no effect on another σ^S-dependent gene, *dsrB* (data not shown). This demonstrates that the effect of Lrp upon *cbpAM* is specific for *cbpAM* and depends upon σ^S, which is the main regulator of *cbpAM* transcription.

Since transcriptional regulation by Lrp is complex and can be either leucine dependent or independent (7), the role of leucine in the Lrp-dependent regulation of *cbpAM* was probed. We repeated the above-mentioned experiments with the addition of 0.4 mM leucine to the growth medium. In wild-type cells, addition of leucine resulted in an ~40% decrease in *cbpA* transcription compared to transcription in the absence of leucine (Fig. 2C). Addition of leucine had no effect on *cbpA* transcription in either Δ*rpoS* or Δ*lrp* cells (Fig. 2C). Furthermore, the decrease in *cbpA* transcription caused by adding leucine to wild-type cultures was almost identical to the decrease in transcription seen in Δ*lrp* cells grown in the absence of leucine (Fig. 2C). This demonstrates that Lrp activates the *cbpAM* promoter only in the absence of leucine. In a control experiment, addition of leucine had no effect on *dsrB* transcription (data not shown). Taken together, these data show that σ^S and Lrp are both necessary for the full activation of the *cbpAM* promoter during stationary phase and that Lrp activates *cbpA* transcription only in cells starved for leucine.

After determining that Lrp is a transcriptional activator of *cbpA* in stationary phase at 30°C, we attempted to replicate a previous study's finding that Lrp represses *cbpA* transcription in exponential phase (26). At 37°C, the temperature used in the prior study, neither leucine nor Lrp had a significant effect on *cbpA* transcription (Fig. 2D). At 30°C, addition of leucine or deletion of *lrp* each reduced *cbpA* transcription by ~20%, and the effects were additive (Fig. 2D). However, the growth temperature did affect *cbpA* transcription; in the absence of leucine, the wild-type cells

grown at 37°C transcribed ~40% less *cbpA* than cells grown at 30°C (Fig. 2D).

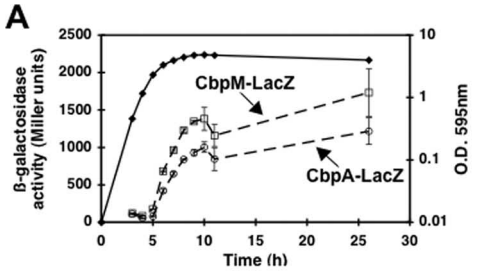
We next examined the effects of Lrp and leucine on stationary-phase cells grown at 30 and 37°C. In contrast to our finding that at 30°C addition of leucine or deletion of *lrp* reduced *cbpA* transcription, at 37°C, either adding leucine or deleting *lrp* resulted in a small but reproducible increase in *cbpA* transcription (Fig. 2E). The transcriptional effects were additive when the conditions were combined (Fig. 2E). Interestingly, wild-type transcription of *cbpA* in the absence of leucine was ~50% lower at 37°C than at 30°C (Fig. 2E). Together, these data demonstrate that Lrp can activate or repress *cbpA* depending upon the conditions. Also, increased temperatures result in decreased transcription of *cbpA* in both exponential and stationary phases.

Translation and accumulation of CbpA and CbpM. Since there was no apparent difference between the transcriptional patterns of *cbpA* and *cbpM*, we wanted to determine if there were effects at the level of translation that might lead to higher levels of CbpA than CbpM, thereby allowing CbpA to function at its full capacity. Translational fusions of *lacZ* to *cbpA* and *cbpM* were constructed in a manner similar to that for the transcriptional constructs and used to determine that the translational patterns of *cbpA* and *cbpM* were similar to the transcriptional patterns (Fig. 3A). For both genes, translation was very low during exponential growth, increased sharply during the transition to stationary phase, and reached a plateau as the cells entered stationary phase. Under the conditions used, the ratio of β-galactosidase activity produced from the CbpM-LacZ relative to the CbpA-LacZ fusion remained stable at 1.5 ± 0.3, very similar to the ratio seen with the transcriptional fusions.

