Cyclic AMP Receptor Protein-Dependent Repression of Heat-Labile Enterotoxin

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Enterotoxigenic Escherichia coli is a major cause of acute diarrheal illness worldwide and is responsible for high infant and child mortality rates in developing nations. Two types of enterotoxins, one heat labile and the other heat stable, are known to cause diarrhea. The expression of soluble heat-labile toxin is subject to catabolite (glucose) activation, and three binding sites for cAMP receptor protein (CRP or CAP) were identified upstream and within the toxin promoter by DNase I footprinting. One CRP operator is centered at −31.5, thus encompassing the promoter’s −35 hexamer. Potassium permanganate footprinting revealed that the occupancy of this operator prevents RNA polymerase from forming an open complex in vitro. However, the operator centered at −31.5 is not sufficient for full repression in vivo because the deletion of the other two CRP binding sites partially relieved the CRP-dependent repression of the heat-labile toxin promoter. In contrast to heat-labile toxin, CRP positively regulates the expression of heat-stable toxin. Thus, the conditions for the optimal expression of one enterotoxin limit the expression of the other. Since glucose inhibits the activity of CRP by suppressing the pathogen’s synthesis of cyclic AMP (cAMP), the concentration of glucose in the lumen of the small intestine may determine which enterotoxin is maximally expressed. In addition, our results suggest that the host may also modulate enterotoxin expression because cells intoxicated with heat-labile toxin overproduce and release cAMP.

The type I heat-labile toxin (LT-I) of enterotoxigenic Escherichia coli (ETEC) is a multimeric A-B5-type toxin that acts on Gsα, a protein that governs the activity of adenylate cyclase in eukaryotic cells. The ADP ribosylation of Gsα results in the overproduction of cyclic AMP (cAMP), which causes increased chloride secretion and disrupts sodium absorption (33). Water is lost to the intestinal lumen as a consequence of these alterations of ion transport (36). Like many extracellular proteins, the toxin subunits have amino-terminal signal peptides that allow the Sec-dependent general secretory pathway to transport them to the periplasm where assembly of the heteromer takes place. Transport across the outer membrane is dependent upon the main terminal branch of the general secretory pathway, otherwise known as type II protein secretion (45). After secretion, a significant portion of the toxin remains associated with the bacterial cell through an interaction between the B subunit and lipopolysaccharide (22). Membrane-bound toxin is destined to become a surface feature of outer-membrane vesicles as they are released from the cell (22, 23). Once released, toxin-coated vesicles are capable of intoxicating eukaryotic cells (25). The delivery of LT-I is also stimulated when ETEC contacts eukaryotic cells, but the stimulatory mechanism, which does not affect the transcription of LT-I genes, is not fully understood (14).

LT-I is structurally and mechanistically similar to cholera toxin of Vibrio cholerae (33, 41). The expression of cholera toxin is positively regulated through a regulatory cascade involving TcpP and TcpH, which activate the expression of toxT in conjunction with ToxR and ToxS. ToxT then activates the expression of the toxin genes ctxAB (30). The expression of cholera toxin is negatively regulated by cAMP receptor protein (CRP or CAP for catabolite activator protein), a homodimeric protein that represses the expression of tcpPH (27, 42).

As with cholera toxin, CRP has also been implicated as a transcriptional regulator of eltAB, the two genes encoding LT-I. The expression of the eltAB bicistronic message is inhibited by glucose (17). As for other catabolite-repressed genes, this glucose effect was shown to be dependent upon CRP and adenylate cyclase, the enzyme that produces cAMP (17). Since CRP cannot bind DNA in the absence of its cAMP cofactor, its ability to activate the expression of eltAB is abolished when glucose inhibits the activity of adenylate cyclase. Curiously, the addition of glucose to culture medium has also been reported to increase the yield of soluble LT-I as determined by toxin activity assays (18). To reconcile these two disparate studies, a model has been proposed wherein the expression of eltAB is positively regulated by CRP, while posttranscriptional events, such as toxin assembly and/or secretion, are stimulated by glucose (17). However, in this study, we disprove this convoluted regulatory model by demonstrating that CRP does not activate the expression of eltAB. Rather, CRP represses eltAB expression, and this repression is mechanistically consistent with the previously reported glucose stimulation of LT-I production. Furthermore, the expression of LT-I is inversely related to that of heat-stable toxin (STA), which suggests that the two toxins may be temporally and spatially separated during the course of an infection.

