Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system

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We show that the two-component signal transduction system of *Escherichia coli*, CpxA–CpxR, controls the expression of genes encoding cell envelope proteins involved in protein folding and degradation. These findings are based on three lines of evidence. First, activation of the Cpx pathway induces 5- to 10-fold the synthesis of DsbA, required for disulfide bond formation, and DegP, a major periplasmic protease. Second, using electrophoretic mobility shift and DNase I protection assays, we have shown that phosphorylated CpxR binds to elements upstream of the transcription start sites of *dsbA*, *degP*, and *ppiA* (*rotA*), the latter coding for a peptidyl-prolyl *cis/trans* isomerase. Third, we have demonstrated increased *in vivo* transcription of all three genes, *dsbA*, *degP*, and *ppiA*, when the Cpx pathway is activated. We have identified a putative CpxR consensus binding site that is found upstream of a number of other *E. coli* genes. These findings suggest a potentially extensive Cpx regulon including genes transcribed by σ^{70} and σ^E , which encode factors involved in protein folding as well as other cellular functions.

[Key Words: DsbA; Cpx; DegP; protein folding; transcription]

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A number of proteins have been identified in bacteria that catalyze different steps in the folding of cell envelope proteins. Periplasmic proteins catalyze disulfide bond formation, the isomerization of peptidyl-proline bonds, and protein degradation, and may be important for the assembly of outer membrane proteins (Wulfig and Pluckthun 1994). Under certain conditions, cell envelope proteins may be more exposed to environmental stresses than cytoplasmic proteins, making the need for protein maintenance and repair systems specific for the cell envelope important for survival (Visick and Clarke 1995). However, very little is known about how bacteria regulate the expression of periplasmic protein folding catalysts.

In *Escherichia coli*, the best understood periplasmic protein folding pathway is the Dsb system that catalyzes disulfide bond formation. The DsbA and DsbB proteins are required for the formation of disulfide bonds in cell envelope proteins (Bardwell et al. 1991, 1993; Kamitani et al. 1992; Jander et al. 1994; Guilhot et al. 1995). The

DsbC protein is apparently also involved in cell envelope protein folding as it mediates the shuffling of disulfide bonds when incorrect disulfide bonds have formed (Rietsch et al. 1996).

In addition to proteins containing disulfide bonds, those containing proline residues may also require the activity of folding catalysts for efficient folding. Peptidyl-proline residues can occur in either the *cis* or *trans* conformations, yet usually only one conformation is suitable to yield a correctly folded protein (Wulfig and Pluckthun 1994). *E. coli* contains at least three periplasmic proteins catalyzing isomerization, peptidyl-prolyl *cis/trans* isomerases (PPIases) or rotamases, each belonging to a different family: PpiA (cyclophilin family) (Liu and Walsh 1990; Hayano et al. 1991), FkpA (FKBP family) (Roof et al. 1994; Wulfig et al. 1994; Horne and Young 1995), and SurA (PpiC family) (Rahfeld et al. 1994; Rudd et al. 1995; Lazar and Kolter 1996; Missiakas et al. 1996; Rouvière and Gross 1996). Although all of the genes encoding these enzymes are nonessential, PpiA is a potent PPIase *in vitro* (Liu and Walsh 1990; Hayano et al. 1991), and SurA stimulates the rate of folding of the outer membrane protein LamB *in vivo* (Lazar and Kolter 1996; Missiakas et al. 1996; Rouvière and Gross 1996).

If any of these periplasmic protein-folding catalysts is regulated, a mechanism (or mechanisms) must exist for

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sensing either cell envelope protein-folding defects or the conditions that lead to folding defects and for transmitting these signals across the inner membrane. A stress response pathway involved in the maturation of cell envelope proteins has been identified recently in *E. coli*. This pathway requires σ^E , a member of the ECF (extra cytoplasmic function) family of transcription factors, as well as at least two Rse (regulator of σ E) proteins. σ^E Activity is induced by heat shock, by the overproduction of outer membrane proteins, and by *surA* mutations (Mecses et al. 1993; Raina et al. 1995; Rouviere et al. 1995). Recent evidence suggests that the transmembrane protein RseA is an anti- σ factor whose activity is regulated by the periplasmic protein RseB (De Las Peñas et al. 1997).

The σ^E regulon includes at least 10 genes, based on the analysis of two-dimensional gels after overproduction of σ^E , but only three of these have been identified; *degP*, *rpoH* (σ^{32}), and *rpoE* (Raina et al. 1995; Rouviere et al. 1995). The *degP* gene encodes a periplasmic protease induced by heat shock (Lipinska et al. 1988; Strauch and Beckwith 1988). Although the cellular substrates of DegP are not known, certain unstable foreign proteins are stabilized by *degP* mutations (Strauch et al. 1989). The *rpoH* gene encodes the heat shock sigma factor, σ^{32} , which directs transcription of the genes encoding the classical heat shock response, such as the chaperones DnaK/J and GroES/EL.

In bacteria, sensing and responding to external stimuli is usually mediated by two-component signal transduction systems (for review, see Parkinson and Kofoid 1992), and one might therefore expect such a system to play a role in both detecting cell envelope defects and coordinating the appropriate response. The Cpx system, composed of the CpxA sensor kinase and the CpxR response regulator (Dong et al. 1993), represents a possible candidate for such a system. The *cpx* locus was first identified by dominant mutations in the *cpxA* gene that adversely affect a number of cell envelope processes, such as the ability to transport small molecules into the cell via proton-driven cotransporters, F plasmid conjugation, and the production of outer membrane proteins (for summary, see Rainwater and Silverman 1990). More recently, these so-called *cpxA*^{*} mutations were found to confer resistance to the toxic effects associated with the expression and export of a hybrid protein (Cosma et al. 1995; Snyder et al. 1995) and lead to constitutively high expression from the *degP* promoter (Danese et al. 1995). Furthermore, the wild-type Cpx proteins stimulate transcription of the *degP* gene in response to overexpression of NlpE (Danese et al. 1995), a lipoprotein of unknown function (Gupta et al. 1995). In addition to CpxA and CpxR, σ^E is required for induction of *degP* transcription (Danese et al. 1995).