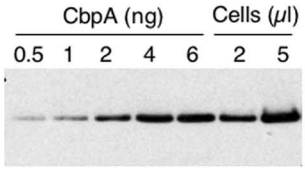
After determining that *cbpA* and *cbpM* transcription and translation followed similar patterns, we wanted to know if the proteins accumulated in the cell to similar levels. Using quantitative Western blot analysis, the levels of CbpA and CbpM in stationary-phase cells were directly determined. We found that cells from 24-h cultures had 2,500 ± 300 CbpA monomers per cell and 2,100 ± 300 CbpM monomers per cell (Fig. 3B and C). This indicates that after being transcribed and translated, CbpA and CbpM remain in the cell at similar levels, suggesting that neither is preferentially removed from the cytoplasm under the conditions tested. Based on the quantitative Western data and the LacZ translational-fusion data, we calculate that approximately 200 monomers each of CbpA and CbpM were present in late-exponential-phase cells prior to the transition to stationary phase.

Taken together, these data demonstrate that regulation of *cbpA* and *cbpM* occurs at the level of transcription and that CbpA and CbpM accumulate rapidly within the cells as growth ceases and then remain at relatively constant levels. There is no evidence for asynchrony in either transcription or translation of *cbpA* and *cbpM* that would lead to different protein levels of CbpA and CbpM. Indeed, our data demonstrate that CbpA and CbpM exist in approximately equal amounts in stationary-phase cells.

CbpM is unstable in the absence of CbpA. In wild-type cells, CbpA and CbpM levels remained constant after the termination of protein synthesis by chloramphenicol treatment (Fig. 4A), consistent with the observation that they remained constant throughout stationary phase despite low levels of transcription (Fig. 2A and data not shown). We wanted to determine if they were stable under other conditions, since differential degradation would provide a possible rationale for the coexpression of CbpA and CbpM. Single-deletion strains were used to examine the level of CbpA in the absence of CbpM and the level of CbpM in the absence of CbpA. Wild-type amounts of CbpA were present in Δ*cbpM* cells at both early (OD_{595}, 2.5; 6 h of growth) and late (OD_{595}, 5.0; 24 h of growth) stationary phase (Fig. 4B). Interestingly, CbpM was

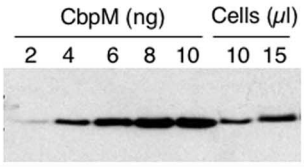


B



Volume (μl)	CFU	Net Intensity	CbpA (ng)	CbpA per cell	AVG
2	$2.2x10^7$	1775	3.39	2700	2500 ± 300

C



Volume (μl)	CFU	Net Intensity	CbpM (ng)	CbpM per cell	AVG
10	$1.1x10^8$	1163	3.76	1830	2100 ± 300
15	$1.6x10^8$	1759	5.00	1600	

FIG. 3. Translation and accumulation of CbpA and CbpM. (A) Translational analysis of CbpA and CbpM utilizing Lac fusions. *E. coli* strains bearing translational fusions were grown in LB at 30°C, and the β-galactosidase activities of strains producing CbpA2-LacZ (open circles) or CbpM2-LacZ (open squares) was determined in cultures throughout the growth cycle. Each time point represents the average ± standard deviation (SD) of three independent assays. Diamonds, OD_{595} measurements. (B) Quantification of CbpA monomers per cell. Wild-type *E. coli* was grown in LB at 30°C for 24 h and analyzed for CbpA content. Varying culture volumes and known amounts of purified protein were separated by SDS-PAGE, detected by Western blotting with specific antisera, and analyzed by densitometry. The resulting data were used to determine the amount of CbpA (ng) per μl of culture. CFU were determined by plating the cells on LB. A representative blot and analysis are shown. The numbers of CbpA monomers per cell were calculated, and the average ± SD of at least three assays is shown in the AVG column. (C) Experiments to quantify CbpM were performed and analyzed as for panel B.