MATERIALS AND METHODS

Plasmids and strains. ETEC strain H10407 (O78:H11 CFA/I STa+ LT-I+) was isolated from an adult patient with acute cholera-like diarrhea in Dacca, Bangladesh (15). The LT-I promoter, eltAp from −410 to +154, was amplified from H10407 with primers SN577 and SN578. The primer sequences are shown in Table 1. The STA promoter, estAp from −218 to +30, was amplified from...
TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence*</th>
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<tbody>
<tr>
<td>SN58</td>
<td>CCCAGTTCGCGGCGACGAT</td>
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<td>SN59</td>
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<td>SN237</td>
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<td>SN577</td>
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<td>SN578</td>
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<td>SN582</td>
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<tr>
<td>SN697</td>
<td>TATATCCGACACTGATTCACATAGTTTTTTATAAAACATTTCACTGCGAGAAGAATTGTACATTGCGGAGTGGGGATCATTTGTTT</td>
</tr>
</tbody>
</table>

* The underlined nucleotides indicate primer-template mismatches that add sites for restriction endonucleases. The nucleotides shown in bold hybridize to pKD13.

H10407 with primers SN58 and SN583. The numbering is relative to the transcription start site of each promoter. The PCR products were digested with BamHI and EcoRI and then ligated into the same sites of pLTLac1 to construct pLTLac1 (eltAp::lacZYA) and pSTaLa ((eltAp::lacZYA). Plasmid pHKLac1 is a promoterless lac reporter vector carrying aadA, attB120002, and the pr-dependent γ origin of replication from R6K (6). Transcription terminators are located upstream and downstream of lacZYA to prevent readthrough from flanking sequences. Reporter plasmids were integrated into the chromosome of K-12 strain M182 [lacX74 gale15 galK16 relA1 spoT1] (11) or its isogenic progeny M182 [eltAp::lacX74 gale15 galK16 relA1 spoT1] (9) and M182 cydA::kan [eltAp::lacX74 gale15 galK16 relA1 spoT1] by Int1077-dependent, site-specific recombination (19). Reporter strains GPM1160 (attB120002::PLTLac1), GPM1162 (eltAp::attB120002::PLTLac1), GPM1159 (attB120002::pTsaLa), and GPM1161 (eltAp::attB120002::pTsaLa) were used in this study. Each strain carried a reporter construct integrated at attB120002 as determined by colony PCR (19). Plasmid pSE196 (21) expresses CRP from the lac promoter of cloning vector pHG165 (43).

Selected regions containing CRP binding sites upstream of lacZYA were amplified by PCR with primers SN577 and SN237, which anneal to the noncoding regions (numbering relative to the lac promoter’s transcription start site as determined in this study) were replaced with a kanamycin resistance cassette flanked by FRT sites. The cassette was assembled from pKD13 (12) by PCR with primers SN695 and SN696 or SN697. The PCR products were then recombined into strain GPM1160(pSM6) (13) by RED-mediated homologous recombination to produce strains GPM1253 and GPM1254. In the second step, the kanamycin cassette was amplified from pKD13 (12) to produce strains GPM1261 [eltAp::lacX74 gale15 galK16 relA1 spoT1] (9) and M182 cydA::kan [eltAp::lacX74 gale15 galK16 relA1 spoT1] by Int1077-dependent, site-specific recombination (19). Reporter strains were grown aerobically at 37°C in LB medium. Ampicillin was used at 100 μg/ml for the positive selection of plasmids pHG165 and pSE186 as appropriate. The cells were lysed and assayed for β-galactosidase activity as previously described (32).

