LexA Represses CTXΦ Transcription by Blocking Access of the α C-terminal Domain of RNA Polymerase to Promoter DNA*

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CTXΦ is a Vibrio cholerae-specific temperate filamentous phage that encodes cholera toxin. CTXΦ lysogens can be induced with DNA damage-inducing agents such as UV light, leading to the release of CTXΦ virions and the rapid dissemination of cholera toxin genes to new V. cholerae hosts. This environmental regulation is directly mediated by LexA, the host-encoded global SOS transcription factor. LexA and a phage-encoded repressor, RstR, both repress transcription from PrstA, the primary CTXΦ promoter. Because the LexA binding site is located upstream of the core PrstA promoter and overlaps with A-tract sequences, we speculated that LexA represses PrstA by occluding a promoter UP element, a binding site for the C-terminal domain of the α subunit of RNA polymerase (RNAP) (αCTD). Using in vitro transcription assays, we have shown that the LexA binding site stimulates maximal rstA transcription in the absence of any added factors. The αCTD of RNAP is required for this stimulation, demonstrating that the LexA site contains, or overlaps with, a promoter UP element. LexA represses rstA transcription by normal RNAP but fails to repress rstA transcription catalyzed by RNAP lacking the αCTD. DNase I footprint analysis mapped the αCTD binding site to the upstream promoter region that includes the LexA binding site. The addition of free α subunits blocked the binding of LexA to rstA promoter DNA, indicating that LexA and the αCTD directly compete for binding to their respective sites. To our knowledge, this is the first report of a repressor blocking transcription initiation by occluding a promoter UP element.

CTXΦ, a lysogenic filamentous phage, has played a critical role in the evolution of toxigenic Vibrio cholerae, the causative agent of the diarrheal disease cholera. The ~6.9-kb CTXΦ genome encodes cholera toxin, the principal virulence factor of this Gram-negative enteric pathogen. Following infection of V. cholerae, the CTXΦ genome integrates in a site-specific fashion near the terminus of chromosome 1 (1–3), generating a CTX prophage. Even though CTXΦ virions are secreted from V. cholerae without cell lysis, the expression of prophage genes required for CTXΦ virion production is ordinarily repressed, as is also the case for temperate phages that lyse their respective hosts upon prophage induction. Most knowledge of prophage induction has been obtained from studies of phage λ and some closely related temperate phages that are unrelated to CTXΦ. We are studying the environmental conditions and molecular processes that control CTXΦ virion production to expand basic understanding of cellular processes that govern phage development and to gain insight into factors that may contribute to the emergence of new pathogenic V. cholerae strains.

Previous studies suggest that the expression of the genes required for CTXΦ production initiates from a single promoter, PrstA. This promoter is located in ig-2, an intergenic region that separates rstA (a gene required for CTXΦ replication) from rstR, the gene encoding the phage repressor (see Fig. 1A). RstR binds to three operators within ig-2 and represses transcription from PrstA (4, 5). In addition to RstR, we recently found that LexA, a host repressor that regulates the SOS regulon, also represses PrstA (6). LexA represses PrstA by binding to an A + T-rich site positioned at −41 to −56 from the start of rstA transcription (Fig. 1A) (6). This 16-bp sequence is nearly identical to the consensus Escherichia coli LexA binding site (7).

The V. cholerae SOS response to DNA damage activates CTXΦ production (6). DNA-damaging agents, such as mitomycin C or UV light, increase transcription from PrstA and production of CTXΦ virions from CTXΦ lysogens in a recA-dependent fashion. No stimulation of transcription from PrstA or CTXΦ production was observed following UV treatment of CTXΦ lysogens that contained a noncleavable LexA (6). We proposed that the activated form of RecA generated by DNA damage provokes the auto-cleavage of LexA, thereby alleviating its repression of PrstA. RstR levels also decline after treatment of CTXΦ lysogens with DNA damaging agents. However, unlike the λ repressor CI, RstR does not appear to undergo RecA-stimulated auto-cleavage, and the mechanism that accounts for the decrease in RstR levels in UV-treated CTXΦ lysogens is unknown.