Because σ^E appears to function as an extracytoplasmic periplasmic stress response pathway and Cpx and σ^E together control expression of *degP* transcription, it has been suggested that the Cpx proteins are also part of a stress response pathway involved in monitoring periplasmic protein folding (Cosma et al. 1995). However, the

relationship between Cpx and σ^E is unclear. For example, Cpx could regulate *degP* expression indirectly by altering the activity or expression of the proposed regulators of σ^E , namely RseAB. Alternatively, Cpx could directly activate σ^E -transcribed genes by binding to sequences adjacent to σ^E -dependent promoters. Finally, Cpx could play a more general role in cell physiology by controlling the transcription of genes transcribed by other sigma factors, such as σ^{70} , in addition to σ^E . Here, we identify new targets of the Cpx signal transduction pathway and provide evidence that CpxA and CpxR serve to directly regulate the expression of genes encoding periplasmic protein-folding catalysts. We also demonstrate that phosphorylated CpxR binds to specific sequences upstream of both σ^E and σ^{70} promoters, allowing the identification of a putative DNA-binding consensus sequence and suggesting that CpxA and CpxR comprise an extracellular stress response that possibly controls the expression of a large number of genes in *E. coli*.

Results

Temperature-regulated expression of DegP in cpx⁺, Δcpx, and cpxA^{} mutants*

Dominant, gain-of-function *cpxA*^{*} mutations that result in pleiotropic phenotypes have been suggested to lock the CpxA protein into a conformation that results in constitutive signaling in the absence of the normal stimulus or ligand. Consistent with this idea, *cpxA*^{*} mutations lead to constitutive, high-level expression of the heat-inducible *degP* gene (Danese et al. 1995). Expression of DegP is greatly increased in mutants containing *cpxA*^{*} mutations (Fig. 1). Because both temperature and Cpx appear to regulate the expression of DegP, we examined the Cpx dependence of the effect of temperature on the expression of DegP (Fig. 1). The rates of synthesis of the DegP protein in a variety of *cpx* mutants at 30°C, 37°C, or after a 10-min shift from 30°C to 42°C were measured in pulse-chase immunoprecipitation experiments, and the quantitated results are shown in Figure 1. In wild-type cultures, synthesis of DegP is slightly elevated (~50%) when grown at 37°C compared with 30°C, and DegP is induced eightfold after 10 min of heat shock. In *cpxA*^{*} mutants, rates of DegP synthesis are significantly elevated at 37°C compared with wild type and increase even further after a shift to 42°C. The degree of regulation of DegP synthesis by temperature in a *cpx* null mutant is identical to that seen with regulation in *cpx*⁺ strains. Hence, the pathway leading to activation of σ^E by temperature appears to be independent of Cpx.

DsbA is overexpressed in mutants containing cpxA^{} mutations*

On the basis of experiments showing that *cpxA*^{*} mutants constitutively express the DegP protease, we reasoned that CpxA may be a sensor of cell envelope defects and that other genes involved in maturation of cell envelope proteins may therefore be regulated by Cpx. One prediction of this model is that periplasmic protein-fold-

Figure 1. DegP expression in *cpx*⁺, Δ *cpx*, and *cpxA*^{*} mutants. Pulse-chase experiments were performed at 30°C (solid bars), 37°C (hatched bars), or after a 10-min shift from 30°C to 42°C (shaded bars) as described in Materials and Methods. Strains are JP406 (*cpxRA*⁺), ECL1212 (Δ *cpxRA*), JP408 (*cpxA9*), JP467 (*cpxA2.1*), and JP468 (*cpxA9.4*). The data shown is from a single, representative experiment. Results using another *cpx*⁺ wild-type strain (JP466) were similar.

ing catalysts will be expressed at higher levels in strains containing *cpxA*^{*} mutations. To test this idea, we examined the expression levels of DsbA and SurA, two proteins important for folding of cell envelope proteins (Bardwell et al. 1991; Lazar and Kolter 1996). The rates of synthesis of the DsbA and SurA proteins were measured in *cpxA*^{*} mutants using pulse-chase immunoprecipitation experiments. Expression of the SurA protein was not elevated in *cpxA*^{*} mutants (data not shown); however, expression of DsbA was greatly affected. As is shown in Figure 2a, DsbA was increased four- to eight-fold by *cpxA*^{*} mutations in strains grown at 37°C, a level of induction comparable to that seen for DegP in the same experiments (Fig. 2b).

DsbA is regulated by the wild-type Cpx signal transduction pathway

In addition to being regulated by *cpxA*^{*} mutations, the *degP* gene is regulated by the wild-type Cpx proteins in response to overproduction of an exported lipoprotein, NlpE (Danese et al. 1995). We examined the ability of NlpE overexpression to induce synthesis of the DsbA and DegP proteins using pulse-chase immunoprecipitation experiments. A plasmid, pND18, expressing NlpE from the arabinose promoter, induces the DegP protein ~10-fold (Fig. 2b). Furthermore, the induction of DegP protein by NlpE was found to be dependent on the Cpx proteins. These results are in accord with those of previous studies based on strains containing a λ phage expressing a *degP-lacZ* transcriptional fusion, suggesting that both methods accurately reflect changes in tran-

scription from the *degP* promoter in vivo. The rates of synthesis of DsbA were increased approximately four-fold after 1.25 hr of production of NlpE (Fig. 2a). Overproduction of NlpE did not result in DsbA induction if the *cpx* operon was deleted. These results suggest that expression of DsbA is regulated by the Cpx pathway.

Cpx regulates transcription of the dsbA gene

The *dsbA* gene is transcribed from two promoters: a proximal promoter (P1) immediately upstream of the *dsbA* open reading frame and a distal promoter (P2) up-

Figure 2. DegP and DsbA expression in *cpxA*^{*} mutants or in *cpx*⁺ and Δ *cpx* null strains when NlpE is expressed from a high-copy-number plasmid. The genotype of each strain is indicated as + for *cpx*⁺, Δ for Δ *cpx*, and * for *cpxA*^{*}. Strains 1–4 contain the plasmid pND18 or pBAD22 and were grown with (solid bars) or without (hatched bars) arabinose. Strains 5, 6, and 7 do not contain plasmids and were not grown in the presence of arabinose. Cultures grown at 37°C in minimal maltose medium were either induced (solid bars) or not induced (hatched bars) with 0.2% arabinose for 1.25 hr and then pulse-labeled as described in Materials and Methods. Strain 1 (JP472); strain 2 (JP474); strain 3 (JP558); strain 4 (JP560); strain 5 (JP406); strain 6 (JP408); strain 7 (JP467). The results shown are from a typical experiment. Similar results were obtained using a different time of induction of NlpE with arabinose (1.5 hr) or using a different strain background in which cells cannot catabolize arabinose (JP313).

stream of an open reading frame (*yihE*) that precedes the *dsbA* gene (Belin and Boquet 1994). The DsbA protein might be regulated by Cpx directly by stimulating transcription of one or both of the *dsbA* promoters or indirectly by another mechanism. We therefore quantitated the *in vivo* levels of RNA transcripts originating from both promoters (P1 and P2) by RNase protection. RNA was harvested from *cpx*⁺- or Δ *cpx*-mutant strains containing either a control plasmid, pBAD22, or a plasmid that overexpresses NlpE, pND18. Expression of the distal promoter (P2) is induced 10-fold by NlpE overproduction, whereas the proximal promoter (P1) is not induced (Fig. 3a). Induction of P2 was not observed in strains containing a *cpx* null mutation (Fig. 3a).

