Autogenous Regulation of Ethanolamine Utilization by a Transcriptional Activator of the eut Operon in *Salmonella typhimurium*

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The genes required for use of ethanolamine as a carbon and nitrogen source are encoded by a single operon (eut) whose expression is induced by the simultaneous presence of both ethanolamine and cobalamin (vitamin B₁₂). The action of B₁₂ as an inducer of this operon reflects the fact that this cofactor is required by the degradative enzyme ethanolamine lyase (eutBC). The eutR gene encodes a protein that activates transcription of the eut operon in response to the simultaneous presence of B₁₂ and ethanolamine. The eutR gene is expressed by a weak constitutive promoter activity (P₁₄) and by the main regulated promoter (Pᵢ). Because it is encoded within the operon that it activates, the EutR protein controls its own production. Initial induction of the eut operon by ethanolamine plus B₁₂ causes an increase in expression of the eutR gene; this increase acts as part of a positive feedback loop that is required for maximal operon expression. Because of this mode of regulation, constitutive regulatory mutations, described here, include mutations that generate new internal promoters and thereby increase the basal level of eutR gene expression. In mutants with an increased level of activator protein, each inducer (B₁₂ or ethanolamine), presented singly, is sufficient for partial operon induction.

*Salmonella typhimurium* LT2 uses ethanolamine as the sole source of carbon and nitrogen when B₁₂ is available to serve as a cofactor for the first enzyme in the pathway, ethanolamine ammonia lyase (7, 11). Unlike most cofactors, B₁₂ is unreliable since it is synthesized only under anaerobic conditions (2, 3, 15, 18). Under both aerobic and anaerobic growth conditions, exogenous B₁₂ can be transported into the cell by a mechanism described by Kadner and coworkers (4, 26) for *Escherichia coli*. Cells growing aerobically have B₁₂ only if they can obtain it from their environment. In view of the fact that B₁₂ may or may not be available, it is logical that transcription of the genes for ethanolamine utilization (the eut operon) is induced only when both ethanolamine and the essential B₁₂ cofactor are available (23). This pattern of regulation is unusual; we know of no other enzyme whose synthesis requires the presence of its cofactor. This study is an initial characterization of the mechanism whereby two effector molecules serve to induce this operon.

The requirement for both ethanolamine and B₁₂ as inducers of synthesis of the ethanolamine ammonia lyase was first shown, in *E. coli*, by Turner and coworkers (5, 6, 25). They have termed this regulatory pattern concerted induction. An *E. coli* regulatory mutant constitutively expresses both ethanolamine ammonia lyase and the second enzyme in the pathway, acetaldehyde dehydrogenase, but the nature of the defect in the *E. coli* mutant is not understood (6, 19, 20).

The genes for degradation of ethanolamine lie in a single operon between the *cysA* and *purC* loci at min 50 of the *S. typhimurium* chromosome (23). The genetic map of this operon is presented in Fig. 1. Complementation tests defined six genes in the operon (24). The *eutB* and *eutC* genes encode the two subunit types of ethanolamine ammonia lyase (ethanolamine→NH₃ plus acetaldehyde), and the eutE gene encodes acetaldehyde dehydrogenase, the second enzyme in the degradative pathway (acetaldehyde→acetyl coenzyme A). The eutD gene encodes an unknown function that, like acetaldehyde dehydrogenase, is required only for use of ethanolamine as the carbon source. The *eutA* gene is required if cells are to use exogenous cyanocobalamin (commercial vitamin B₁₂) or hydroxy-B₁₂ as a source of cofactor for the lyase. Current evidence suggests that the eutA function prevents inhibition of lyase by these forms of B₁₂ and does this by in part by formation of adenosyl-B₁₂, the true cofactor of ethanolamine ammonia lyase (26a). Null mutations in the eutR gene prevent induction of transcription of the operon (23).

