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Multiple Regulators Control Expression of the Entner-Doudoroff Aldolase (Eda) of Escherichia coli

Elizabeth L. Murray\(^1\) and Tyrrell Conway\(^2\)\(^*\)

Comprehensive Cancer Center and Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio,\(^1\) and Advanced Center for Genome Technology, University of Oklahoma, Norman, Oklahoma\(^2\)

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The *Escherichia coli* eda gene, which encodes the Entner-Doudoroff aldolase, is central to the catabolism of several sugar acids. Here, we show that Eda synthesis is induced by growth on glucuronate, glucuronate, or methyl-β-D-glucuronide; phosphate limitation; and carbon starvation. Transcription of *eda* initiates from three promoters, designated P1, P2, and P4, each of which is responsible for induction under different growth conditions. P1 controls *eda* induction on glucuronate and is regulated by GntR. P2 controls *eda* induction on glucuronate and galacturonate and is regulated by KdgR. P4 is active under conditions of phosphate starvation and is directly controlled by PhoB. In addition, CsrA activates Eda synthesis, apparently by an indirect mechanism that may be involved in the modest changes in expression level that are associated with carbon starvation. The complex regulation of *eda* is discussed with respect to its several physiological roles, which apparently accommodate not only sugar acid catabolism but also detoxification of metabolites that could accumulate during starvation-induced stress.

Sugar acids are metabolized via the Entner-Doudoroff and Ashwell pathways (1, 5, 8, 20). The Entner-Doudoroff aldolase, encoded by the *eda* gene, is necessary for growth on several mucus-derived sugar acids (20), which may define a niche for *Escherichia coli* colonization of the mammalian large intestine (4). Mutants lacking the key enzyme of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase (encoded by *edd*), are compromised in their ability to colonize the mouse intestine (4), as are *eda* mutants (29).

Eda cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDPG) to pyruvate and triose-3-phosphate is a common step of hexonate and hexuronate catabolism (20). Eda has also been found to have a role in the degradation of 2-keto-4-hydroxyglutarate (KHG) to pyruvate and glyoxylate (16). Although this reaction has not been shown to occur in vivo, cleavage of KHG could serve in tricarboxylic acid (TCA) cycle regulation or, when operating in the reverse direction, in the detoxification of glyoxylate. Cloning and sequencing the genes encoding these functions revealed that KHG aldolase and Eda are the same enzyme (7, 18). However, this study is the first to extensively characterize *eda* regulation.

The *eda* gene is located immediately downstream of *edd*, at 40.5 min on the *E. coli* chromosome (Fig. 1) (6). Previously, primer extension analysis of the *eda* regulatory region revealed four putative *eda* promoters (7). P1 drives cotranscription of *edd* and *eda* and is induced by growth on glucuronate (7); P1 appears to be controlled by GntR (20, 21, 24), although binding of GntR to the P1 regulatory region was not established prior to this work. A second promoter, P2, is located within the terminal one-third of the *edd* coding sequence, and the closely spaced third and fourth putative promoters (P3 and P4) are located at the distal end of the *edd* coding region (7). High basal expression is characteristic of *eda* (20). Although it is known that the P2, P3, and P4 promoters are inducible above basal levels, the specific inducers have not been established prior to this study (7). This organization of promoters suggests that regulation of *eda* transcription is complex. Hexuronates induce Eda, perhaps mediated at the transcriptional level through the actions of KdgR (22). Two dimensional gel electrophoresis revealed induced synthesis of Eda under conditions of phosphate limitation (31). Eda appears to be induced during carbon starvation or stationary phase, but these have not been distinguished with respect to control of Eda (17).