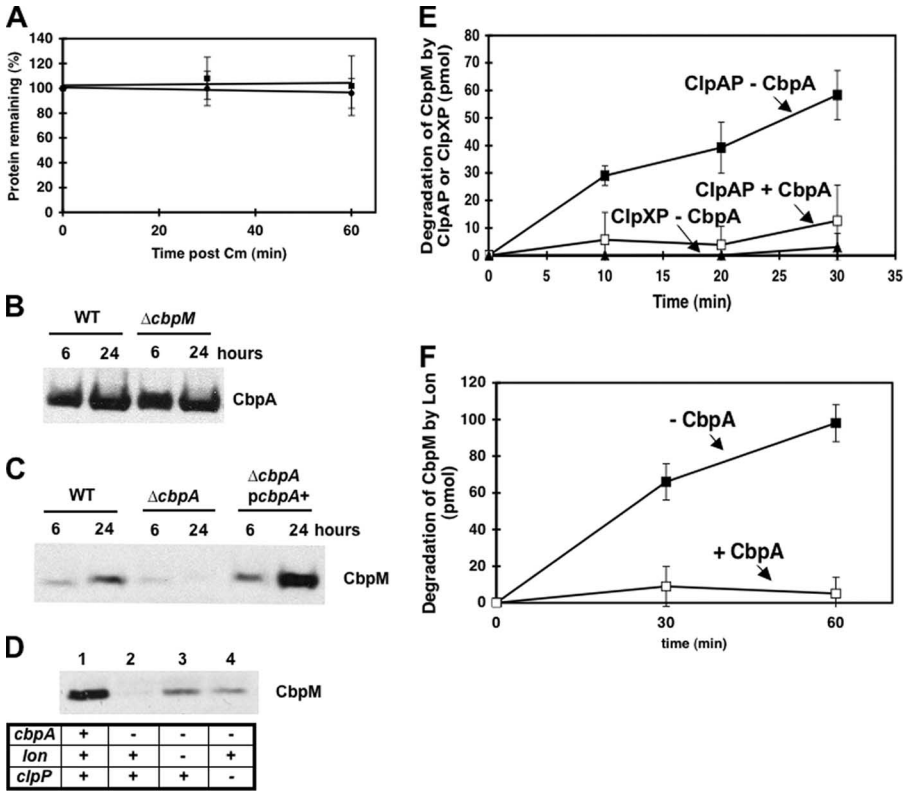


FIG. 4. Stability of CbpA and CbpM. (A) In vivo stability determined by chloramphenicol chase. Cultures were grown in LB at 30°C for 24 h, and then chloramphenicol (100 μg/ml) was added to halt protein synthesis. Samples were precipitated with trichloroacetic acid (TCA) at the indicated time points, and the CbpA (circles) and CbpM (squares) levels were determined by Western blotting, followed by densitometry. The values are the average ± standard deviation (SD) of at least three independent experiments. (B) Stability of CbpA in the presence and absence of CbpM in vivo. Wild-type (WT) or Δ*cbpM* cells were grown to early (6 h) or late (24 h) stationary phase in LB at 30°C, TCA precipitated, separated by SDS-PAGE, and analyzed by Western blotting for CbpA levels. (C) Stability of CbpM in the presence and absence of CbpA in vivo. Wild-type, Δ*cbpA*, and Δ*cbpA* p*cbpA*+ cells were grown to early (6 h) or late (24 h) stationary phase, separated by SDS-PAGE, and analyzed by Western blotting for CbpM levels. The strain bearing the p*cbpA*+ plasmid was supplemented with 50 μg/ml ampicillin and induced with 0.02% arabinose. (D) In vivo roles of Lon and ClpP in the degradation of CbpM. *E. coli* strains were grown to late stationary phase (24 h) in LB at 30°C, and the CbpM content was determined by Western blotting with CbpM antiserum. The relevant genotypes are indicated below lanes 1 to 4. (E) In vitro degradation of CbpM by ClpAP. Reaction mixtures (50 μl) containing CbpM (352 pmol) were incubated with (open squares) or without (filled squares) 181 pmol CbpA dimers in the presence of 17 pmol ClpP tetradecamers, 10.3 pmol ClpA hexamers, 5 mM ATP, and 20 mM $MgCl_2$ in buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM dithiothreitol [DTT], 0.005% Triton X-100) at 25°C. Reaction mixtures containing 13 pmol ClpX hexamers instead of ClpA were carried out under identical conditions (filled triangles). Samples were precipitated with TCA at the indicated times, separated by SDS-PAGE, stained with Coomassie blue, and analyzed by densitometry using ImageJ software. The values are the averages ± SD of at least three independent experiments. (F) In vitro degradation of CbpM by Lon. Reaction mixtures (20 μl) containing CbpM (317 pmol) were incubated with (open squares) or without (closed squares) 181 pmol CbpA dimers in the presence of 3.8 pmol Lon hexamers, 4 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, and an ATP-regenerating system (10 mM phosphocreatine and 0.2 mU creatine phosphokinase) in 50 mM Tris-HCl, pH 8.0. Samples were precipitated with TCA at the indicated times, and CbpM was quantified as in panel E. The values are the average ± SD of three independent experiments.