Evidence that the eutR gene is located within the eut operon and encodes a transcriptional activator that mediates activation of the operon in response to ethanolamine plus B₁₂ is presented here. Because it is encoded within the operon it activates, the EutR protein controls its own production. The increase in EutR protein level caused by autoinduction appears to be required for maximal operon expression. Several sorts of regulatory mutations affect this control mechanism. Mutations that increase the basal level of eutR gene expression appear to partially circumvent the requirement for two effectors; in these mutants, the operon is partially induced by either one of the normal inducers.

**MATERIALS AND METHODS**

**Bacterial strains and transposons.** All strains used in this study are derivatives of *S. typhimurium* LT2 (Table 1). Two transposition-defective derivatives of Tn10, Tn10*del16* del17 Tet' (29) and Tn10dCam (14), were used. The Tn10*del16 del17* Tet' element is referred to as Tn10dTet. Transcriptional fusions were made with two transposition-defec-
tive derivatives of the specialized transducing phage Mu d1(Amp' Lac cts) of Casadaban and Cohen (8). The derivatives Mud1 1734 Kan' (9) and Mud1-8 Amp' (16) are referred to here as MudJ and MudA, respectively. MudA insertion mutations were converted to MudJ insertion mutations by recombination between sequences common to each element in a cross previously described (17); the insertion site and lac operon fusion were unchanged when a MuA prophage is converted to a MudJ prophage. The MudA-to-MudJ conversions were made to eliminate the residual transposition activity of MudA that interferes with use of the lac fusions to select regulatory mutations.

Media and growth conditions. The complex medium was nutrient broth (0.8%; Difco Laboratories) with NaCl (0.5%). The minimal medium was the E medium of Vogel and Bonner (28) with glucose (0.2%) as the carbon and energy source. The carbon-free minimal medium was NCE medium (13), and the carbon- and nitrogen-free minimal medium was NCN medium (22). Ethanolamine hydrochloride (0.2%; Aldrich) was used as the carbon source in NCE medium, as the nitrogen source in NCN medium with glycerol (0.2%), or as both the carbon and nitrogen source in NCN medium. Cyanocobalamin (Sigma Chemical) was used as the exogenous B12 source (0.1 µg/ml). Amino acids were added to minimal media as required at the concentrations previously described (13). Antibiotics were added to media as previously described (24). Solid medium contained agar (1.5%; Difco) or, when ethanolamine was used as the sole nitrogen source, Noble agar (1.5%; Difco). Cells were grown aerobically at 37°C.

Chromogenic indicator plates for detecting β-galactosidase activity contained NCE medium with glycerol and 25 µg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per ml. The X-Gal was dissolved in N,N-dimethylformamide before addition to the medium.

Genetic techniques and strain construction. Transductional crosses were performed with the high-frequency, generalized transducing phage mutant P22 HPI 1051/eut-201 (27) as previously described (23). Transductants were purified and made phage free by streaking for single colonies on nonselective indicator plates (10). Conjugational crosses were performed directly on selective medium as previously described (24).

The plasmids F' 606 his' eut' and F' T80 his' (in strains TT14082 and TR35) were used in constructing merodiploid strains. Plasmid F' 606 was constructed from F' T80 by addition of the eut operon sequences (24). Various eut::Tn10 insertion mutations were placed on plasmid F' 606 by transduction of strain TT14082 to Tet' as described previously (24). All F' plasmids were maintained in strains with a his deletion mutation on medium lacking histidine to select against loss of the F' plasmid. To prevent recombination between plasmid and chromosomal eut sequences, the recA1 mutation was introduced to all strains destined to carry an F' plasmid. This was done by cotransduction of the recA1 mutation with the selectable srl-203::Tn10/Cam insertion mutation from donor strain TT11289; recA4 mutant transductants were identified by UV light sensitivity.