We then investigated the effect that various mutations in the *cpx* locus have on expression from P2. The amount of RNA produced from the P2 promoter was assayed by RNase protection in strains containing either the wild-type *cpx* operon, the defined *cpxRA* deletion, or one of two *cpxA*^{*} mutations. As is shown in Figure 4a, strains containing *cpxA*^{*} mutations produced from four- to sevenfold more of the transcript originating from P2 relative to the amount produced in the *cpx*⁺ parent strain. This difference is highly significant, as determined by standard *t*-test analysis.

Kinetics of activation of the Cpx pathway by NlpE overproduction

NlpE overproduction is routinely used as an activator of the Cpx pathway, but the mechanism underlying such activation is unknown. To gain further insight into the phenomenon, we determined the kinetics of activation of the Cpx pathway following induction of NlpE synthesis. A rapid activation of Cpx may reflect a more direct role for NlpE in activating the Cpx pathway. However, if the kinetics of activation of Cpx were found to be slow and the strength of the signal to increase with time of synthesis, NlpE overproduction may result in an indirect effect on cell envelope structure. NlpE synthesis was induced by the addition of arabinose to exponentially growing cultures, and the expression from the *dsbA* P2 promoter was measured at various times by RNase protection. The *dsbA* P2 transcript is rapidly and maximally induced within 5 min of induction of NlpE (Fig. 5). Overproduction of another exported protein, alkaline phosphatase (AP), did not significantly induce *dsbA* transcription, despite the fact that AP was more highly expressed than NlpE in these experiments, as revealed by Coomassie staining of total proteins at the end of the experiment (data not shown). These results are consistent with those of Danese et al. (1995) in which the overproduction of several other exported proteins did not stimulate the Cpx response and suggest that NlpE is a specific and potent activator of the Cpx pathway.

Binding of His₆-CpxR and His₆-CpxR-P proteins to the transcriptional regulatory region of the *degP* gene

To determine whether the CpxR protein, or more likely the phosphorylated form (CpxR-P), serves directly as a

Figure 3. Expression of *dsbA* and *ppiA* RNA in strains in which the Cpx pathway has been activated by NlpE overproduction. Transcription from the *dsbA* P1 (a, open bars) and P2 (a, solid bars) promoters, and the *ppiA* P1 promoter (b) was measured by RNase protection. Strains contain the plasmids pND18 or pBAD22 and were grown at 37°C in minimal maltose medium and then either induced (+) or not induced (-) with 0.2% arabinose for 1.5 hr. The strains used were JP472 (*cpx*⁺/pBAD22), JP474 (Δ *cpx*/pBAD22), JP558 (*cpx*⁺/pND18), and JP560 (Δ *cpx*/pND18). The results are expressed relative to the values obtained for JP472 and grown in the absence of arabinose.

transcriptional regulator of the *degP* locus, we tested whether binding of the CpxR protein to DNA fragments containing the transcriptional regulatory region of the *degP* gene could be detected. For this study, a derivative of the CpxR protein (His₆-CpxR) bearing an amino-terminal, oligohistidine extension was used prior to or after incubation with acetyl phosphate.

In a preliminary search for specific DNA-binding sites for the His₆-CpxR and His₆-CpxR-P proteins, electrophoretic mobility shift (EMS) and DNase I footprinting assays were performed using a 362-bp fragment (derived from pBTKSdegP1) that includes 301 bp of the region

Figure 4. Expression from the *yihE-dsbA* P2 promoter and the *ppiA* P1 promoter in *cpx* mutants. Three independent cultures of each strain were grown to an OD_{600} of ~0.3. Samples were then taken, and expression of mRNA from each promoter was assayed by RNase protection. The average value for each strain (labeled 1–5) and the s.d. (error bars) for that average are expressed relative to the values obtained for strain 1. Results for the *yihE-dsbA* P2 promoter are shown in *a* (top) and those for the *ppiA* P1 promoter are shown in *b* (bottom). Strain 1 (JP406); strain 2 (ECL1212); strain 3 (JP408); strain 4 (JP467); strain 5 (JP466).

upstream of the initiation codon of DegP. The results obtained were indicative of the presence of two distinct and well-separated binding sites, at which the phosphorylated form of the protein appeared to bind with a significantly higher affinity (data not shown).

Figure 6a shows DNase I footprinting analysis of the binding of the His₆-CpxR and His₆-CpxR-P proteins to a 249-bp fragment (derived from pBTKSdegP2) that includes the σ^E promoter of the *degP* gene and that contains the stronger of the two binding sites identified in the preliminary analysis. Clear DNase I footprints are only readily apparent with the His₆-CpxR protein at the highest concentration of protein employed (720 nM; lanes 12,24). In contrast, clear DNase I footprints are

observed with the His₆-CpxR-P protein at a significantly lower concentration (180 nM; lanes 4,16). As the concentration of the His₆-CpxR-P protein is increased further, the regions of protection from DNase I cleavage expand in a unidirectional fashion to include sequences located downstream of the promoter.

Figure 6b shows a similar analysis of protein binding to a 213-bp fragment containing the weaker His₆-CpxR-P-binding site, which is located upstream of the σ^E promoter-proximal site described above. In this case, DNase I footprints are not apparent with the His₆-CpxR protein even at the highest concentration of protein employed (720 nM; lanes 9,18). In contrast, clear DNase I footprints are observed on both strands of the template with the His₆-CpxR-P protein at a concentration of 360 nM (lanes 3,12).

The core His₆-CpxR-P footprints at both sites are indicated in Figure 6c (labeled as “CpxR-P binding site I” or “CpxR-P binding site II”). Each site corresponds to the sequences at which DNase I cleavage is most significantly affected by His₆-CpxR-P protein binding (i.e., when His₆-CpxR-P is present at only relatively low concentrations).