Maximal expression of rstA requires sequences upstream of the −10 and −35 binding sites for the σ subunit of RNA polymerase (RNAP)2 in PrstA (6). We hypothesized that these sequences, which are rich in runs of A/T or T-A base pairs and overlap with the LexA binding site in PrstA, function as a promoter UP element. UP elements are A + T-rich sequences found upstream of the −35 element in many highly active promoters, where they function to increase promoter strength by binding the C-terminal domain (αCTD) of RpoA, the σ subunit of RNAP (8–11). UP elements have also been characterized for

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2 The abbreviations used are: RNAP, RNA polymerase; WT, wild type.

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LexA Regulation of Bacteriophage Transcription

In Vitro Transcription—Briefly, 25-μl reaction mixtures contained 0.5 nm supercoiled plasmid DNA in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM MgCl₂, 1.0 mM dithiothreitol, 100 μg/ml bovine serum albumin, 500 μM CTP, 100 μM ATP, 100 μM GTP, and 10 mM UTP with [α-32P]UTP (PerkinElmer Life Sciences) at a specific activity of ~30 Ci/mmol. The control plasmid pRLG593 was added to each reaction at a concentration of 0.5 nm. RNA was added to initiate transcription, and the reaction proceeded for 15 min at room temperature (25°C). In experiments with added repressors, template DNA was preincubated at room temperature for 15 min with LexA and/or RstR in reaction buffer prior to the addition of RNAP. With the exception of experiments utilizing RstR or LexA, reactions were terminated with the addition of an equal volume of stop solution (7 mM urea, 10 mM EDTA, 1% SDS, 2 mM Tris-borate-EDTA, 0.5% bromphenol blue, 0.025% xylene cyanol). In experiments that analyze RstR and LexA repression, reactions were terminated by ethanol precipitation. Pellets were air-dried and resuspended in a formamide loading buffer (25 mM Tris-HCl, pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50% glycerol), concentrated by centrifugation in Centricon filters (Millipore), and stored in aliquots at ~80°C. His-α preparations were estimated to be >90% pure by Coomassie Blue-stained SDS-polyacrylamide gels. For reconstituted RNAP, His-α and His-αΔ235 were overexpressed from plasmids pRLG3538 and pRLG3545, respectively, and purified by nickel affinity chromatography as described previously (13). Protein fractions were dialyzed against storage buffer (25 mM Tris-HCl, pH 7.9, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50% glycerol), concentrated by centrifugation in Centricon filters (Millipore), and stored in aliquots at ~80°C. His-α preparations were estimated to be >90% pure by Coomassie Blue-stained SDS-polyacrylamide gels. For reconstituted RNAP, His-α and His-αΔ235 were overexpressed from plasmids pRLG3538 (WT) or pRLG3545 (Δ235) and purified by nickel affinity chromatography. RNA polymerase β, β', and σ⁺ subunits were overexpressed, and holoenzyme was reconstituted in vitro as described previously (13). The specific activities of reconstituted RNAP was determined using a promoter binding assay on lacUV5 (17).

RESULTS

The LexA Binding Site in PₙrstA Is Required for Maximal Transcription—To explore the possibility that the LexA binding site in PₙrstA overlaps with or contains a UP element, we replaced the LexA operator region (positions −40 to −58) (Fig. 1A) with SUB, a 19-bp sequence that was previously shown not to function as a UP element (8, 15). The influence of this substitution on PₙrstA activity was assessed by in vitro transcription assays using purified E. coli RNA polymerase. The use of E. coli RNAP is justified by our previous findings that 1) The rstA promoter is efficiently transcribed in both E. coli and V. cholerae (5, 6), 2) LexA repression of PₙrstA transcription is observed in both V. cholerae and E. coli (6), and 3) the αCTD of E. coli and V. cholerae RNAP (including all of the determinants important for transcription).
for DNA binding) are highly conserved (12). A plasmid containing the lacUV5 promoter that lacks a UP element (14) was included in the same reaction as a control. The quantity of rsaA transcribed in vitro was reduced ~4-fold when the DNA template carried the SUB sequence in place of the normal DNA sequence upstream of P_{rstA} (Fig. 1B, lanes 1 and 2). These observations indicate that the LexA binding site in P_{rstA} includes sequences that stimulate transcription in the absence of additional factors, consistent with the presence of a UP element in the substituted region (~40 to ~58).