Isolation and mapping of regulatory mutations. Regulatory mutants were selected by using a parent strain (TT10271) with a lac operon fusion in the first gene of the eut operon (eutD18::MudJ). Mutant derivatives that expressed the lacZ gene of the fusion without one of the two normally required inducers, ethanolamine and B12, were identified. The regulatory mutations were separated from the parental MudJ insertion by transduction into a eut deletion mutant, selecting Eut' transductants. The Eut' transductants must have acquired donor material to repair the recipient deletion but not the donor's eut::MudJ insertion. If the regulatory mutation is located within the deletion, then all Eut' transductants must acquire the donor regulatory mutation. To test for the presence of the regulatory mutation in the Eut' recombinants, the eutD18::MudA insertion was reintroduced into the Eut' recombinants by transduction, selecting for Amp' recombinants on X-Gal indicator medium containing ethanolamine but no B12. If the recipient contains the regulatory mutation, some of the Amp' transductants will form blue colonies on X-Gal because of constitutive expression of the eutD18::MudA fusion. Recipients that do not contain the regulatory mutation will form white transductant colonies on the indicator medium.

To geneti caliy map the location of a regulatory mutation, several different deletion mutations were used and, in each case, at least 60 eut' recombinants were tested for the presence of the regulatory mutation. If all 60 eut' transductants contained the regulatory mutation, the mutation was

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**FIG. 1.** Genetic map of the eut operon. The horizontal line in the middle of the figure represents the chromosome on which are indicated the positions of the deletions and insertion mutations used here. The solid triangle denotes a Tn10 insertion; open triangles denote insertions of the Mud/Jac Kα) element, all of which form operon fusions that express the lacZ gene from one or both of the eut promoters. The arrows above the horizontal line indicate the two transcripts provided by the P1 and P2 promoters. Letters above these arrows indicate the eut genes. Below the horizontal map are listed the regulatory mutations described here; dashed lines indicate the regions of the map that include the mutations indicated. Mutation numbers in parentheses are assigned to unstable mutations, inferred to be duplications whose join points lie in the designated region.
inferred to map within the deletion or to be greater than 98% linked by P22 transduction to the deletion end point. This cotransduction frequency corresponds to a distance of about 200 bp of DNA, calculated with the formula of Wu (30). Since this is a forced cross, in which a recombination event must occur between the deletion and the donor Mud insertion, the fraction of recombinants inheriting the donor regulatory mutation should reflect the position of the regulatory mutation as a fraction of the distance from the deletion to the eutD18::MudJ insertion.

One of the deletions used in the mapping experiments, eut-230, enters the operon from the left and includes part of the eutD gene; deletions DEL742, DEL760, DEL758, and DEL863 enter the operon from the right. These deletions were included in a genetic map of the eut operon previously described (24) and are included in Fig. 1.

One regulatory mutant (eut-262) was identified by screening on X-Gal ethanolamine indicator medium for mutants with increased expression of the eutD::lac fusion following localized mutagenesis of the eutR region. Phage was prepared on strain TT14847, which carries a Tn10 insertion outside the promoter-distal end of the eut operon and deletion mutation eut-229 which enters the operon from the left side and removes the eutD and eutE genes. This phage lysate was mutagenized with hydroxylamine as described previously (23) and then used to transduce strain TT10271, which carries the mutation eutD18::MudJ. Transductants resistant to tetracycline were selected on NCE glycerol ethanolamine medium containing tetracycline, kanamycin, and X-Gal. On this medium one selects for inheritance of the donor Tn10 element and retention of the recipient eutD::MudJ insertion. Inclusion of the donor eut deletion ensures that mutations within the operon must lie distal to the eutE gene. The eut-262 mutant was isolated as a blue transductant colony that expressed the eut::lac fusion despite the presence of only ethanolamine as an inducer.

β-Galactosidase assays. Strains to be assayed for β-galactosidase activity were grown with vigorous shaking in NCE medium supplemented with a carbon source (0.2% glucose or glycerol or 0.6% succinate), ethanolamine (0.2%), and/or B12 (0.1 μg/ml); cyclic AMP (cAMP), when used, was added to a final concentration of 5 mM. Cells were harvested at a density of 80 to 110 Klett units by centrifugation and were resuspended in 0.85% NaCl. β-Galactosidase was assayed in permeabilized cells as described by Miller (21). Activity is expressed as nanomoles of product produced per minute per
The eutR gene product is required only for induction of eut operon transcription. It was previously shown that eutR mutants fail to induce transcription of the eut operon in response to added ethanolamine and B_{12} (23). This suggested that the eutR gene might encode a positive activator of transcription that mediates both B_{12} and ethanolamine effects on eut operon expression. Alternatively, the eutR gene product might catalyze a step of ethanolamine degradation needed for uptake of ethanolamine or synthesis of the true regulatory effector. If the sole function of the eutR protein is to activate eut operon transcription, then expression of the operon by a eutR-independent promoter would permit ethanolamine utilization without the eutR function.