The LT-I promoter from H10407 was cloned into pBR322 as follows. LM322 was grown aerobically at 37°C in TY broth (5 g/liter tryptone, 5 g/liter yeast extract, 2.5 g/liter NaCl, 100 μg/ml ampicillin). CRP expression was induced for 3 h by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.7 mM when the optical absorbance of the culture reached 0.6 at 600 nm. The bacterial cells were collected by centrifugation and suspended in 10 ml of ice-cold buffer A (20 mM Tris-Cl [pH 7.6], 500 mM NaCl, 5 mM imidazole). All subsequent purification steps were conducted at 4°C. Bacteria were lysed by passage through a French press. The lysate was lyophilized by high-speed centrifugation, and the soluble material was loaded onto a 5-mL nickel-Sepharose column equilibrated with buffer A. The column was washed with several column volumes of buffer A, and the concentration of imidazole was increased linearly from 5 to 500 mM. Under these conditions, CRP eluted from the column at 80 mM imidazole. The purified protein was stored at −20°C after equilibrium dialysis with 40 mM Tris-Cl (pH 7.6), 200 mM KCl, 4 mM dithiothreitol, 20% (vol/vol) glycerol. The concentration of CRP was determined by the Bradford method relative to a standard curve of bovine serum albumin (7). The CRP preparation was determined to be 89% pure by microfluidic, lab-on-chip analysis with a 2100 bioanalyzer (Agilent Technologies).
RESULTS

CRP negatively regulates the LT-I promoter. It has been previously reported that the two genes eltAB encoding LT-I are catabolite repressed, while paradoxically, another study has reported that glucose increases the yield of soluble toxin (17, 18). This peculiar inverse relationship between LT-I promoter activity and toxin production led us to reconsider the methods and conclusions of both studies. We began by investigating how the production of soluble toxin is affected by glucose. For this study, we chose ETEC strain H10407 because it has been extensively characterized since its initial isolation in the early 1970s (15), and more recently, its genome has been fully sequenced (http://www.sanger.ac.uk/Projects/E_coli_H10407/). This strain was cultured in LB medium with or without glucose.

The medium was also buffered with phosphate to pH 7.5 because toxin release is inhibited below pH 7.0 (28). Culture aliquots were collected during the log phase of growth (Fig. 1A), and the concentration of soluble LT-I was determined by GM1-ELISA (44). Since glucose altered the growth kinetics of H10407 (Fig. 1A), we compared the culture aliquots with similar turbidity readings (Fig. 1B). After bacteria were removed by centrifugation, we found that the supernatants from cultures with glucose contained two- to threefold more soluble toxin than the supernatants from cultures without glucose (Fig. 1B). These statistically significant differences were observed at early, mid-, and mid-/late log phases of growth. Thus, as has been reported previously for toxin activity assays (18), we find by GM1-ELISA that glucose enhances the expression and/or secretion of LT-I.

We also investigated the transcriptional regulation of LT-I by cloning its promoter, eltAp, from H10407 into a promoterless Lac reporter vector. The reporter constructs were then integrated into the chromosome of K-12 strain M182 and its isogenic progeny M182 Δcrp and M182 cyaA::kan using a site-specific recombinase as previously described (6, 19). When the expression of β-galactosidase was quantified from log- and stationary-phase cultures, we found statistically significant (P ≤ 0.0001 by Student’s t test) derepression of eltAp in the Δcrp and cyaA::kan strains compared to that in the isogenic wild-type strain (Fig. 1C). We also complemented the crp deletion with a plasmid, pSE186 (21), that expresses CRP (Fig. 1D). This plasmid is a derivative of the low-copy-number cloning vector pHG165 and has an expected 10 to 20 copies per cell (43). In the Δcrp strain, the expression of β-galactosidase was 2.7-fold lower with crp in trans than that in the same strain transformed with the vector control, thus demonstrating the complementation of the crp deletion (Fig. 1D). However, when the wild-type eltAp reporter strain was transformed with the CRP expression plasmid, we also observed a 2.7-fold reduction in the expression of β-galactosidase compared to that for the vector control (Fig. 1D). This indicates that CRP expressed from its chromosomal locus does not fully repress eltAp under these laboratory conditions since we were able to increase the level of eltAp repression by expressing CRP in trans. Neverthe-
less, our results demonstrate that the expression of LT-I is transcriptionally repressed by CRP. Although these results contradict a previous study that reported the CRP-dependent activation of \(\text{eltAp}^{(17)}\), they are consistent with the results of our GM1-ELISA (Fig. 1B) and those of P. Gilligan and D. Robertson (18). The observed glucose-dependent increase of soluble toxin can now be explained, at least partially, by the derepression of \(\text{eltAp}^{(114)}\) when glucose inhibits the synthesis of the CRP cofactor cAMP.