The objectives of this study were to characterize the promoters and conditions that initiate transcription of *eda*, as well as the regulatory factors and mechanisms involved. We show here that three functional promoters drive transcription of *eda* and that each promoter responds to different growth conditions. We show that P1 is induced by growth on glucuronate, which is mediated by GntR. P2 is induced by growth on glucuronate, galacturonate, and methyl-β-D-glucuronide and is mediated directly by KdgR. Phosphate starvation results in induction of P4, which is directly mediated by PhoB. Eda levels are slightly elevated during carbon starvation but not stationary phase. The mRNA binding protein CsrA is also implicated in the control of Eda synthesis.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The *E. coli* strains, plasmids, and phages used in this study are listed in Table 1. The *eda*\(^*\)-lac\(^Z\) fusions were created in *E. coli* BH90C; other *eda*-lac\(^Z\) fusions were created in *E. coli* W1485 \(\Delta\text{lac-pro}\). *E. coli* DH5\(^{\alpha}\) was used for constructions and propagation of plasmids. *E. coli* strains were grown at 37°C, and growth was monitored with a Spectronic 601 spectrophotometer (Milton Roy Co.). Culture media used were Luria-Bertani medium (LB) (13), M63 minimal medium, MOPS (morpholinepropanesulfonic acid minimal medium; phosphate-replete medium contained 1.32 mM KH\(_2\)PO\(_4\); phosphate-limiting medium contained 0.066 mM KH\(_2\)PO\(_4\)) (15), and Kornberg medium (12). Antibiotics were used at the follow-
100 ml of PBST (10 mM sodium phosphate [pH 7.2], 0.9% sodium chloride, 80°C for 15 min or until dry in a vacuum oven, blocked by incubation for 1 h with a Bio-Rad transfer apparatus. Following transfer, membranes were baked at 100°C for 1 h. Western blot analysis. Samples for Western blot analysis (approximately 14 mg of cell biomass) were removed from the culture, harvested by centrifugation, and frozen at −80°C. Thawed cell pellets were resuspended in 500 µl of buffer (20 mM Tris-HCl, 1 mM EDTA, 5 mM β-mercaptoethanol [pH 8]) and disrupted by sonication. Samples (50 µg of total-cell protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (11). Proteins were transferred from PAGE gels to Immobilon-P membranes (Millipore) with a Bio-Rad transfer apparatus. Following transfer, membranes were baked at 80°C for 15 min or until dry in a vacuum oven, blocked by incubation for 1 h with 100 ml of PBST (10 mM sodium phosphate [pH 7.2], 0.9% sodium chloride, 0.05% Tween 20, 2.5 g of dry milk), and washed five times in PBST. Blocked membranes were incubated in 1 ml of PBST containing 50 µl of primary antibody (chicken anti-Eda) and washed as before, followed by incubation in 100 ml of PBST containing 8 mg of secondary antibody (horseradish peroxidase-labeled goat anti-chicken immunoglobulin Y) and another washing. Membranes were air dried and visualized by chemiluminescence with Western blotting detection reagents and ECL (Amersham Pharmacia Biotech) and exposure to Kodak Biomax MS film for 1 to 30 s.

DNA sequencing. DNA was prepared for sequencing by using the QiAprep Spin Miniprep kit (Qiagen) and sequenced according to the manufacturer’s instructions with either Sequenase, version 2.0 (USB/Amersham Life Science), for α-[32P]ATP sequencing or the double-stranded DNA cycle sequencing system (Invitrogen) for α-[32P]ATP sequencing.

RNA isolation, Northern hybridization, and primer extension. Total RNA for northern hybridization and primer extension analysis was isolated by the hot-phenol method (19) from E. coli cultures grown to mid-exponential or stationary phase under the conditions described above. For mRNA half-life determinations, cells were grown overnight in 3 ml of Kornberg medium containing 0.5% glucuronate and then subcultured in 400 ml of Kornberg medium plus 0.5% glucuronate and grown at 37°C to an A600 of 0.5. Rifampin was added to the culture to a final concentration of 200 µg/ml, and samples were taken at 0 (control), 2, 4, 8, and 12 min postaddition. Samples (10 ml) were diluted 1:1 in RNAlater (Ambion), and RNA was isolated with the Qiagen RNA/DNA Maxi kit. Contaminating DNA was removed by treatment with RNase-free DNase (Qiagen). For Northern hybridization, gels were loaded with 5 µg of total RNA and were treated as described previously (2, 30). Radioactively labeled RNA probes were synthesized with α-[32P]UTP by using T7 RNA polymerase with pT7 Carp (HincII-digested) DNA as the template. The membranes were visualized by exposure to Kodak X-ray film at room temperature. Methods for transcript end mapping by primer extension were described previously (30).

Purification of KdgR. KdgR was prepared by using the His tag modification system from Qiagen. An 840-bp fragment containing the complete kdgR coding sequence was amplified by PCR with primers that contained BamHI restriction sites at the 5’ end and ligated into pPCR-Script Amp (SK+) to create pBMEskK. KdgR was overproduced and purified on a nickel-nitriilotriacetate column, as described previously (19). The protein contents of column fractions were quantified by using the method of Bradford (5).

Cell mobility shift assays for KdgR. The 115-bp DNA probe for the mobility shift assay was generated by PCR with primers flanking the promoter and