To test this possibility, we selected for Eut\(^+\) revertants of the eutR156::MuDJ insertion strain TT13738. Some revertants, designated Eut\(^+\)(N\(^+\)C\(^-\)), could use ethanolamine as a nitrogen source but not as a carbon source; these were isolated at a frequency of approximately 1\(^{-8}\) without mutagenesis. These revertants could complete the first step in the ethanolamine degradation pathway (cleavage of ethanolamine to acetaldehyde and ammonia) but not the second step (conversion of acetaldehyde to acetyl coenzyme A). The first step of ethanolamine utilization requires the eutA, eutB, and eutC gene products, while the second step requires the eutD and eutE gene products (24). Revertants that are able to use ethanolamine as both a carbon and nitrogen source, Eut\(^+\)(N\(^+\)C\(^+\)) revertants, must express all five of the above eut genes. No revertants of this second type (frequency of less than 10\(^{-10}\)) were found among spontaneous mutants, but a single example (eut-241) was obtained after nitrosoguanidine mutagenesis. All of the mutations allowing operon expression independent of EutR function are located in or near the eut operon. Six mutations conferring the Eut\(^+\)(N\(^+\)C\(^-\)) phenotype were tested and found to be cotransducible with the eutR156::MuDJ insertion at frequencies ranging between 48 and 75%; this is consistent with a location in the first portion of the operon, upstream of the eutABC genes. The single mutation conferring the Eut\(^+\)(N\(^+\)C\(^+\)) phenotype cotransduced with the eutR::MuDJ insertion at a frequency of 44%, consistent with a location near the P\(_1\) promoter.

In addition to permitting eutR-independent ethanolamine utilization, the revertant mutations caused the lac genes of the parental eutR156::MuDJ insertion to be expressed constitutively. In the Eut\(^+\)(N\(^+\)C\(^+\)) revertant, the active promoter must be located to the left of the first gene in the operon (eutD), while the new promoters in the Eut\(^+\)(N\(^+\)C\(^-\)) revertants appear to be located within the operon at some point to the left of the eutA, eutB, and eutC genes since they permit use of ethanolamine as a nitrogen source only. Since the Eut\(^+\)(N\(^+\)C\(^-\)) strain is able to use ethanolamine as a carbon source, it must express all eut genes; this revertant may have a modified main promoter, allowing it to act without the EutR function, or may have fused the operon to a foreign promoter.

Since all revertants express ethanolamine lyase and one can successfully use ethanolamine as both a carbon and nitrogen source without the eutR function, we conclude that the EutR protein plays no essential direct role in transport or degradation of ethanolamine or in generation of the adenyl-B_{12} cofactor required for lyase activity. We infer that EutR protein plays a purely regulatory role in operon expression.

**Inducible transcription of the eutR gene from the primary eut operon promoter.** The eut enzymes are encoded by a single transcription unit (eutDEABC); insertion mutations within this gene cluster block transcription of genes located farther to the right (promoter distal) as the map is generally presented (23, 24) (Fig. 1). The eutR gene is located at the distal end of this eutDEABC operon, but previous work did not address how the regulatory gene is expressed.

Because the eutR gene is located very close to the eutC gene, it seemed possible that the eutR gene is included in the eutDEABC transcript and expressed from the primary inducible promoter. Transcription of eutR was measured by using a chromosomal eutR::lac operon fusion (eutR156::MuDJ); a functional eutR gene was supplied on a derivative of the F\(')\) his\(^+\) plasmid which carries the complete eut operon of S. typhimurium (F\(')\) 606). The latter plasmid was constructed by Tn10-mediated transposition of the entire eut\(^+\) operon onto the F\(')\) his\(^+\) plasmid (24).