To contrast the transcriptional regulation of LT-I with that of STa, we also constructed wild-type and \(\Delta\text{crp}\) Lac reporter strains with the STa promoter \(\text{estAp}^{(176)}\). Quantitative enzymatic assays revealed that the expression of \(\beta\text{-galactosidase from estAp}^{(210)}\) was lower in a \(\Delta\text{crp}\) strain than in a wild-type strain in both log and stationary phases of growth (Fig. 1C). These latter results were expected because previous studies have shown that the expression of heat-stable toxin is subject to catabolite repression and thus activated by CRP (3, 10, 29). Thus, by using the STa promoter as a point of reference, we clearly demonstrate that the two toxin promoters are inversely regulated by CRP in our side-by-side comparison (Fig. 1C).

Identification of CRP binding sites within and near \(\text{eltAp}^{(530)}\). Since our in vivo results demonstrate that CRP represses \(\text{eltAp}^{(529)}\), we used in vitro DNase I footprinting with purified CRP to determine if the repressor binds at or near the LT-I promoter. Our analysis revealed three CRP binding sites (Fig. 2A). The

FIG. 2. Locations of CRP binding sites and the transcription start site of \(\text{eltAp}^{(219)}\). Numbering is relative to the transcription start site of \(\text{eltAp}^{(319)}\), which is represented by a wavy arrow. (A) Representative DNase I footprint of CRP homodimers bound to the coding and noncoding strands of \(\text{eltAp}^{(52)}\) in the presence of 200 \(\mu\text{M cAMP}\). Two independent DNase I footprinting experiments were done for each strand; therefore, each CRP binding site was visualized four times. For primer extensions, 103 \(\mu\text{g of total RNA from strain GPM1160 (eltAp::lacZYA)}^{(310)}\) was used but only 62 \(\mu\text{g from strain GPM1162 (eltAp::lacZYA)}^{(394)}\). Primer extensions were repeated twice. Lanes labeled GA and TC contain Maxam-Gilbert sequencing ladders. (B) Nucleotide sequence of \(\text{eltAp}^{(62)}\) from ETEC strain H10407. The \(\text{eltAp}^{(184)}\) sequences are bound by rectangles and have been previously characterized by site-directed mutagenesis (30). Each CRP binding site is compared to the CRP consensus sequence, and the nucleotides shown in bold are part of spaced inverted repeats. Over- and underlines indicate the approximate extent of each DNase I footprint.
footprint of CRP bound to site eltA1o encompasses a spaced inverted repeat centered at $-31.5$, relative to the eltAp transcription start site, with 69% identity to a CRP binding site consensus sequence (Fig. 2B). Although it has been previously postulated that this spaced inverted repeat is a CRP binding site (17), this is the first experimental verification of the site’s authenticity. Two additional CRP binding sites were identified further upstream of eltA1o. Site eltA2o contains a spaced inverted repeat centered at $-132$, and eltA3o contains an imperfect spaced inverted repeat centered at $-261$. Our qualitative DNase I footprints revealed that higher concentrations of CRP were required to saturate eltA3o than eltA1o, suggesting that CRP has a higher affinity for eltA1o than eltA3o (Fig. 2A). Of the three binding sites, eltA2o appears to be the lowest affinity binding site because it was not fully saturated even at 250 nM CRP. Sites eltA2o and eltA3o have 75% and 63% identity, respectively, to the CRP consensus sequence. However, this level of identity was achieved by allowing for atypical 7-bp spacers between their consensus elements (Fig. 2B). The majority of the known CRP binding sites have 6-bp spacers, as exemplified by eltA1o. The 7-bp spacers of eltA2o and eltA3o may account, at least partially, for CRP’s apparent lower affinity for these binding sites relative to eltA1o.