FIG. 1. (A) Genetic map of the edd-eda coding region. Arrows, transcription start sites confirmed by primer extension (P1, P2, and P4). Relative locations of operator sites (boxes) are shown above the line. DNA fragments used for lacZ fusions are shown below the line. P1, P2 operon fusion (E. coli NP304) and P1 operon fusion with mutant gntO site (E. coli NP305); P2, P2 operon fusion (E. coli BM106); P4, P4 protein (E. coli BM105) and operon (E. coli BM111) fusions. (B) Primer extension analysis of the 5’ end of the eda transcript originating from P4. Lanes G, A, T, and C, eda sequence ladder (generated with the same primer used for extension); lanes 1 and 2, RNA isolated from phosphate-starved cells; lanes 3 and 4, RNA isolated from glucose-starved cells; lanes 5 and 6, RNA isolated from stationary-phase cells. Lanes 1, 3, and 5 were extended with Omniscript reverse transcriptase; lanes 2, 4, and 6 were extended with Superscript reverse transcriptase.
pBM204 as the template. The PCR products were cleaned with the QIAquick PCR purification kit (QIAGEN) and labeled with [γ-32P]ATP by using T4 polynucleotide kinase. Binding reaction mixtures (20 µl) contained labeled probe (0.025 pmol), reaction buffer (12 mM HEPES, 4 mM Tris-HCl [pH 8], 70 mM KCl, 1 mM EDTA, 10% glycerol, 1 µg of salmon sperm DNA, 4 µg of bovine serum albumin, 1 mM dithiothreitol), and KdgR in elution buffer. Reaction mixtures were incubated at room temperature for 30 min, loading buffer was then added, and the mixtures were electrophoresed on a 5% native polyacrylamide gel containing 2% glycerol. DNA-protein complexes were visualized directly by fluorescence with a Typhoon variable-mode imager (Molecular Dynamics).

**RESULTS**

**Primer extension analysis of eda promoters.** To determine the locations of promoters that are active under specific conditions, we mapped the 5′ ends of transcripts originating from P2, P3, and P4 in cells grown in conditions hypothesized to induce these promoters (Fig. 1). E. coli W1485 was grown in M63 minimal medium containing 0.4% glucuronate, and RNA was harvested during exponential growth. Primer extension analysis of cells grown on glucuronate revealed a transcription start site identical to that which was previously identified for the P2 promoter (7); the P2 transcription start site is located at position −26 with respect to the eda start codon (data not shown). Next, E. coli W1485 was grown under conditions of glucose or phosphate starvation and during the transition to stationary phase in Luria broth. Primer extension analysis of the P3-P4 promoter region revealed a single eda 5′ transcript end corresponding to the previously mapped eda P4 promoter transcription start site, which is located at position −353 with respect to the eda start codon (data not shown). Next, E. coli W1485 was grown under conditions of glucose or phosphate starvation and during the transition to stationary phase in Luria broth. Primer extension analysis of the P3-P4 promoter region revealed a single eda 5′ transcript end corresponding to the previously mapped eda P4 promoter transcription start site, which is located at position −353 with respect to the eda start codon (data not shown). Next, E. coli W1485 was grown under conditions of glucose or phosphate starvation and during the transition to stationary phase in Luria broth. Primer extension analysis of the P3-P4 promoter region revealed a single eda 5′ transcript end corresponding to the previously mapped eda P4 promoter transcription start site, which is located at position −353 with respect to the eda start codon (data not shown).
tional regulation described below can be explained by a single promoter in this region, we conclude that P4 regulates eda expression during phosphate and carbon starvation.

**P1 regulates induction by gluconate.** Western analysis showed that Eda was produced in *E. coli* W1485 cells grown in M63 minimal glucose (glc), glucuronate (glr), or gluconate (gnt) medium. The transcriptional promoter fusion strains, when grown on gluconate, were not induced above the basal level of expression (Table 2). Induced promoter fusions. The transcriptional fusion to P1 (edaP1-lacZ) in *E. coli* NP304 was expressed at very low levels when cells were grown on glucose and induced 100-fold when cells were grown on glucuronate (Table 2). No induction of edaP1-lacZ was observed in cells grown on glucuronate, galactonate, 5-ketogluconate, N-acetylglucosamine, or N-acetylneuraminic acid; however, edaP1-lacZ was induced 25-fold when cells were grown on idonate and was induced 7-fold when cells were grown on 5-ketogluconate, conditions also known to induce the Entner-Doudoroff pathway (2). The P2 and P4 eda promoter fusion strains, when grown on glucuronate, were not induced above the basal level of expression (Table 2). Induction of eda by glucuronate was confirmed by Northern blot analysis (Fig. 2B), which showed increased expression of a 2,600-nucleotide (nt) transcript that was shown previously to correspond to P1 (7). Thus it is clear that P1 is responsible for induction of eda by glucuronate, but not for high basal expression.

**P2 regulates induction by hexuronates.** Western blots showed that Eda was induced in *E. coli* W1485 cells grown on M63 minimal medium containing 0.4% glucose or methyl-β-D-glucuronide (data not shown), which is another substrate of the Ashwell pathway. To determine which promoter was responsible for glucuronate-dependent Eda induction, β-galactosidase activity in various eda-lacZ fusion strains was measured. The edaP2-lacZ operon fusion in *E. coli* BM106 was induced 2.5-fold in cells grown on glucuronate, compared to induction in cells grown on glucose, and 1.7-fold in cells grown on galacturonate (Table 2). The edaP2-lacZ fusion was not induced by glucuronate, 5-ketogluconate, N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid, or glycerol. Expression of edaP2-lacZ in cells grown on glucuronate with added glucose, but not with added glucuronate, was repressed slightly compared to that in cells grown on glucuronate alone (Table 2). This suggests that eda P2 is subject to catabolite repression or is simply not induced to the same extent when alternative substrates are available, in keeping with the preference of *E. coli* for glucose over glucuronate (22). Induction of eda by glucuronate was confirmed by Northern blot analysis (Fig. 2B), which showed increased expression of a 1,000-nt transcript that was shown previously to correspond to P2 (7). From these results we conclude that P2 is responsible for induction of eda by hexuronates.