The chromosomal eutR::lac fusion strain with only the parental F\(')\) his\(^+\) plasmid showed no induction of \(\beta\)-galactosidase in response to addition of ethanolamine plus B_{12} (Table 2, line 1). However, the same fusion was induced 25-fold in strains with the F\(')\) 606 his\(^+\) eut\(^+\) plasmid which could provide the EutR function in trans (line 2). Data shown later will demonstrate that the same F\(')\) plasmid with a eutR mutation cannot provide for this induction. This inducible expression of the eutR::lac fusion is dependent on the primary operon promoter (P\(_1\)) for the eutDEABC genes (Fig. 1). This was shown by adding a polar eut::Tn100Tet insertion mutation to the chromosome of the diploid strain to

### TABLE 2. Expression of eutR::lac fusions by the primary promoter (P\(_1\)) and the internal promoter (P\(_2\))

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Strain</th>
<th>Relevant chromosomal markers(^a)</th>
<th>Relevant F(^+) plasmid marker(s)</th>
<th>(\beta)-Galactosidase activity (U) in cells grown in minimal medium supplemented with B_{12}</th>
<th>Induction (fold)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TT14488</td>
<td>eutR156::lac</td>
<td>his(^+)</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>TT1489</td>
<td>eutR156::lac</td>
<td>his(^+) eut(^+)</td>
<td>1.6</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>TT14490</td>
<td>eutA::Tn100Tet eutR156::lac</td>
<td>his(^+)</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>TT14491</td>
<td>eutA::Tn100Tet eutR156::lac</td>
<td>his(^+) eut(^+)</td>
<td>2.6</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>TT10674</td>
<td>eut-38::lac(^a)</td>
<td>None</td>
<td>5.0</td>
<td>210</td>
</tr>
<tr>
<td>6</td>
<td>TT14492</td>
<td>eutA::Tn100Tet eut-38::lac(^a)</td>
<td>None</td>
<td>8.0</td>
<td>42</td>
</tr>
</tbody>
</table>

\(^a\) All F\(^+\) plasmid strains are recA mutants. See Table 1 for complete genotype.

\(^b\) Calculated by dividing the activity with ethanolamine plus B_{12} by the activity with neither ethanolamine nor B_{12}.

\(^c\) This lac fusion is located promoter distal to the eutR gene at the end of the operon.

\(A_{650}\) unit of cells. Measured activity in Lac\(^-\) strains was 0.2 U or less.
block transcription of the eutR fusion from the primary promoter. The eutA insertion prevented induction of β-galactosidase (lines 3 and 4), indicating that the inducible transcription of eutR originates upstream of the eutA gene (presumably from the primary promoter).

Expression of the eutR gene from the primary promoter was confirmed by using the eutR::MudA insertion. This insertion is located on the promoter-distal side of the eutR gene (Fig. 1); since the insertion causes no obvious Eut phenotype, maps distal to eutR mutations, and provides regulated β-galactosidase, we infer that it lies in a 3' untranslated region promoter distal to the eutR coding sequences. Unlike the haploid eutR::lac fusion strain (Table 2, line 1) and like the eutR::lac fusion in strains with an added eutR+ gene (line 2), expression of β-galactosidase in the haploid eutR::MudA strain was induced upon addition of ethanolamine plus B12 (line 5). Thus, the eutR gene appears not to be damaged by the eutR::MudA insertion. The inducible transcription of the eutR::MudA fusion originates from the primary promoter because inducibility was abolished when the polar eutA::Tn10Tet insertion mutation was introduced (line 6). Since the eutR::MudA element is inserted distal to the eutR gene, EutR function can be provided by the intact chromosomal eutR gene.

**Basal transcription of the eutR gene.** In addition to the inducible transcription of the eutR gene from P1, we infer the existence of a weak constitutive promoter for the eutR gene. We presume that this promoter (P0) is located between the eutC and eutR genes and provides a basal level of EutR protein that allows the cell to sense the appearance of the inducers.