To further investigate whether the LexA binding site includes a UP element, we performed in vitro transcription experiments using RNAP reconstituted with αΔ235, an α subunit that is deleted for the αCTD that directly contacts UP element DNA (18–20). In contrast to WT RNAP, the relative level of rsaA transcripts generated by αΔ235 RNAP was reduced ~4-fold (Fig. 1B, compare lanes 1 and 5). Furthermore, in reactions with αΔ235 RNAP, the relative level of rsaA transcripts was not reduced when the LexA binding site was substituted by SUB in the template DNA (Fig. 1B, lanes 5–8). Thus, maximal transcription from P_{rstA} requires both the upstream LexA binding site and the intact αCTD of RNAP. Taken together, these observations suggest that P_{rstA} contains a UP element within or overlapping the LexA binding site.

RNAP Binding to P_{rstA}—DNase I protection assays were performed to physically define the sites in ig-2 bound by RNAP. Because specific binding of RNAP to promoter sequences is often obscured by nonspecific DNA binding, RNAP-DNA complexes were treated with heparin sulfate, a polyanion that irreversibly binds free RNAP, prior to DNase I treatment. Protection of the P_{rstA} promoter by WT RNAP was observed from ~−60 to ~+25, including the core promoter elements (~−10, −35, and transcription start site +1) and the region upstream of the core promoter elements (from ~−45 to ~−60; Fig. 2, see arrows). Similar to WT RNAP, αΔ235 RNAP protected the core promoter elements (although higher concentrations were required). However, αΔ235 RNAP did not protect sequences upstream of the −35 element (Fig. 2). Thus, the αCTD of RNAP is responsible for the upstream footprint. This region of the promoter overlaps the LexA binding site (~41 to ~56) (Fig. 2A).

DNase I protection assays were also performed on a promoter construct containing the SUB sequence in place of the LexA box (SUB RstaA). WT RNAP protected the core promoter regions but protected sequences upstream of the −35 element weakly or not at all (Fig. 2), consistent with the footprints observed at other promoters lacking UP elements (e.g. lacUV5 and λpr, (21, 22)). Together, the data suggest that the upstream region of P_{rstA} contains sequence-specific binding sites for the αCTDs.

To confirm that the protections observed upstream of the −35 element resulted from interactions with the RNAP α subunit, as observed in previous studies with the rrnB P1 promoter (8), DNase I protection experiments were also carried out with purified E. coli α subunits (Fig. 3). Free α protected upstream sequences that include those protected by RNAP (Fig. 2), and protection was observed at the α concentrations similar to those required for binding to other UP elements (2 μM; Ref. 8). No protection was observed with αR265A (Fig. 3, lanes 7 and 8), a mutant α that does not bind DNA (8, 20). The sequences protected by purified α extended further upstream than those observed with RNAP holoenzyme (Fig. 2). Similar extended footprints have been observed previously with purified α at other promoters (8, 19). There is evidence in some promoters for binding of αCTD to DNA minor grooves upstream of the interactions detected in the rrnB P1 UP element (17, 23). However, it is also possible that the extended footprint with free α simply reflects nonphysiologically relevant interactions of additional α subunits with additional A + T-rich upstream sequences. Although small effects on the transcription of α binding to rsaA sequences upstream of ~−60 cannot be ruled out, the effects of the SUB mutation on P_{rstA} transcription (Fig. 1B) suggests that the ~−41 to ~−58 region is sufficient to account for the major effects of αCTD binding on rsaA transcription stimulation.