**P4 regulates induction by phosphate starvation.** Previously, two-dimensional gel electrophoresis of protein gels indicated that Eda is induced when *E. coli* was starved for phosphate (31). To directly determine if *E. coli* W1485 synthesizes larger amounts of Eda under phosphate-limiting conditions, cells were grown overnight in MOPS minimal medium containing 1.32 mM K2HPO4 and then transferred to the same medium containing 0.066 mM K2HPO4. The culture began to starve for phosphate after approximately 2.6 h. Western blots showed increased production of Eda upon phosphate starvation (Fig. 3A). To determine which promoter was responsible for eda induction upon phosphate starvation, the eda-lacZ fusions were tested under this condition; in this experiment the culture began to starve for phosphate after approximately 3.5 h. *E. coli* BM111, containing the edaP4-lacZ operon fusion, was induced two- to threefold after 2 h of phosphate starvation (Fig. 3B). Addition of phosphate to starved cells in stationary phase restored exponential growth of the culture and reduced edaP4-lacZ expression to prestarvation levels (data not shown). The

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**TABLE 2. Carbon source-dependent regulation of eda-lacZ fusions**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>β-Galactosidase activity (Miller units) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1 fusionb</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 +/- 6</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>1034 +/- 148</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>13 +/- 7</td>
</tr>
<tr>
<td>Galacturonate</td>
<td>16 +/- 7</td>
</tr>
<tr>
<td>Glucose + glucuronate</td>
<td>818 +/- 53</td>
</tr>
<tr>
<td>Glucose + glucuronate</td>
<td>12 +/- 6</td>
</tr>
<tr>
<td>Glucose + glucuronate</td>
<td>904 +/- 160</td>
</tr>
<tr>
<td>Idonate</td>
<td>244 +/- 59</td>
</tr>
<tr>
<td>5-Ketogluconate</td>
<td>68 +/- 58</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>15 +/- 7</td>
</tr>
<tr>
<td>N-Acetylneuraminic acid</td>
<td>5 +/- 6</td>
</tr>
<tr>
<td>Glycerol</td>
<td>ND</td>
</tr>
</tbody>
</table>

* 0.2% carbon source in M63 minimal medium.
* edaP1-lacZ fusion, *E. coli* NP304.
* edaP2-lacZ fusion, *E. coli* BM106.
* edaP4-lacZ fusion, *E. coli* BM111.
* ND, not determined.
P1 and P2 fusions were not induced by phosphate starvation (data not shown). Induction of eda by phosphate starvation was confirmed by Northern blot analysis (Fig. 3C), which showed increased expression of a 750-nt transcript that was shown previously to correspond to P4 (7). Thus, we conclude that eda P4 is responsible for the induction of eda that occurs upon starvation for phosphate.

**Eda is modestly induced by starvation, but not by stationary phase.** To test the effect of starvation on Eda expression, *E. coli* W1485 was grown in MOPS-glucose minimal medium and Eda synthesis was measured by Western blotting. M63 minimal medium containing 0.2% glucose is designed such that *E. coli* is starved for both carbon and nitrogen (ammonia) when growth ceases (15). Eda levels were elevated upon exhaustion of 0.2% glucose (Fig. 4A and B). To test whether this increase was due to exhaustion of glucose or nitrogen, the experiment was repeated with 0.1% glucose, conditions which do not starve the cells for nitrogen; the same result was obtained (Fig. 4B). To examine Eda synthesis during non-starvation-induced stationary-phase conditions during exponential phase, stationary phase (5 h after inflection of the growth curve), or the transition into stationary phase (at inflection of the growth curve).

![FIG. 3. Induction of Eda during phosphate starvation.](image-url)

**FIG. 3.** Induction of Eda during phosphate starvation. (A) Western blot analysis of Eda synthesis in *E. coli* BW14087 (phoB) and *E. coli* BW13711 (wild type [wt]; isogenic phoB+). Cells were grown in phosphate-limited minimal medium and harvested at the indicated times. (B) β-Galactosidase activity of an eda-lacZ fusion (*E. coli* BM111; P4 operon fusion). Black circles, growth on phosphate-replete medium; black bars, eda-lacZ fusion expression under these conditions; open circles, growth on phosphate-limited medium; gray bars, eda-lacZ fusion expression under these conditions. A_{420} is shown in logarithmic scale. (C) Northern blot analysis of eda transcription in *E. coli* BW14087 (phoB) and *E. coli* BW13711(wt) grown on phosphate-replete (hi PO_4) or -limited (lo PO_4) medium. Size markers in thousands of nucleotides are shown on the right.