Polar mutation insertions (Mud and Tn10 elements) in any of the eutDEABC genes do not abolish the eutR function in complementation tests, suggesting that the basal expression of the eutR gene is independent of the primary operon promoter (P0) (24). All operon fusions created by Mud insertions in the eutDEABC region show eutR-dependent induction of β-galactosidase by ethanolamine plus B12; this induction is lost if a eutR mutation is added (23). This demonstrates that at least some EutR protein is synthesized when the operon is fully repressed and that this synthesis is not eliminated by any of the insertion elements we have placed promoter proximal to the eutR gene. We will describe the basal transcription activity in terms of an internal promoter, located between the eutC and eutR genes (Fig. 1). However, we cannot eliminate the possibility that all of the Tn10Tet and Mud insertion elements we have used introduce a weak promoter that provides eutR gene expression. If this were true, the basal transcription of the eutR gene in wild-type strains could come from the primary promoter P1. We cannot distinguish between a single internal promoter and a series of sites within the operon at which a low level of transcription might start.

In strains with a eutA::Tn10Tet insertion, described above, the eutR::lac and the eutR::lac fusions should be expressed only from the internal promoter. The low level of lac expression in these strains was similar in the eutR+ (Table 2, lines 4 and 6) and eutR (line 3) strains, indicating that the internal promoter (P1) does not require EutR function and appears to be constitutively expressed. The β-galactosidase levels measured in the eutR::MudA fusion strain (line 6) are severalfold higher than in the eutR156::MudJ strain (line 4); this seems to be simply a characteristic of the particular lac fusions.

**Positive autogenous regulation of the eutR gene.** The maximum induced activity of the primary promoter is greater in strains that can induce expression of the eutR gene. A chromosomal eutD1::MudJ insertion was used to measure primary promoter activity. This insertion blocks transcription of downstream genes (including the eutR gene) from the primary promoter; the eutR gene is expressed only from the weak constitutive internal promoter. In this strain, the primary promoter showed a 390-fold induction in response to ethanolamine and B12; this induction was completely dependent on an intact eutR gene (Table 3, lines 1 and 2).

A larger induction of the primary promoter was observed when eutR was expressed by both the internal promoter and the regulated primary promoter. Transcription of the same chromosomal eutD::lac fusion was induced 1,300-fold in a strain carrying a second, intact copy of the eut operon on plasmid F' 606 his+ eutR+ (Table 3, line 3). This level is not changed by removal of the chromosomal eutR gene (line 4), but it was reduced when transcription of the plasmid eutR gene from the primary promoter was blocked by the polar eutD46::Tn10 insertion (Table 3, line 5). Elimination of the eutR gene from the plasmid (line 6) abolishes the plasmid's ability to complement a chromosomal eutR mutation; the eutR(eutR) diploid is unable to induce transcription from P1.

It should be noted that when all EutR protein production is due to the plasmid's internal promoter, the level of induction seen (Table 3, line 5) is higher than that seen for a strain with only a chromosomal copy of eutR under the same promoter (line 1). This is probably due to a slightly higher copy number of the eutR gene on the F' plasmid. This further supports the idea that the basal level of eutR gene expression provided by the internal promoter provides a

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**TABLE 3. Regulation of a eutD::lac fusion**

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Strain</th>
<th>Relevant chromosomal markers*</th>
<th>Relevant F plasmid marker(s)</th>
<th>β-Galactosidase activity (U) in cells grown in medium supplemented with---</th>
<th>Induction (fold)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No inducer</td>
<td>EA</td>
</tr>
<tr>
<td>1</td>
<td>TTI4476</td>
<td>eutD::lac</td>
<td>his+</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>TTI4478</td>
<td>eutD::lac eutR::Tn10</td>
<td>his+</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>TTI4477</td>
<td>eutD::lac</td>
<td>his+ eut+</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>TTI4479</td>
<td>eutD::lac eutR::Tn10</td>
<td>his+ eut+</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>TTI4484</td>
<td>eutD::lac eutR::Tn10</td>
<td>his+ eutD::Tn10</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>TTI4487</td>
<td>eutD::lac eutR::Tn10</td>
<td>his+ eutR::Tn10</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>TTI4483</td>
<td>eutD::lac</td>
<td>his+ eutR::Tn10</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* All F' plasmid strains are recA mutants. See Table 1 for complete genotypes.