Binding of His₆-CpxR and His₆-CpxR-P proteins to the transcriptional regulatory region of the yihE-dsbA operon

Preliminary studies suggested that the CpxR protein binds in a phosphorylation-stimulated manner to a

Figure 5. Kinetics of induction of the *yihE-dsbA* operon by NlpE. Strains containing the plasmids pND18 (solid bars; overexpresses NlpE), pDB3 (hatched bars; overexpresses AP), or pBAD22 (open bars; control plasmid) were grown aerobically in minimal maltose medium with ampicillin at 37°C. At time zero (corresponding to an OD_{600} of ~0.2), a sample from each culture was taken and then arabinose added to a final concentration of 0.2%. Samples were taken at 5, 10, 20, and 40 min after the addition of arabinose. mRNA originating from the P2 promoter of the *yihE-dsbA* operon was quantitated by RNase protection. The results are expressed relative to the values obtained for each culture at time zero. The strains were JP444 (*cpx*⁺/pDB3), JP445 (*cpx*⁺/pBAD22), and JP446 (*cpx*⁺/pND18). Similar results were obtained using the *ara*⁺ strains JP472 and JP558.

Figure 6. CpxR-P binding at sites located in the region upstream of the *degP* gene. (a) DNase I protection assays of the binding of His₆-CpxR and His₆-CpxR-P to *degP* site I. A 249-bp DNA fragment (obtained by PCR amplification of the *degP* promoter region of pBTKSdegP2 with primers DEGP3 and DEGP2) was end-labeled with ³²P at its 5' termini by treatment with T4 polynucleotide kinase, and uniquely labeled substrates were generated by *Eco*RI (lanes 1–12) or *Bam*HI (lanes 13–24) digestion. The substrate DNA was present at ~2 nM in reactions that also contained no added protein (lanes 1,7,13,19), 36 nM His₆-CpxR-P (lanes 2,14), 90 nM His₆-CpxR-P (lanes 3,15), 180 nM His₆-CpxR-P (lanes 4,16), 360 nM His₆-CpxR-P (lanes 5,17), 720 nM His₆-CpxR-P (lanes 6,18), 36 nM His₆-CpxR (lanes 8,20), 90 nM His₆-CpxR (lanes 9,21), 180 nM His₆-CpxR (lanes 10,22), 360 nM His₆-CpxR (lanes 11,23), or 720 nM His₆-CpxR (lanes 12,24). The sequencing ladders were generated by extension of ³²P-end-labeled DEGP3 primer for the coding strand or DEGP2 primer for the noncoding strand using pBTKSdegP2 as a template. The core His₆-CpxR-P binding site I is indicated by the thick line; the thin line indicates the limits of the sequence shown in the lower half of c. (b) DNase I protection assays of the binding of His₆-CpxR and His₆-CpxR-P to *degP* site II. A 213-bp DNA fragment (obtained by amplification of the *degP* promoter region of pBTKSdegP3 with primers DEGP4 and DEGP5) was end-labeled with ³²P, and uniquely labeled substrates were generated by *Eco*RI (lanes 1–9) or *Bam*HI (lanes 10–18) digestion. The substrate DNA was present at ~2 nM in reactions that also contained no added protein (lanes 5,14), 90 nM His₆-CpxR-P (lanes 1,10), 180 nM His₆-CpxR-P (lanes 2,11), 360 nM His₆-CpxR-P (lanes 3,12), 720 nM His₆-CpxR-P (lanes 4,13), 90 nM His₆-CpxR (lanes 6,15), 180 nM His₆-CpxR (lanes 7,16), 360 nM His₆-CpxR (lanes 8,17), or 720 nM His₆-CpxR (lanes 9,18). Sequencing ladders were generated by extension of ³²P-end-labeled DEGP4 primer for the coding strand or DEGP5 primer for the noncoding strand using pBTKSdegP3 as a template. The core His₆-CpxR-P binding site II is indicated by the thick line; the thin line indicates the limits of the sequence shown in the upper half of c. (c) The location of CpxR-P binding sites in the region upstream of the *degP* gene. Elements of the σ^E promoter (Erickson and Gross 1989) are indicated. The core CpxR-P binding sites I and II, as defined by the patterns of altered DNase I reactivity observed on both strands in the presence of a limiting concentration of His₆-CpxR-P, are indicated by the brackets.

single site in the transcriptional regulatory region of the *yihE-dsbA* operon. Figure 7a shows DNase I footprinting analysis of the binding of the His₆-CpxR and His₆-CpxR-P proteins to a 275-bp fragment (derived from pBTKSdsbA2) that includes the P2 promoter of the *yihE-dsbA* operon. DNase I footprints are readily apparent on the noncoding strand with the lowest concentration of His₆-CpxR-P used (36 nM), whereas equivalent footprints are only observed with the His₆-CpxR protein at the highest concentration of protein employed (720 nM). As the concentration of the His₆-CpxR-P protein is increased, the region of protection from DNase I cleavage expands bidirectionally. Significant differences in the apparent affinities of the His₆-CpxR and His₆-CpxR-P proteins are also indicated by the DNase I footprints obtained on the coding strand (lanes 1–12); a core His₆-CpxR-P footprint is indicated in Figure 7b.

Computer-assisted alignments of the DNA sequences present in the three core footprints characterized to this point yielded only a pentamer motif (5'-GTAAA-3'; indicated with arrows in Figs. 6c and 7b) as the most significant primary sequence element present in all three sequences. In the case of the *yihE-dsbA* site, it was noted that a second imperfect copy of the motif (5'-GTAAG-3') was present in close proximity with only 5 bp (or half a helical turn) located between them (also indicated in Fig. 7b). A computer search of the *E. coli*

genomic DNA sequences present in GenBank (release 96.0) with the sequences 5'-GTAA(N)₆GTAA-3' and 5'-GTAA(N)₇GTAA-3' revealed the presence of a number of these elements, as would be expected given the relatively low complexity of the search motifs. However, subsequent analysis indicated that the presence of a subset of the motifs identified may be of physiological significance because they were present in known (or putative) transcriptional regulatory regions of genes encoding products related to protein folding.