![FIG. 4. Synthesis of Eda under carbon starvation and stationary-phase conditions.](image-url)

**FIG. 4.** Synthesis of Eda under carbon starvation and stationary-phase conditions. (A) Growth of *E. coli* W1485 in M63 minimal medium supplemented with 0.1% (squares) or 0.2% (diamonds) glucose. (B) Eda synthesis in *E. coli* W1485 as measured by Western blot analysis of cells harvested during exponential phase (E) or stationary phase (S; 2 h after inflection of growth curve) or during the transition into stationary phase (T; at inflection of the growth curve). (C) Simulation of non-starvation-induced stationary phase by growth of *E. coli* W1485 in one-eighth-strength (diamonds), one-fourth-strength (squares), one-half-strength (triangles), 1× (circles), or 2× (asterisks) LB. (D) Western blot analysis of Eda synthesis in *E. coli* W1485 under non-starvation-induced stationary-phase conditions during exponential phase, stationary phase (5 h after inflection of the growth curve), or the transition into stationary phase (at inflection of the growth curve).
starvation, perhaps as a result of the toxic buildup of metabolic by-products (17). Eda synthesis in cells grown on one-eighth- or one-fourth-strength LB increased slightly during the transition to stationary phase brought on by starvation (Fig. 4D). There was no increase in Eda production by cells during non-starvation-induced stationary phase when grown on one-half-strength LB. Thus, there appears to be a correlation between starvation and Eda induction, but no correlation between Eda expression and stationary phase was established.

**GntR controls transcription from the** **eda** **P1 promoter.** To determine if the glucuronate repressor protein GntR regulates Eda induction, Eda synthesis by the gntR mutant strain *E. coli* HT216 was monitored. Levels of Eda were high when cells were grown on either glucose or gluconate, indicating that Eda synthesis is derepressed in *E. coli* HT216 (Fig. 5A). The *eda* P1 promoter region contains two putative GntR binding sites (Fig. 1), which have a consensus sequence of ATGGTTACCGGTA (24). Gel mobility shift analysis was used to determine if *eda* is regulated directly by GntR binding. Purified GntR retarded the mobility of a labeled DNA probe corresponding to the *eda* P2 promoter region, which contains the putative GntR binding right half-site GTGTTTCAAA (Fig. 1), a close match to the consensus sequence, AAATGAAACAnTGTTTCATTT (24) (Fig. 6A). The *eda* probe was shifted with as little as $1.4 \times 10^{-3}$ pmol of GntR and was shifted completely by 1.4 pmol of GntR. A probe containing a GTTT-to-GGTT mutation in the right half-site was not shifted by GntR (data not shown). These results indicate that GntR binds to the GntR right half-site in the *eda* P2 promoter region.

**PhoB-dependent regulation of P4.** Many proteins induced in response to phosphate starvation are under the control of the two-component PhoB-PhoR regulatory system. Since Eda is apparently induced by phosphate starvation (Fig. 3), we sought to determine if Eda synthesis is under PhoR control. Eda levels in Western blots of *E. coli* phoB mutant strain BW14087 were compared with levels in blots of the isogenic wild-type strain, *E. coli* BW13711. Both strains were grown overnight in MOPS-glucose minimal medium containing 0.32 mM $K_2HPO_4$ and then subcultured by dilution into MOPS-glucose minimal medium containing 0.066 mM $K_2HPO_4$. Cells grown in phosphate-limiting conditions began to starve after 2.6 h and grew more slowly thereafter (data not shown). Western blot analysis revealed higher levels of Eda in the *phoB* mutant prior to starvation and at all times during growth than in the isogenic wild-type strain (Fig. 3A). Derepression of Eda synthesis in the *phoB* mutant suggests that PhoB may act as a repressor of *eda* transcription in a fashion that could be described as exponential silencing. Northern analysis of cells starved for phosphate showed slightly larger amounts of the 750-nt transcript originating from *eda* P4 in the *phoB* mutant than in the wild-type strain and a higher ratio of the 750- to 1,000-nt transcripts (Fig. 3C). Examination of the DNA sequence of the *eda* P4 region revealed a putative PHO box (Fig. 1), CTTGCGTGAAAAA CTGTCCG, upstream of the *eda* P4 promoter (32). Gel mo-

**KdgR controls transcription from the** **eda** **P2 promoter.** To determine if KdgR controls Eda synthesis, *E. coli* CT110, a *kdgR* mutant, was grown in minimal medium containing 0.4% glucose or gluconate. In the wild-type strain, Eda was induced by growth on gluconate. In the *kdgR* mutant, Eda was produced at high levels on both glucose and gluconate (Fig. 2A). Transcription of *eda* in *E. coli* W1485 and *E. coli* CT110 grown in minimal medium containing 0.4% glucose or gluconate was measured by Northern blot hybridization (Fig. 2B). The 1,000-nt transcript initiated from the P2 promoter was present under all conditions. This transcript was derepressed in *E. coli* CT110 grown on glucose and induced by gluconate in the wild type. These data indicate that *eda* P2 has a high basal level of transcription, is induced by gluconurate, and is repressed by KdgR. A 750-nt transcript was also present under all growth conditions, and its abundance tended to correlate with that of the 2,600- and 1,000-nt transcripts, depending on which one was induced (Fig. 2B). This suggests that the 750-nt band in Northern blots may result, at least in part, from the processing of longer transcripts initiated from upstream promoters, although a band of this size can also result from transcription initiation from P4, which is not induced by hexuronates (Table 2).