The transcription start site of eltAp was determined by the primer extension of RNA isolated from wild-type and $\Delta$crp reporter strains (Fig. 2A). In both strains, the start site mapped to a deoxyadenosine 55 bp upstream of the eltA initiating codon. This transcription start site differs from the previously reported eltAp start site at the deoxyadenosine at $-56$, relative to the initiating codon (50). One possible reason for this discrepancy is that we accounted for the fact that Maxam-Gilbert reactions are excision reactions; therefore, the sequence ladders are 1 nucleotide shorter than primer extension products. It is not clear that this offset was accounted for previously (50). Nevertheless, this is a minor difference that does not affect the assignment of LT-I promoter elements or the interpretation of our results. We also note that 103 $\mu$g of RNA isolated from the wild-type strain yielded fewer primer extension products than 62 $\mu$g of RNA from the $\Delta$crp strain (Fig. 2A). Although these results are qualitative, they are consistent with our quantitative $\beta$-galactosidase assays which demonstrate that eltAp is repressed by CRP (Fig. 1C).

CRP prevents RNA polymerase from forming an open complex at eltAp. Since eltAp is repressed by CRP in vivo and the $-35$ hexamer of eltAp is embedded within eltA1o (Fig. 2B), it seemed likely that the occupancy of eltA1o prevents RNA polymerase from recognizing the $-35$ hexamer and, subsequently, the formation of an open complex. To test this, we equilibrated CRP with an LT-I promoter fragment, from $-91$ to $+154$ (eltAp$^{-}$ DeltaeltA2o DeltaeltA3o), with the noncoding strand radiolabeled is shown. Hyperreactive nucleotides within the RNA polymerase open complex are indicated by black dots. The final concentrations of CRP, cAMP, and RNA polymerase were 100 nM, 500 $\mu$M, and 50 nM, respectively. Numbering is relative to the eltAp transcription start site, which is designated by a wavy arrow. The ability of CRP to inhibit open complex formation in the presence of cAMP was visualized in three independent potassium permanganate footprinting experiments of which one representative gel image is shown. Lanes labeled GA and TC contain Maxam-Gilbert sequencing ladders. +, presence; Ø, absence.

The products on a sequencing gel, we found that nucleotides from $-10$ to $+4$ on the noncoding strand were hyperreactive with KMnO$_4$ when RNA polymerase was present (Fig. 3). Based upon the transcription start site of the promoter (Fig. 2), this region of reactivity is consistent with the formation of an open complex at eltAp. As expected from our in vivo results (Fig. 1B), we found that the formation of the open complex was prevented by CRP but only when cAMP was also included in the reactions. Since the DNA fragment lacked binding sites eltA2o and eltA3o, these results indicate that the occupancy of the CRP operator eltA1o is sufficient to block RNA polymerase’s recognition and isomerization of eltAp in vitro. We also observed that $-66T$ and $-71T$ were hyperreactive with KMnO$_4$ when CRP, cAMP, and RNA polymerase were present (Fig. 3). These hypersensitive positions do not corre-
late with a CRP binding site or known promoter elements; therefore, their biological significance, if any, is currently unclear. These sensitive positions might indicate a distortion of the DNA helix or perhaps the driving of RNA polymerase to a pseudopromoter when eltAp is blocked by CRP (38).