Binding of His₆-CpxR and His₆-CpxR-P proteins to the transcriptional regulatory region of the *ppiA* gene

In the *yihE-dsbA* operon promoter, the 5'-GTAAA-(N)₅GTAA-3' sequence is located 6 bp upstream of the -35 element of the σ^{70} promoter. This same element was found to be located 5 bp upstream of the -35 element of the major σ^{70} promoter (P1) of the *ppiA* gene (Norregaard-Madsen et al. 1994) that encodes a periplasmic PPIase of the cyclophilin family (Liu and Walsh 1990; Hacker and Fischer 1993; Clubb et al. 1994). A definitive role for the PpiA protein in protein folding in the periplasmic compartment has yet to be demonstrated (Kleerebezem et al. 1995); however, as the products encoded by the two other transcriptional activation targets of CpxR-P (*degP* and *dsbA*) have distinct roles in the

Figure 7. (a) DNase I protection assays of the binding of His₆-CpxR and His₆-CpxR-P to the promoter of the *yihE-dsbA* operon. A 275-bp DNA fragment (obtained by amplification of the *yihE-dsbA* promoter region of pBTKSdsbA2 with primers DSBA3 and DSBA2) was end-labeled with ³²P, and uniquely labeled substrates were generated by *Eco*RI (lanes 1–12) or *Bam*HI (lanes 13–23) digestion. The substrate DNA was present at ~2 nM in reactions that also contained no added protein (lanes 1,7,13,18), 36 nM His₆-CpxR-P (lanes 8,19), 90 nM His₆-CpxR-P (lanes 9,20), 180 nM His₆-CpxR-P (lanes 10,21), 360 nM His₆-CpxR-P (lanes 11,22), 720 nM His₆-CpxR-P (lanes 12,23), 36 nM His₆-CpxR (lane 2), 90 nM His₆-CpxR (lanes 3,14), 180 nM His₆-CpxR (lanes 4,15), 360 nM His₆-CpxR (lanes 5,16), or 720 nM His₆-CpxR (lanes 6,17). Sequencing ladders were generated by extension of ³²P-end-labeled DSBA3 primer for the coding strand or DSBA2 primer for the noncoding strand using pBTKSdsbA2 as a template. The core His₆-CpxR-P binding site is indicated by the thick line; the thin line indicates the limits of the sequence shown in b. (b) The location of CpxR-P binding sites in the promoter region of the *yihE-dsbA* operon. Elements of the putative σ^{70} promoter (Belin and Boquet 1994) are indicated; the core CpxR-P binding site is indicated by the brackets.

processing or maturation of proteins in the periplasm, it was of interest to determine whether the *ppiA* gene may also be a member of the Cpx regulon.

EMS and DNase I footprinting studies suggested that phosphorylated CpxR binds to a single site in the transcriptional regulatory region of the *ppiA* gene. Figure 8a shows DNase I footprinting analysis of the binding of the His₆-CpxR and His₆-CpxR-P proteins to a 267-bp fragment (derived from pBTKSp*ppiA*1) that includes the P1 promoter of the *ppiA* gene (Norregaard-Madsen et al. 1994). Under the conditions used in this analysis, clear DNase I footprints are readily apparent on the noncoding strand with the lowest concentration of His₆-CpxR-P (36 nM), whereas equivalent footprints are only observed with the highest concentration of unphosphorylated His₆-CpxR (360 nM). As the concentration of the His₆-CpxR-P protein is increased, the region of protection from DNase I cleavage again expands in a bidirectional fashion; a core His₆-CpxR-P footprint is indicated in Figure 8b.

Cpx regulates transcription of the ppiA gene

If the CpxR-P binding site in the *ppiA* promoter region is of physiological significance, then changes in expression of the *ppiA* gene may be expected to be observed under

conditions that activate the Cpx pathway. We therefore studied changes in the levels of RNA transcripts originating from the P1 promoter after induction of NlpE overexpression; the quantitated data is shown in Figure 3b. Expression from the *ppiA* P1 promoter is induced 2.5-fold when NlpE is overexpressed in strains containing a wild-type *cpxRA* operon but not in strains in which the *cpx* operon is deleted. This level of induction is lower than that seen for the *yihE-dsbA* operon, which was induced 5- to 10-fold in the same experiment.

To more definitively establish the role that the Cpx system plays in regulating the expression of the *ppiA* gene, we then examined the effect that various *cpx* mutations have on the expression of the *ppiA* P1 promoter. Three independent cultures of each strain containing either the wild-type *cpx* operon, the defined *cpxRA* null mutation, or one of two *cpxA** mutations were grown at 37°C, and the in vivo level of expression of the mRNA originating from the P1 promoter determined by RNase protection. The quantitated results were averaged and are shown in Figure 4b. Two strains containing different *cpxA** mutations both express significantly more (~2.5-fold; $P < 0.01$, $n = 3$) of the *ppiA* P1 transcript relative to the *cpx** parent strain. In addition, we also reproducibly observed a two- to threefold decrease ($P < 0.005$, $n = 3$) in expression of the P1 promoter in the *cpx* null mutant

Figure 8. (a) DNase I protection assays of the binding of His₆-CpxR and His₆-CpxR-P to the *ppiA* promoter region. A 267-bp DNA fragment (obtained by amplification of the *ppiA* region of pBTKSp*ppiA*1 with primers PPIA1 and PPIA2) was end-labeled with ³²P, and uniquely labeled substrates were generated by *Eco*RI (lanes 1–9) or *Bam*HI (lanes 10–18) digestion. The substrate DNA was present at ~2 nM in reactions that also contained no added protein (lanes 1,10), 36 nM His₆-CpxR-P (lanes 2,11), 90 nM His₆-CpxR-P (lanes 3,12), 180 nM His₆-CpxR-P (lanes 4,13), 360 nM His₆-CpxR-P (lanes 5,14), 36 nM His₆-CpxR (lanes 6,15), 90 nM His₆-CpxR (lanes 7,16), 180 nM His₆-CpxR (lanes 8,17), 360 nM His₆-CpxR (lanes 9,18). Sequencing ladders were generated by extension of ³²P-end-labeled PPIA1 primer for the coding strand or PPIA2 for the noncoding strand using pBTKSp*ppiA*1 as a template. The core His₆-CpxR-P binding site is indicated by the thick line; the thin line indicates the limits of the sequence shown in b. (b) The location of CpxR-P binding sites in the σ⁷⁰ P1 promoter region of the *ppiA* gene. Elements of the putative σ⁷⁰ P1 promoter (Norregaard-Madsen et al. 1994; this paper) are indicated; the core CpxR-P binding site is indicated by the brackets.

(Fig. 4b). The effects that *cpx* mutations have on the expression of the *ppiA* P1 promoter (Figs. 3b and 4b), taken together with results from DNase I footprinting (Fig. 8), suggest that the *ppiA* gene is a member of the Cpx regulon.

Discussion

New targets of the Cpx signal transduction pathway

Evidence presented here suggests that CpxA and CpxR directly regulate the expression of genes encoding periplasmic proteins required for protein folding and degradation. When the Cpx pathway is activated by *cpxA*^{*} mutations or by NlpE overexpression, DsbA and DegP are coordinately induced. Elevated expression of DsbA is attributable to increased transcription of the P2 promoter of the *yihE-dsbA* operon. The phosphorylated form of CpxR binds with significantly higher affinity than the unphosphorylated form to specific sequences within the transcriptional regulatory regions of the *degP* gene, the *ppiA* gene, and the *yihE-dsbA* operon. Transcriptional activation of these genes is therefore likely to be mediated by phosphorylation of CpxR by CpxA.