Gel mobility shift analysis was used to determine if *eda* is regulated directly by KdgR binding. Purified KdgR retarded the mobility of a labeled DNA probe corresponding to the *eda* P2 promoter region, which contains the putative KdgR binding right half-site GTGTTTCAAA (Fig. 1), a close match to the consensus sequence, AAATGAAACAnTGTTTCATTT (24) (Fig. 6A). The *eda* probe was shifted with as little as $1.4 \times 10^{-3}$ pmol of KdgR and was shifted completely by 1.4 pmol of KdgR. A probe containing a GTTT-to-GGTT mutation in the right half-site was not shifted by KdgR (data not shown). These results indicate that KdgR binds to the KdgR right half-site in the *eda* P2 promoter region.

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**FIG. 5.** GntR control of transcription from the *eda* P1 promoter. (A) Western blot analysis of Eda synthesis in *E. coli* W1485 (wild type) or *E. coli* HT216 (*gntR*) grown in glucose (glc) or gluconate (gnt) minimal medium. (B) Transcription of the *edaP1-lacZ* fusion in *E. coli* CT110 grown on glucose or gluconate. In Western blots of *E. coli* phoB mutant strain BW14087, both strains were grown overnight in MOPS-glucose minimal medium containing 0.32 mM $K_2HPO_4$ and then subcultured by dilution into MOPS-glucose minimal medium containing 0.066 mM $K_2HPO_4$. Cells grown in phosphate-limiting conditions began to starve after 2.6 h and grew more slowly thereafter (data not shown). Western blot analysis revealed higher levels of Eda in the *phoB* mutant prior to starvation and at all times during growth than in the isogenic wild-type strain (Fig. 3A). Derepression of Eda synthesis in the *phoB* mutant suggests that PhoB may act as a repressor of *eda* transcription in a fashion that could be described as exponential silencing. Northern analysis of cells starved for phosphate showed slightly larger amounts of the 750-nt transcript originating from *eda* P4 in the *phoB* mutant than in the wild-type strain and a higher ratio of the 750- to 1,000-nt transcripts (Fig. 3C). Examination of the DNA sequence of the *eda* P4 region revealed a putative PHO box (Fig. 1), CTTGCGTGAAAAA CTGTCCG, upstream of the *eda* P4 promoter (32). Gel mo-
the shown). Western blots showed decreased Eda production in
similar rates and reached similar final densities (data not
tween the turnover rates for
PhoB. CsrA appears not to affect
the turnover of eda transcripts but does have a generally positive
effect on eda transcription.

**DISCUSSION**

The first goal of this study was to establish the number of
promoters that drive the transcription of eda. We obtained
evidence for three functional promoters located in the edd-eda
region, designated P1, P2, and P4, and conclude that the pre-
viously identified P3 is not a functional promoter. The second
gold of this study was to establish which promoters are respon-
sible for eda expression under inducing conditions. Our results
indicate that P1 is induced by growth on gluconate and ido-
nate, P2 is induced by growth on hexuronates, and P4 is in-
duced by phosphate starvation. Starvation for carbon, but not
the transition to stationary phase, slightly increased Eda syn-
thesis. The third goal of this study was to identify the regula-
tory factors that control eda transcription. We obtained evi-
dence that P1 is under negative control of GntR; P2 is under
negative control of KdgR; and P4 is under negative control of
PhoB. CsrA appears not to affect eda transcript stability but
appears to activate eda, since eda transcription was 1.6- to
1.8-fold lower in a csrA mutant strain grown on glucuronate
(csrA/H9252) strain during the transition to stationary phase. Taken
tgether, these results indicate that CsrA does not affect the
turnover of eda transcripts but does have a generally positive
effect on eda transcription.