Although our potassium permanganate footprinting indicates that CRP’s occupancy of eltA1o is sufficient to prevent the formation of an open complex at eltAp, this does not exclude the possibility that sites eltA2o and eltA3o have a function in vivo. To test this, we replaced eltAp sequences from −407 to −120 or −407 to −74 with a 79-bp remnant sequence by the recombinering of eltAp (−407 to +154)::lacZ/YA with λRED and FLP recombinase (12, 13). Like the full-length construct with all three CRP binding sites, the two shorter reporters were derepressed in Δcrp strains compared to the isogenic wild-type strains (Table 2). This indicates that the replacement of eltA2o, eltA3o, and flanking sequences with an unrelated fragment of DNA does not abolish the CRP-dependent repression of eltAp. However, we did observe a statistically (P ≤ 0.017 by Student’s t test) higher level of β-galactosidase activity with the construct encompassing nucleotides −120 to +154 compared to the full-length construct in both the wild-type and Δcrp strains. This indicates that the deletion of eltA2o and/or eltA3o partially relieves the repression of eltAp. This trend was not apparent with the shortest construct, which had essentially the same level of expression as the full-length construct in the crp+ strain (P = 0.093 by Student’s t test) but a lower level of expression in the Δcrp strain (P = 0.051 by Student’s t test). Since the three constructs produced different absolute levels of enzymatic activity in the wild-type and Δcrp strains, the data were normalized by calculating the repression of each construct. When the repression levels of the three constructs are compared, a small but statistically significant (P = 0.0022 by analysis of variance) derepression of eltAp is apparent with the two constructs lacking eltA2o and eltA3o compared to that of the full-length eltAp (Table 2). Thus, we conclude that although binding site eltA1o is sufficient for the repression of eltAp in vitro, binding site eltA2o or eltA3o or both are required for the full CRP-dependent repression of eltAp in vivo.

**DISCUSSION**

In this study, we have shown that the expression of LT-I is repressed at the level of transcription by CRP. The −35 hexamer of eltAp is a σ70-dependent consensus hexamer (TTG ACA) that is embedded within a CRP operator, eltA1o, centered at −31.5 relative to the eltAp transcription start site (Fig. 2B). The location of eltA1o is consistent with CRP functioning as a repressor of eltAp because CRP-repressed promoters typically have binding sites centered downstream of −41 (2, 49). In contrast, CRP-activated promoters typically have one or more binding sites centered, relative to the transcription start site, near −42, −62, −72, −83, or −93 (8). As expected from its location and our in vivo results showing the CRP-dependent repression of eltAp, CRP’s occupancy of eltA1o prevents RNA polymerase from forming an open complex at eltAp in vitro. However, it is likely that CRP acts at an earlier step of promoter initiation by sterically occluding RNA polymerase’s access to the −35 hexamer of eltAp. Although ETEC strains are heterogeneous, it is likely that LT-I will be negatively regulated by CRP in most, if not all, LT-I+ strains because the CRP binding site centered at −31.5 is a conserved feature of the toxin promoter (39).

Our DNase I footprinting also revealed two additional CRP binding sites which are centered at −132, site eltA2o, and −261, site eltA3o. CRP may have a lower affinity for these sites than for eltA1o because the latter appeared to be fully saturated by lower concentrations of CRP than eltA2o and eltA3o in our DNase I footprinting experiments (Fig. 2A). In addition, the conserved region of LT-I promoters extends only as far as −181 (39); therefore, site eltA3o may not be present in some ETEC strains. Nevertheless, the deletion of eltA2o and eltA3o partially relieved the CRP-dependent repression of eltAp in vivo, even though eltA1o was sufficient to prevent the formation of an open complex at eltAp in vitro. This difference was most likely produced by the saturating concentrations of CRP used for in vitro potassium permanganate footprinting. In vivo, binding site eltA1o is probably only partially saturated because we observed expression from eltAp even under repressing conditions. In addition, we observed the further repression of eltAp when CRP was expressed from a multicopy plasmid, even in a crp+ strain (Fig. 1D). Binding site eltA2o and/or binding site eltA3o may contribute to the occupancy of eltA1o through cooperative interactions, although this was not investigated in this study.

H-NS has also been reported to repress the transcription of eltAB through two silencer regions located downstream of eltAp (50). The first silencer region maps to a region between +30 and +109 while the second region lies between +459 and +555, relative to the eltAp transcription start site identified in this study. When bound to these regions, H-NS functions synergistically to prevent RNA polymerase from either clearing eltAp or elongating the eltAB mRNA (50). Thus, the mechanism of H-NS-mediated repression is clearly distinct from that of CRP, which we have shown prevents RNA polymerase from forming an open complex at eltAp. Moreover, the H-NS-mediated repression of eltAp is greater at 18°C than 37°C (46, 47, 50); therefore, the repression of eltAp by CRP may be more relevant during an infection than repression by H-NS.