unstable in the Δ*cbpA* strain (Fig. 4C). When CbpA was supplied via a multicopy plasmid, CbpM was stabilized in late stationary phase (Fig. 4C). Induction with low levels of arabinose that produced CbpA in quantities similar to wild-type levels was sufficient to prevent almost all CbpM degradation (data not shown). Taken together, these results show that CbpA is stable in the absence of CbpM and is required to stabilize CbpM.

After observing that CbpM was unstable in the absence of CbpA, we wanted to determine which proteases were responsible for the degradation of CbpM. Lon, one of the major proteases in *E. coli*, and ClpP, the peptidase component of the ClpAP and ClpXP proteases, were tested to determine if they played a role. Deletions of the *lon* and *clpP* genes were transduced into a Δ*cbpA* background, resulting in Δ*cbpA* Δ*lon* and Δ*cbpA* Δ*clpP* double-deletion strains. Compared to cells lacking only CbpA, cells lacking both CbpA and Lon contained significant amounts of CbpM in late stationary phase, although ~5-fold less than the wild type (Fig. 4D, lanes 1 to 3). A lesser, but significant, amount of CbpM remained stable in the Δ*cbpA* Δ*clpP* strain (Fig. 4D, lane 4). These results suggest that CbpM is likely recognized and acted upon by a number of proteases when it is not protected by CbpA.

To confirm the in vivo results, the degradation of CbpM was examined in vitro. We found that ClpAP degraded CbpM at a rate of ~1.9 pmol/min (Fig. 4E). The addition of CbpA to the reaction mixtures reduced the degradation rate of CbpM ~5-fold, to ~0.4 pmol/min (Fig. 4E). In a control experiment, CbpA did

not significantly inhibit ClpAP degradation of another ClpAP substrate, green fluorescent protein-SsrA (data not shown). CbpM was not detectably degraded by ClpXP (Fig. 4E). Next, we tested Lon and found that it degraded CbpM at a rate of 1.6 pmol/min (Fig. 4F). Addition of CbpA to the reaction mixtures prevented CbpM degradation by Lon. These results indicate that the protection of CbpM from proteolysis by CbpA was likely due to a specific interaction between CbpA and CbpM. Taken together with the in vivo data, this suggests that free CbpM is a substrate for proteolysis but that CbpM complexed to CbpA is not.