To assess whether LexA and α compete for binding to the same site, both proteins were combined with the DNA template at concentrations sufficient to protect their respective binding sites (Fig. 3). Lane 9 shows the LexA footprint in the absence of
α. As the α concentration was increased (Fig 3, lanes 11 and 12), protection of the LexA site was reduced, indicating that α inhibits LexA binding. In regions distal to the position −64, where LexA binding does not obscure the α footprint, α binding was also reduced when both proteins were added to the DNA. The simplest interpretation of these footprint data is that the two proteins directly compete for binding to overlapping DNA sites (see also "Discussion"). Such mutual inhibition likely occurs by a direct steric mechanism.

The αCTD Is Required for LexA Repression of PrstA in Vitro—Our discovery of a UP element at PrstA plus our finding that free α and LexA compete for binding to upstream DNA, suggests that the mechanism of LexA repression is to physically occlude αCTD from its binding site upstream of PrstA. To further test this model, we investigated whether LexA could repress PrstA transcription catalyzed by reconstituted αΔ235 RNAP, which lacks the αCTD. The plasmid templates for these experiments were p770ig-2 (−160 to +92) and pRLG593 carrying the lacUV5 promoter as a control. LexA specifically reduced PrstA transcription catalyzed by WT RNAP (Fig. 4, lanes 1–4). At high LexA concentrations, there was an ~5-fold reduction in the level of PrstA transcripts, whereas it had little or no effect on transcription from the control lacUV5 template. This partial repression by LexA in vitro is similar to previous in vivo measurements of LexA repression (6) and is strikingly similar to the 4-fold enhancement of PrstA transcription provided by the UP element (Fig. 1B). RstR was a more potent repressor of PrstA transcription than LexA, reducing transcription to nearly undetectable levels (Fig. 4, lane 5). In contrast to WT RNAP, PrstA transcription by αΔ235 RNAP was not repressed by LexA (Fig. 4, lanes 6–9). Identical results were obtained when the concentration of αΔ235 RNAP was lowered from 40 nM to 20 nM (data not shown). These data indicate that LexA repression of PrstA transcription results from inhibition of αCTD binding to the PrstA UP element.

Transcription by αΔ235 RNAP was slightly stimulated (~50%) by LexA (Fig. 4, lanes 6–9). This weak stimulation could be due to fortuitous protein-protein contacts between LexA and αΔ235 RNAP, or through structural changes in promoter DNA upon LexA binding.

RstR was still a potent repressor of PrstA transcription catalyzed by αΔ235 RNAP (Fig. 4, lane 10), indicating that RstR functions by blocking RNAP binding to the core promoter elements or represses a later step in transcription initiation. These observations demonstrate that LexA and RstR independently repress PrstA in the absence of other cellular factors and that LexA-mediated repression of transcription from PrstA requires the C-terminal domain of α.
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FIGURE 3. DNase I protection analysis of purified α and LexA binding to
ig-2 DNA. His-α, His-R265α, or LexA were incubated with 32P-labeled pro-
moter DNA at room temperature for 30 min prior to DNase I treatment. Lane
1, probe DNA only. Lanes 2–6, His-α added at 0.22, 0.72, 1.3, 1.8, and 2.4 μM.
Lanes 7 and 8, His-R265α at 1 and 5 μM, respectively. Lanes 9–12, LexA added
in 3.7 μM. Lanes 10–12, His-α added at 0.22, 1.8, and 2.4 μM. The region pro-
tected by His-α is depicted by the white bar, and the region protected by LexA
is depicted by the black bar. Numbering to the right indicates positions relative
to the transcription start site of PrstA.