In 1979, P. H. Gilligan and D. C. Robertson published their study showing that LT-I production is higher in culture media with glucose than without (18). We have corroborated their study by GM1-ELISA and shown that this effect can be explained by the CRP-dependent repression of eltAp. Since CRP

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**TABLE 2. Deletion of eltA2o and eltA3o partially relieves the CRP-dependent repression of eltAp**

<table>
<thead>
<tr>
<th>Reporter construct</th>
<th>β-Galactosidase activity</th>
<th>P value</th>
<th>Fold repression</th>
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<tr>
<td>eltAp (−407 to +154)::lacZ</td>
<td>920 ± 72</td>
<td>1,700 ± 181</td>
<td>&lt;0.0001</td>
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<tr>
<td>eltAp (−120 to +154)::lacZ</td>
<td>1,384 ± 227</td>
<td>2,087 ± 278</td>
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<td>eltAp (−74 to +154)::lacZ</td>
<td>1,008 ± 91</td>
<td>1,487 ± 151</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
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a CRP binding sites eltA1o, eltA2o, and eltA3o are centered at −31.5, −132, and −261, respectively.

b β-Galactosidase activity is reported in Miller units (mean ± standard deviation) (n = 6).

c By Student’s t test.
cannot bind DNA in the absence of its cAMP cofactor, the repression of eltAp is relieved when glucose suppresses the synthesis of cAMP. However, we do not exclude the possibility that CRP also regulates other steps in the production of LT-I, such as its secretion or the production of toxin-laden outer membrane vesicles (23, 45). The report of the CRP-dependent expression of eltAB is not reproducible, even though we cloned the LT-I promoter from the same strain, H10407, as in the previous study (17). It is possible that the previous study was reporting gene dosage effects rather than LT-I promoter activity, because they used high-copy-number Lac reporter plasmids (17). It is known that plasmid copy number can vary as a nonlinear function of bacterial growth rates (1, 26). To avoid gene dosage artifacts, we and others routinely use single-copy Lac reporters stably integrated into the bacterial chromosome (19, 21, 37, 40). With a chromosomally integrated reporter, we found that the LT-I promoter is repressed two- to fourfold by CRP. Since this is not a particularly large signal, it may have been lost by plasmid copy number fluctuations.

Unlike LT-I, the expression of STa is positively regulated by CRP (Fig. 1C and 4A). For strains of ETEC that have the capacity to express both enterotoxins, this differential regulation is likely to temporally and spatially separate the maximal expression of the two toxins during the course of infection (Fig. 4B). Since glucose inhibits the synthesis of cAMP in E. coli, and thus the activity of CRP, our studies predict that the expression of LT-I will be highest in the duodenum when glucose is present. As glucose is absorbed by the small intestine (16), LT-I production will decrease while STa production increases. However, this model may be overly simplistic since exogenous cAMP can override catabolite repression, or catabolite activation in the case of LT-I. In fact, several studies, when considered in aggregate, provide compelling evidence for a cAMP feedback loop that is completed when the pathogen receives cAMP produced by LT-I-intoxicated host cells (4, 20, 24). If this feedback loop exists, it would be a form of enterotoxin negative autoregulation, since the expression of LT-I is repressed by CRP and cAMP. Although this potential cAMP feedback loop will require additional research to fully explore its validity, it seems no mere coincidence of evolution that LT-I-intoxicated cells hyperproduce the same molecule, cAMP, that negatively regulates the toxin’s expression.

FIG. 4. Differential regulation of ETEC enterotoxins by CRP. (A) In the presence of its ligand cAMP, CRP activates the expression of heat-stable toxin STa and represses the expression of heat-labile toxin LT-I. High concentrations of glucose suppress the synthesis of cAMP, rendering CRP inactive. (B) As a result, the expression of the enterotoxins may change relative to one another as the pathogen moves through the small intestine because glucose concentrations are typically higher in the duodenum than in the ileum. CRP without its cAMP cofactor is represented as CRPapo.