**CsrA exerts indirect negative control of Eda levels.** CsrA is
a global regulatory protein that controls a variety of genes,
including those encoding many central metabolism enzymes.
To determine if CsrA regulates eda, synthesis of Eda in wild-
type and csrA mutant strains grown in LB with or without
gluconate or glucuronate was measured. All cultures grew at
similar rates and reached similar final densities (data not
shown). Western blots showed decreased Eda production in the
csrA mutant, E. coli BM106c, by comparison to that by the
parent strain, E. coli BM106, upon transition to stationary
phase (Fig. 7A). Since CsrA affects the mRNA stability of
target genes (26), we investigated the turnover rate for the eda
transcript (Fig. 7B). There was no significant difference be-
tween the turnover rates for eda transcripts in E. coli BM106c
and E. coli BM106 grown on rich (Kornberg’s) medium con-
taining 0.4% glucuronate. To determine if CsrA had an effect
on the transcription of eda from any of its promoters, β-galac-
tosidase activity in reporter strains with csrA mutant or wild-
type backgrounds was assayed. Expression of the edaP1-lacZ
fusion was decreased 1.6-fold in the csrA mutant strain grown
on gluconate, compared to that in the wild-type (csrA+) strain
(Fig. 7C). Expression of the edaP2-lacZ fusion was decreased
1.8-fold in the csrA mutant strain grown on glucuronate (Fig.
7D). Expression of the edaP4-lacZ fusion was also decreased
1.8-fold in the csrA mutant strain grown on glucose (Fig. 7E).
In addition, E. coli csrA strain BM106c (Fig. 6D) did not
induce eda during the transition to stationary phase. Taken
for any of its promoters, eda

**together, these results indicate that CsrA does not affect the
turnover.**

**FIG. 6.** (A) KdgR binding to the eda P2 promoter region. Shown is a
gel mobility shift assay of the binding of recombinant KdgR to a
radioactively labeled DNA fragment containing eda P2 and the put-
ative KdgR binding site. Top band, bound probe; bottom band, free
probe. Lanes 1 to 10, 0, 0.07, 0.14, 0.21, 0.28, 0.35, 0.49, 0.63, 1.41, and
2.11 pmol of KdgR, respectively. (B) PhoB binding. Shown is a gel
mobility shift assay of PhoB binding to the eda and phoA regulatory
regions. Lanes 1 to 13, 0, 1.99, 8.3, 24.9, 49.8, 58.1, 66.4, 49.8, 0, 1.66,
24.9, 58.1, and 59.1 pmol of PhoB, respectively. Arrows: a, bound-eda
probe; b, free-eda probe; c, bound-phoA probe; d, free-phoA probe.
FIG. 7. CsrA control of eda expression. (A) Synthesis of Eda in E. coli BM106 (wild type) and BM106c (csrA) grown in LB, LB plus gluconate (gnt), or LB plus glucuronate (glr). Cells were harvested during exponential phase (E), stationary phase (S), or the transition from exponential to stationary phase (T). (B) Northern blot analysis of eda transcripts following rifampin treatment of E. coli BM106 (wild type) or BM106c (csrA). Cells were harvested at the indicated times. (C) β-Galactosidase activity and growth of edaP1-lacZ fusion strains E. coli NP304 (csrA) grown on glucose (black bars and open circles) and gluconate (dark gray bars and filled circles) and E. coli BM304c (csrA) grown on glucose (dark gray bars and open inverted triangles) and gluconate (light gray bars and filled inverted triangles). (D) β-Galactosidase activity and growth of eda P2-lacZ fusion strains E. coli BM106 (csrA) grown on glucose (black bars and open circles) and glucuronate (light gray bars and filled circles) and E. coli BM106c (csrA) grown on glucose (dark gray bars and open inverted triangles) and glucuronate (light gray bars and filled inverted triangles). (E) β-Galactosidase activity and growth of edaP4-lacZ fusion strains E. coli BM105 (csrA) grown on glucose (black bars and open circles) and E. coli BM105c (csrA) grown on glucose (gray bars and filled circles).

Phosphate-starvation genes (32). Although PhoB typically acts as a transcriptional activator, it is a repressor of eda expression (Fig. 3). Also, in contrast to typical PhoB-dependent control, we observed the binding of the P4 promoter region by unphosphorylated PhoB. While unphosphorylated PhoB possesses DNA binding capability, phosphorylated PhoB dimerizes, which increases its affinity for PhoB binding sites (9). Under phosphate-replete conditions, unphosphorylated monomeric PhoB may bind to its P4 operator to repress eda expression. If phosphate becomes limiting, phosphorylated PhoB dimers may have a higher affinity for other PhoB binding sites and thereby derepress the eda promoter. Negative control of target genes by PhoB, although rare, has been observed by others (Barry Wanner, personal communication). While the mechanism of Eda regulation by PhoB remains to be elucidated, it is clear that Eda is induced by phosphate limitation in a PhoB-dependent manner.

that P4 is active under phosphate and carbon starvation conditions (Fig. 1). No primer extension product corresponding to P3 was found under the conditions tested (Fig. 1). P4 is located in the intergenic region between edd and eda, 26 bp upstream of the eda start codon. Both P2 and P4 are responsible for the high basal level of eda expression. Northern analysis showed that a 750-nt transcript is expressed in cells grown on gluconate (Fig. 2). Since this transcript increases when upstream promoters are highly expressed, it seems likely that there is a transcript-processing site in this region, thus complicating the interpretation of Northern blots. However, β-galactosidase assays of eda-lacZ fusions showed there are no glucuronate-inducible promoters in this region. Thus, we conclude that the eda P4 promoter is responsible for inducing a 750-nt transcript under phosphate starvation conditions.