DISCUSSION

Our previous studies of the regulation of PrstA revealed that a host repressor, LexA, and the CTXΦ-encoded repressor RstR both repress transcription from P_{rstA}. Using in vitro transcription assays, we showed here that each of these repressors acts directly on P_{rstA} to inhibit transcription. The LexA binding site in ig-2 is centered −48.5 bp from the start of rstA transcription; given its location and high A + T content, we speculated that it overlaps with a binding site for the C-terminal domain (αCTD) of the α subunit of RNAP. Such α binding sites in other highly active promoters are known as UP elements (8, 11). Our experi-
mental observations support this hypothesis. High level transcrip-
tion from P_{rstA} was dependent on specific sequences between positions −40 and −58 overlapping the LexA binding site. Replacement of the LexA box in this region with a sequence (SUB) that does not bind to the αCTD significantly reduced transcription from P_{rstA}. The stimulatory effect of this promoter site on P_{rstA} transcription was dependent upon the αCTD of RNAP; in vitro transcription with reconstituted RNAP deleted for the αCTD yielded a pronounced reduction in the level of transcripts from wild-type P_{rstA} but did not reduce

transcript levels from a mutant P_{rstA} containing the SUB sequence in place of the LexA box.

In DNase I protection experiments, RNA polymerase holoenzyme protected several sites clustered around positions −46 and −58 that likely represent interactions with the αCTD (Fig. 2). α subunit interactions in the context of the RNAP holoenzyme have been observed at a series of positions in the minor groove upstream of the −35 hexamer (23–25). Typically, the interactions most significant for function are just upstream of the −35 hexamer. In the E. coli rrnB P1 UP element, these sites are centered at −41 and −52 and are referred to as the proximal and distal subsites, respectively (10, 26). Because the SUB mutation, which extends from −41 to −58 in the rstA promoter, eliminated effects of upstream sequences on transcription, it is likely that this region contains the α binding sites corresponding to the proximal and distal subsites. Determining the precise limits of the α-DNA interactions in the rstA promoter region will require the use of other reagents (e.g. hydroxyl radical footprinting).

LexA and free α competed for binding to this region. Our findings strongly suggest that LexA represses transcription from P_{rstA} by blocking the binding of the αCTD to the promoter UP element. It is likely that αCTD binds to successive minor grooves in the P_{rstA} UP element (e.g. from −40 to −60), similar to its position in other characterized UP elements (8, 23, 26). The LexA dimer interacts with two successive major grooves using a winged helix-turn-helix DNA binding motif (27). With its binding site centered at position −48.5, it is likely that LexA would occupy the same face-of-the-helix as the αCTD and would overlap with and/or occlude DNA backbone positions along the minor groove that would be required for αCTD binding.
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Although UP elements sometimes overlap with binding sites for other DNA binding proteins (28) and it has been proposed previously that repressors could act by blocking binding of the $\alpha$ subunits of RNAP to a UP element (26, 29), to our knowledge our findings represent the first direct demonstration of this type of transcription regulation. This mechanism could, at least in part, explain SOS control of some other promoters. For example, the ssb gene in *E. coli* has a LexA box centered at position $-46.5$ (30), where LexA binding may also block access of the cCTD to a promoter UP element. A rationale for LexA-mediated repression of high level transcription of SOS genes could be to enable transient bursts of expression of DNA repair genes whose function is important for the amelioration of cellular stress. LexA cleavage during an SOS response would allow for transient high level expression (promoted by cCTD binding to the unoccupied LexA box) of SOS genes; high level expression would be relatively short-lived because resynthesis of LexA after repair of DNA damage would restore expression of these genes to their basal state.

The cCTD is a common target for regulatory factors in bacteria. Usually these transcription factor-cCTD interactions are positive, leading to increased transcription (9), but in some cases, these interactions result in transcription repression. Examples include IclR of *E. coli* (which has been proposed to repress the aceB promoter by relocation of the cCTD to an upstream position less favorable for stimulating transcription (31)), GalR (where cCTD-GalR interactions inhibit a step in transcription after closed complex formation (32, 33)), and Spx of *Bacillus subtilis* (which has been described as an “anti-$\alpha$” factor that binds to the cCTD and inhibits transcription initiation (34, 35)). Furthermore, phage T4 encodes factors that ADP-ribosylate the residue in $\alpha$ that is most critical for UP element binding, resulting in inhibition of host transcription and thus an increase in T4 early transcription (36). The data presented here indicate that LexA, the global SOS repressor, can inhibit transcription initiation in yet another manner, by selectively competing for $\alpha$ subunit binding to a UP element in the primary promoter of CTXΦ.

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