Identification of a consensus sequence for CpxR binding

Analysis of the sequences of the *degP* and *dsbA* promoters to which the His-tagged CpxR-P protein bound resulted in the identification of a putative consensus binding site for CpxR at σ^{70} promoters of 5'-GTAAN₍₆₋₇₎GTAA-3'. Using this sequence, we identified the *ppiA* gene, encoding a periplasmic PPIase, as a potential regulatory target. The results of DNA binding and expression studies suggest that the phosphorylated form of the CpxR protein (CpxR-P) directly serves as a transcriptional activator of the P1 (σ^{70}) promoter of the *ppiA* gene. However, Cpx-dependent induction of the *ppiA* transcript is lower than that observed for the *dsbA* or *degP* genes, and deletion of the *cpx* operon leads to a 2.5-fold decrease in *ppiA* P1 promoter expression. Hence, the Cpx system appears to contribute to the basal level of expression of the *ppiA* P1 promoter under normal growth conditions, suggesting that the unphosphorylated form of CpxR may be capable of binding to, and activating transcription from, the *ppiA* P1 promoter in vivo. Alternatively, a low level of the phosphorylated form of CpxR may be present in cells in "unstimulated" growth conditions. A similar conclusion can be drawn from the results of a previous study, as expression of the *degP* promoter was observed to be reproducibly lower in strains deleted for the *cpx* operon (Danese et al. 1995).

Mechanism of CpxR-mediated transcriptional activation

The results obtained are compatible with the notion that CpxR-P serves in a direct fashion as a transcriptional

activator of the σ^E promoter of the *degP* locus. By analogy with observations on the mechanisms by which other prokaryotic transcriptional activators function (Busby and Ebright 1994), it seems likely that CpxR-P bound at the *degP* promoter-proximal binding site (site I) may facilitate recruitment of σ^E -containing RNA polymerase holoenzyme by interacting with the carboxy-terminal domain of the α -subunit. The upstream CpxR-P-binding site (site II) is absent in the Φ (*degP-lacZ*) construct used in previous studies (Danese et al. 1995). Because we find no obvious discrepancies between our results obtained by examining the expression of DegP protein and those obtained using the *degP-lacZ* fusion, we cannot currently say how site II contributes to *degP* expression.

The location of the DNase I footprints in the *ppiA* and *yihE-dsbA* promoter regions suggests that transcriptional activation may be mediated by interaction of CpxR-P with the σ^{70} subunit of RNA polymerase holoenzyme, as is proposed for λ C1 protein-mediated activation at the λ_{PRM} promoter (Joung et al. 1994; Li et al. 1994), or via interaction with both the carboxy- and amino-terminal domains of the α -subunit as proposed for the catabolite activator protein at class II promoters (Niu et al. 1996). Hence, CpxR-P appears able to activate transcription of promoters recognized by both σ^E (*degP*) and σ^{70} (*ppiA* and *yihE-dsbA*) containing RNA polymerase holoenzymes.

Are other periplasmic protein-folding catalysts members of the Cpx regulon?

The identification of additional genes containing the consensus CpxR-binding site may help to further elaborate the Cpx regulon. For example, the regulatory region of the *surA* gene, the expression of which was not affected by *cpxA*^{*} mutations, does not contain the putative consensus sequence. Other genes such as *dsbB*, *dsbC*, and *dsbD* also lack obvious matches to the consensus sequence. However, a search of the *E. coli* genome database demonstrates that this sequence is located in the transcriptional regulatory regions of a number of potentially relevant genes, such as *rpoE*, *rpoH*, *groE*, and *skp* (*ompH*). As is shown in Figure 9, the 5'-GTAAN₍₅₎GTAAA-3' motif is also present in the upstream regulatory region of the *cpxRA* operon, suggesting that the operon maybe subject to positive feedback regulation.

Potential significance of the Cpx regulon

Several results suggest that increased expression of periplasmic folding catalysts effected by CpxA and CpxR may promote the folding or repair of cell envelope proteins. For example, overexpression of DsbA can suppress many of the phenotypes associated with a *dsbB* null mutation (Bardwell et al. 1993). Thus, although mutants deleted for the *cpx* operon are not defective in disulfide bond formation, it seems likely that under some conditions, DsbA induction by Cpx may promote disulfide

Figure 9. Alignment of the σ^{70} promoters of the *yihE-dsbA*, *ppiA*, and *cpxRA* operons. The putative “-35” and “-10” elements of the promoters are underlined, as are the bases corresponding to the sites of transcription initiation (*yihE-dsbA* and *ppiA*). Identity between the sequences are indicated (through the -35 element) with a “:” symbol.

bond formation. Furthermore, in vitro experiments show that the presence of a PPIase can increase the efficiency of a disulfide bond isomerase (Schonbrunner and Schmid 1992). Hence, the PPIase PpiA, which appears to be coordinately regulated with DsbA by Cpx, may enhance the catalytic efficiency of DsbA or other as yet to be identified protein disulfide isomerases. Increased expression of DegP may be necessary to degrade proteins that are inappropriately folded or which cannot be repaired following oxidative (or other) damage.

Does Cpx play a role in virulence?

The Cpx system may come into play when the cell experiences environmental conditions that affect protein folding, such as those that are thought to be encountered by certain *enterobacteriaceae* in host tissues during infection. For example, a variety of immune cells produce reactive oxygen species ($\text{NO}^\cdot$, $\text{O}_2^\cdot$) that can cause significant oxidative damage to proteins (Badwey and Karnovsky 1980; Nathan and Hibbs 1991; Babior 1992; Marletta 1993). Several studies have demonstrated that DegP is required for normal resistance to reactive oxygen species and is necessary for both survival in macrophages

and full virulence in mice (Johnson et al. 1991; Baumler et al. 1994; Elzer et al. 1994). Hence, it seems possible that the Cpx-dependent expression of DegP may contribute to the protection of the cell from the damaging effects of free radical oxygen species by proteolytically degrading damaged proteins in the cell envelope (Boucher et al. 1996; Govan and Deretic 1996). It will be of interest to ascertain if free radical damage of cell envelope proteins is one of the stimuli that leads to activation of the CpxRA system.