DISCUSSION

To study the regulation of CbpA activity by CbpM, we examined several phenotypes of a Δ*dnaJ* Δ*cbpAM* strain coexpressing CbpA and CbpM from a plasmid with the expectation of finding conditions under which CbpA might be active in the presence of CbpM. Cooverexpression of CbpA and CbpM restored viability at low temperatures but was not sufficient to support growth at high temperatures. Additionally, cooverexpression of CbpA and CbpM partially relieved the cell division defect in Δ*dnaJ* Δ*cbpAM* cells and supported limited λ replication in Δ*dnaJ* cells, suggesting that cooverexpression of *cbpM* with *cbpA* results in incomplete inhibition of the CbpA activity. Taken together, these data imply that CbpM functions more to modulate CbpA activity than to shut it down completely.

Three of the *E. coli* DnaJ homologs, DnaJ, CbpA, and DjlA, have significant functional overlap, although it is unclear why this redundancy exists (11–13, 27). Conceivably, each homolog may recognize its own specific set of substrates that are preferentially targeted for remodeling by DnaK. If this is the case, then CbpM inhibition of CbpA may prevent DnaK from interacting efficiently with CbpA's set of substrates under certain growth or stress conditions. In this way, CbpA may act as an adaptor for the DnaK system, facilitating delivery of particular high-affinity substrates at specific times. CbpM may act as an antiadaptor, protecting those same substrates from DnaK action at other times. In this scenario, CbpM holds CbpA in a state of limited activity until a cellular or environmental signal leads to dissociation. When the CbpA-CbpM complex separates, fully active CbpA is released and CbpM could be removed by selective degradation by cellular proteases. It is tempting to speculate that other DnaJ homologs have their own inhibitory partners to further adjust the DnaK/Hsp70 chaperone system, although none have been isolated yet.

Recently, another adaptor-antiadaptor pair was described in *E. coli*. This system is comprised of RssB and IraP, and it controls the turnover of σ^S (5). RssB acts as an adaptor that targets σ^S for degradation by ClpXP. In response to phosphate starvation, IraP binds to RssB and prevents it from delivering σ^S to the protease, thereby stabilizing σ^S. Such systems add yet another layer to the levels of regulation available to control cellular processes.

Our examination of expression of the *cbpAM* operon showed that CbpA and CbpM, like other stationary-phase proteins, were mainly expressed at the end of the exponential phase as cells ceased active growth. This is in agreement with a previous transcriptional study of *cbpA* (29) but contrasts with a different study, which found that CbpA accumulated only in very late stationary phase (3). The reason for the disparity is unclear, although strain and technique variations likely are involved.

We determined that CbpA and CbpM accumulation was controlled at the level of transcription and that the genes were transcribed from the same promoter. Previously, transcription of *cbpA* was shown to be driven by σ^S (29). Here, we demonstrated that the *cbpAM* promoter was dependent upon both the σ^S and Lrp global regulators for full activity. σ^S activated *cbpAM* transcription independently of Lrp, but Lrp required σ^S to activate *cbpAM* transcription, suggesting that the Lrp effect is indirect and occurs upstream of σ^S. Activation of the *cbpAM* promoter by Lrp was also dependent on leucine, a gauge of amino acid availability (7). Additionally, transcription of the *cbpAM* operon was dependent upon the growth temperature, with more transcription at 30°C than at 37°C. This was likely due to the increased stability of σ^S at lower temperatures (24).

Our data showed that CbpA accumulated ~2,500 monomers per cell and CbpM accumulated ~2,100 monomers per cell in late stationary phase. The number of molecules per cell differed significantly from a previously published estimate of 15,000 CbpA monomers per cell (1). The strains and techniques used to arrive at the different numbers were not identical, possibly explaining the discrepancy.

Although CbpA and CbpM were both stable in strains expressing both proteins under the conditions tested, CbpM was degraded in the absence of CbpA by Lon, either ClpAP or ClpXP or both, and possibly other proteases. This lends support to our model, which proposes that the limited posttranslational control CbpM exerts over CbpA is alleviated by selective degradation of the inhibitor. Studies are ongoing to determine what signal leads to the dissociation of the CbpA-CbpM complex and which substrates are specifically recognized by CbpA.

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