In this study we identified three transcription factors that directly affect the transcription of eda. The first is GntR, a negative regulator of P1 (20). Our results confirm that Eda synthesis is derepressed in a gntR strain (Fig. 5A). There are two putative GntR binding sites located adjacent to P1 (21, 24), but these had not been previously analyzed. Mutation of the downstream GntR binding site caused derepression of the edaP1-lacZ fusion (Fig. 5B). GntR binding and the effector molecule gluconate most likely regulate P1 by a mechanism analogous to that described for gntT (19). Thus, eda is confirmed to be a member of the GntR regulon (20). The second factor affecting eda transcription is KdgR. Derepression of eda in a kdgR strain (Fig. 2) and the binding of KdgR to the P2 promoter in vitro (Fig. 6A) are in keeping with the role of the KdgR regulon in hexuronate catabolism (20, 22).

A third factor found to affect eda transcription is PhoB. E. coli W1485 induces Eda in response to phosphate limitation (Fig. 3), and the P4 promoter region is bound by PhoB in vitro (Fig. 6B). Phosphate starvation causes the sensor kinase PhoR to phosphorylate the response regulator PhoB, which in turn activates phosphate starvation genes (32). Although PhoB typically acts as a transcriptional activator, it is a repressor of eda expression (Fig. 3). Also, in contrast to typical PhoB-dependent control, we observed the binding of the P4 promoter region by unphosphorylated PhoB. While unphosphorylated PhoB possesses DNA binding capability, phosphorylated PhoB dimerizes, which increases its affinity for PhoB binding sites (9). Under phosphate-replete conditions, unphosphorylated monomeric PhoB may bind to its P4 operator to repress eda expression. If phosphate becomes limiting, phosphorylated PhoB dimers may have a higher affinity for other PhoB binding sites and thereby derepress the eda P4 promoter. Negative control of target genes by PhoB, although rare, has been observed by others (Barry Wanner, personal communication). While the mechanism of Eda regulation by PhoB remains to be elucidated, it is clear that Eda is induced by phosphate limitation in a PhoB-dependent manner.
We found that, in addition to induction by phosphate starvation, Eda was induced by carbon starvation in minimal glucose medium and in dilute LB (Fig. 4). The results suggest that carbon starvation is the inducing signal and not entry into stationary phase per se, since Eda was not induced when growth ceased in higher-strength LB. The mechanism underlying Eda induction by carbon starvation was not established, but it is interesting to speculate that CsrA might be involved. CsrA, the carbon storage regulator, was originally identified as a regulator of glycogen biosynthesis, although later it was found that CsrA regulates gluconogenesis, flagellum production, cell surface properties, and motility, indicating that it functions as a global regulator (25). It is also clear that CsrA is an activator of several glycolytic enzymes (25, 27). In keeping with this role, Eda expression in a csrA mutant strain was low. In addition, Eda was no longer induced during the transition to stationary phase in the csrA strain (Fig. 7). The mechanism underlying this control of eda transcript levels did not appear to involve CsrA-dependent modification of transcript stability, as it was the same in wild-type and csrA strains, which suggests that CsrA activates eda indirectly.

Finally, we consider the several physiological roles of Eda (KDPG aldolase) which are suggested by its regulation. Since Eda is required for growth on hexonates and hexuronates, its induction by these sugar acids and inclusion of eda in both the GntR and KdgR regulons are easily understood. This dual control facilitates the induction of Eda only for growth on hexuronates, which is mediated by KdgR and does not require Edd, which is only necessary for converting 6-phosphoglucononate to KDPG; coordinate induction of Edd and Eda for growth on hexonates is controlled by GntR. It may also be important that Eda does not vary in expression by more than threefold and is constitutively produced. It has been proposed that the high basal level of Eda is a mechanism to protect the cell from accumulation of toxic metabolites, such as KDPG (the substrate of Eda), which is formed by sugar acid catabolism (10), as well as glyoxylate, which is formed as a by-product of the TCA cycle (16, 18). In this respect, Eda might be regarded as a member of the glucose starvation stimulus, which includes coordinate repression of the TCA cycle and activation of glycolysis (17). The role of Eda in detoxification may explain why it is expressed at high basal levels; this might also explain why it is induced by starvation for carbon and phosphate. Increased Eda synthesis during phosphate limitation may supply phosphorylated metabolites where they are needed by draining the cytoplasmic pool of KDPG. In other words, imbalances in metabolism created by starvation for carbon or phosphate may lead to accumulation of toxic metabolites that are substrates for degradation by Eda. In this way, Eda may function as a stress response protein, in addition to its role in sugar acid catabolism.

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