Mutations in some of the other genes of the Cpx regulon also result in attenuation of pathogenesis in vivo. In *Shigella sonnei*, the Cpx system regulates expression of the *virF* gene in response to pH changes (Nakayama and Watanabe 1995). VirF is a transcriptional activator that positively regulates expression of the IpaB, IpaC, and IpaD proteins, which are important for invasion of epithelial cells (Menard et al. 1996). Expression and proper folding of these proteins requires DsbA, and *S. flexneri dsbA* mutants are attenuated for invasion (Watarai et al. 1995). A DsbA homolog in *Vibrio cholera* is needed for the maturation of secreted virulence factors (Peek and Taylor 1992). It therefore seems possible that the Cpx system also regulates a variety of genes encoding products that are not directly involved in processes related to

Figure 10. A model showing the Cpx regulon and other proteins implicated in responding to cell envelope stress. See Discussion for details.

protein folding but that play important roles during pathogenesis.

Relationship between the Cpx and σ^E pathways

These findings and how they relate to results in the accompanying paper (Danese and Silhavy, this issue) are summarized in Figure 10. *E. coli* contains at least two periplasmic stress response systems, the Cpx pathway and the σ^E pathway. The σ^E pathway is induced by conditions that lead to misfolding or misassembly of outer membrane proteins (Omps), such as overexpression of Omps or inactivation of the *surA* gene (Mecenas et al. 1993; Rouviere et al. 1995). Stress signals from the cell envelope are communicated to σ^E by the Rse proteins (De Las Peñas 1997). In response to these stresses, σ^E transcribes the *degP* gene and a gene encoding another periplasmic PPIase, *fkpA* (see Danese and Silhavy, this issue). The Cpx pathway responds to cell envelope stresses such as changes in pH or NlpE overexpression and activates the transcription of genes transcribed from both σ^{70} and σ^E promoters (Danese et al. 1995; Nakayama and Watanabe 1995; Danese and Silhavy, this issue). The Cpx regulon includes at least *dsbA*, *degP*, and *ppiA* and possibly also *virF* and *cpxRA*, all of which contain sequences similar to the CpxR consensus sequence (solid boxes in Fig. 10) upstream of their promoters. The Cpx and σ^E pathways, which appear to respond to different stresses and generally regulate different genes, overlap to control the expression of the *degP* gene. The presence of a putative CpxR-binding site in the regulatory regions of several members of the σ^E regulon may indi-

cate that the Cpx and σ^E regulons form a network of overlapping and possibly closely integrated regulatory pathways.

Materials and methods

Bacterial strains

The relevant genotypes of the bacterial strains used in this study are listed in Table 1. Three different *cpxA*⁺ alleles were used in this study. The *cpxA9* mutant is temperature sensitive, exhibiting *cpxA*⁺ phenotypes at 42°C but not at 30°C. The other two mutants, *cpxA2.1* and *cpxA9.4*, are phenotypically more severe than the *cpxA9* mutant and exhibit *cpxA*⁺ phenotypes at both 23°C and 42°C.

Immunoprecipitation of radiolabeled proteins

The rates of synthesis of DsbA, DegP, SurA, MBP, and OmpA were determined by pulse-chase immunoprecipitation experiments. Strains were grown at the indicated temperatures in M63 medium containing 0.2% maltose supplemented with 18 amino acids (50 µg/ml each). Cultures were given a 20-sec pulse of [³⁵S]methionine (100 µCi/ml) and chased with excess, unlabeled methionine. Samples were taken after 1 min of chase, and the labelings were stopped on ice. Immunoprecipitation of radiolabeled proteins was carried out essentially as described (Pogliano and Beckwith 1993). Rabbit polyclonal antibodies specific for each protein, DsbA (Bardwell et al. 1993), DegP, SurA (a gift of S. Lazar and R. Kolter, Harvard Medical School, Boston, MA), and OmpA were used for immunoprecipitations. DegP, SurA, MBP, and OmpA were separated by 10% SDS-PAGE. DsbA and OmpA were separated by 12.5% SDS-PAGE. The counts in each radioactive band were quantitated using a Molecular Dynamics PhosphorImager. Counts in the DegP and DsbA bands were normalized to OmpA as an internal control

Table 1. Strains and plasmids

	Relevant genotype	Reference
Strain		
ECL525	MC4100 Δ (<i>argF-lac</i>) <i>U169 araD139</i>	Iuchi and Lin (1988)
ECL3000	MC4100 Δ (<i>rha-cpxRA-pfkA</i>)15 <i>zig-1::Tn10</i>	A.S. Lynch and E.C.C. Lin (unpubl.)
JP406	ECL525 F'pOXgen	J. Dong, J. Pogliano, and E.C.C. Lin (unpubl.)
ECL1212	JP406 Δ (<i>cpxRA</i>)2	J. Dong, J. Pogliano, and E.C.C. Lin (unpubl.)
JP408	JP406 <i>zii::Tn10 cpxA9</i>	this study
JP313	MC4100 <i>ara</i> Δ 714	this study
JP444	JP313/pDB3	this study
JP445	JP313/pBAD22	this study
JP446	JP313/pND18	this study
JP466	JP406 <i>argE::Tn10</i>	this study
JP467	JP406 <i>argE::Tn10 cpxA2.1</i>	this study
JP468	JP406 <i>argE::Tn10 cpxA9.4</i>	this study
ECL1215	JP406 <i>ara</i> ⁺	this study
ECL1216	ECL1212 <i>ara</i> ⁺	this study
JP472	ECL1215/pBAD22	this study
JP474	ECL1216/pBAD22	this study
JP558	ECL1215/pND18	this study
JP560	ECL1216/pND18	this study
Plasmid		
pBAD22	<i>bla</i> , pBR ori, Pbad promoter	Guzman et al. (1995)
pND18	overexpresses NlpE	Danese et al. (1995)
pDB3	overexpresses alkaline phosphatase	Guzman et al. (1995)

and expressed in arbitrary units. OmpA is expressed constitutively at high levels, and its expression is not affected by the conditions used here; similar results were obtained if DegP or DsbA expression was normalized to the culture OD₆₀₀ at the time of sampling. To ensure that the DegP protein immunoprecipitated in these experiments is encoded by the *degP* gene, a *cpxA9 degP::Tn5* double mutant was also examined; no protein that migrates at the position of DegP was seen in this strain (data not shown).

Plasmid constructions

Plasmids pQE30 and pREP4 were obtained from Qiagen Ltd. To create pQE30CpxR, a 700-bp region of DNA containing the *cpxR* open reading frame (positions 1246–1946 in GenBank file L14579) was amplified by PCR with primers CPXR5 (5'-CCCGGATCCCATATGAATAAAATCCTGTTAGTTGATG-3') and CPXR3 (5'-CCCGGATCCTGCAGTTATCATGAAGCAGAAACCATCAG-3') using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Pst*II, and the resulting 710-bp fragment was cloned between the corresponding sites of pQE30. pQE30CpxR, expresses a CpxR fusion protein, composed of full-length CpxR with an amino-terminal Met-Arg-Gly-Ser-[His]₆-Gly-Ser-His-extension, under transcriptional control of an isopropyl-thiogalactoside (IPTG)-regulated phage T5 promoter.

In the following series of constructions, the pairs of oligonucleotides described were used to amplify the indicated regions of *E. coli* MC4100 genomic DNA, and the PCR products were cloned between the *Bam*HI and *Eco*RI sites of pBlue-script II KS(-); sequence verification of the cloned, amplified DNA was performed in all cases. pBTKSdegP1 contains a 353-bp segment of the *degP* promoter region (positions 69180–69533 in GenBank file D26562), amplified with primers DEGP1 (5'-CCGGATCCGCCATCGGCTGGCCTATGTC-3') and DEGP2 (5'-CCGAATTCGCCAAACCTAAACTCAGACC-3'). pBTKSdegP2 contains a 235-bp segment of the *degP* promoter region (positions 69298–69533), amplified with primers DEGP3 (5'-GGGATCCGACCTCTATGCGTGGGATG-3') and DEGP2 (5'-CCGAATTCGCCAAACCTAAACTCAGAGCC-3'). pBTKSdegP3 contains a 201-bp segment of the *degP* promoter region (positions 69143–69344), amplified with primers DEGP4 (5'-CGGATCCCTATTGAATCGCGCTTATTCAC-3') and DEGP5 (5'-GGGAATTCTACGGCCATCAGACGTCGG-3'). pBTKSdsbA1 contains a 350-bp segment of the region upstream of the *yihE-dsbA* operon (Belin and Boquet 1994; positions 760–1110 in GenBank file X80762), amplified with primers DSBA1 (5'-CCGGATCCTGAATGAAGTTATTGAACCTCTCC-3') and DSBA2 (5'-CCGAATTCGTAAGCGCTGTTATTCATCCC-3'). pBTKSdsbA2 contains a 260-bp segment of the region upstream of the *yihE-dsbA* operon (positions 850–1110) amplified with primers DSBA3 (5'-CCGGATCCAGGTTTTGACGGCGAACTGGC-3') and DSBA2 (5'-CCGAATTCGTAAGCGCTGTTATTCATCCC-3'). Finally, pBTKSppiA1 contains a 253-bp segment of the *ppiA* promoter region (Kawamukai et al. 1989; positions 363–616 in GenBank file M28363) and was amplified with primers PPIA1 (5'-GGGATCCAGGCGGCGATTTTAGGTG-3') and PPIA2 (5'-GGGAATTCATCGCCGCCAGGGTCG-3').

Plasmid pDB7601 contains the 146-bp *Hind*III–*Bam*HI fragment of pPB2234 (Belin and Boquet 1994) cloned in pBSKS; the DNA was linearized with *Hind*III and transcribed with T7 RNA polymerase. The 193-nucleotide cRNA yields a 95-nucleotide S1 nuclease-protected fragment after hybridization to *yihE-dsbA* mRNA initiated at the upstream, P2 promoter. Plasmid pDB5601 contains the 520-bp *Hind*III–*Bgl*II fragment of

pPB3224 cloned between the *Bam*HI and *Hind*III sites of pSP65; the DNA was linearized with *Hpa*I and transcribed with SP6 RNA polymerase. The 414-nucleotide cRNA yields a 310-nucleotide protected fragment after hybridization to *dsbA* mRNA initiated at the downstream, P1 promoter. A PCR-amplified *ppiA* DNA fragment (positions 273–697; Kawamukai et al. 1989; GenBank accession no. M28363) was restricted with *Eco*RI and *Pvu*II and subcloned between the *Eco*RI and *Sma*I sites of pBSKS. Plasmid DNA was digested with *Dra*I and transcribed with T7 RNA polymerase. The 355-nucleotide cRNA yields a 147-nucleotide protected fragment after hybridization to *ppiA* mRNA initiated at the P1 promoter [Norregaard-Madsen et al. 1994].

RNA isolation

Cells were grown in M63 medium supplemented with 18 amino acids (50 µg/ml each), 0.2% maltose and 0.4% glycerol, and induced with 0.2% arabinose for the indicated times. Plasmid-bearing cells were grown in medium supplemented with 100 µg/ml of ampicillin. Aliquots were labeled for 30 sec with [³⁵S]methionine at the time of RNA isolation, and the lysates were immunoprecipitated with appropriate antibodies as described (Bost and Belin 1995). Culture samples (2 ml) were lysed by boiling for 2 min after addition of 0.2 ml of 0.5 M Tris-HCl (pH 8.1), 0.4% SDS, and 20 mM EDTA (Belin et al. 1979). The lysates were made 0.2 M with NaOAc (pH 4.7), extracted twice with phenol/chloroform, and precipitated with ethanol after addition of one-sixth volume of glacial acetic acid. Nucleic acids were resuspended in 10 mM Tris-HCl (pH 7.5), 20 mM NaCl, and 5 mM MgOAc, digested for 20 min at 37°C with 30 units of RNase-free DNase (Promega), extracted with phenol/chloroform, and precipitated with ethanol. The RNA yield was ~50 µg of cells (*A*₅₄₀); the integrity of the RNAs was verified by electrophoresis.

RNase protection

The preparation of probes, hybridization, digestion of the unhybridized RNAs with RNase A, and analysis of the protected fragments were performed as described (Belin 1996a,b). The probes were labeled with [³²P]UTP in the presence of 100 µM unlabeled UTP. For each sample, 2 µg of cellular RNA was hybridized to 4 ng of cRNA probe.

DNA-binding studies

Purification of the His₆-CpxR protein from IPTG-induced *E. coli* ECL3000 cells transformed with pREP4 and pQE30CpxR was performed as described previously for a similarly tagged protein (His₆-ArcA) of the two-component response regulator family (Lynch and Lin 1996). On average, 2.5 mg of purified His₆-CpxR was obtained from 1 gram of cell paste. Phosphorylation of the protein for use in DNA-binding studies used a standard reaction in which His₆-CpxR (100 µg/ml of final concentration) was incubated for 1 hr at 30°C in a buffer containing 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 125 mM KCl, and 50 mM acetyl phosphate (lithium, potassium salt from Sigma). In parallel, similar reactions lacking acetyl phosphate were used to prepare His₆-CpxR for use in DNA-binding studies. The conditions used for in vitro DNA-binding assays by EMS and DNase I footprinting assays were as described previously (Lynch and Lin 1996).

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