* The carbon source was glycerol, and the nitrogen source was ammonia in NCE medium. EA, ethanolamine.

* The specific activity with ethanolamine plus B12 divided by the specific activity without ethanolamine or B12.
limiting concentration of activator protein and confirms the idea that induction of the eutR gene is necessary to achieve full eut operon expression.

**Extra control regions reduce operon expression.** In a strain expressing the eutR gene only from the chromosomal internal promoter, induction of the chromosomal eutD fusion was reduced by a plasmid that provided a eut operon but carried the eutR205::Tn10 mutation (compare lines 1 and 7 in Table 3). This effect may be due to the extra copies of the eut primary control region present on the plasmid; these may titrate some of the limiting EutR protein made from the chromosome and reduce the amount of EutR protein reaching the assayed chromosomal control region.

**Catabolite repression of the eut operon.** Catabolite repression was examined by using a eutD::MudJ fusion and the eut-38::lac fusion on the 3' end of the operon. Both fusion strains showed substantially lower levels of induced expression during growth on glucose than during growth on succinate as the carbon source, and catabolite repression of both fusion strains was overcome by adding exogenous cAMP to the glucose growth media (Tables 4, lines 1 and 4).

The cya and crp genes encode the enzyme adenyl cyclase and the catabolite activator protein; these proteins mediate catabolite repression. Insertions of Tn10 in either gene reduced eut operon expression substantially (Table 4, lines 2, 3, 5, and 6). Addition of exogenous cAMP relieved the reduction caused by the cya::Tn10 insertion, as expected if the defect is due to a lack of cAMP (lines 2 and 5), but had no effect in the crp::Tn10 strains (lines 3 and 6). The significant residual expression of the eut operon during growth on glucose may explain the ability of S. typhimurium to use ethanolamine as the sole nitrogen source even during growth on glucose. However, growth on glucose medium with ethanolamine as the sole nitrogen source is eliminated in cya insertion mutants, and growth is substantially slower in crp insertion mutants. This suggests that the catabolite activation pathway is required for ethanolamine utilization.

Note that the eut-38 fusion shows a high induced level of expression in the presence of glucose (Table 4). We suspect that once high levels of EutR protein are produced in the eut-38 strain by autoinduction, cells may escape the effect of catabolite repression. In the eutD fusion strain, EutR protein is produced only by the inducible internal promoter; these high levels of EutR protein are not attained.

The influence of catabolite repression on the internal promoter was examined by using a eut-38::lac fusion strain containing an upstream eutA::Tn04Tet insertion to block transcription of lacZ from the primary promoter (Table 4, line 7). No significant difference in internal promoter activity was detected when the strain was grown in media with succinate, with glucose, or with glucose plus cAMP, indicating that the internal promoter is not subject to catabolite repression. Therefore, catabolite repression is most likely mediated via the primary promoter.

**Isolation of regulatory mutants.** To identify regulatory elements that mediate expression of the eut operon in response to ethanolamine plus B12, we isolated mutant derivatives of the eutD::lac operon fusion strain (TT10271) that are Lac⁺ on medium containing ethanolamine but not B12; this strain expresses the eutR gene only from the internal promoter. Cells were plated on NCE lactose medium containing ethanolamine; selection was made for growth on lactose as the sole carbon source. Spontaneous Lac⁺ derivatives were found at a frequency of approximately 10⁻⁶.

One exceptional regulatory mutant (eut-262) was identified by screening on X-Gal indicator medium for mutants with increased expression of the eutD::lac fusion on ethanolamine alone following localized mutagenesis of the eutABC region (as described in Materials and Methods). All of the regulatory mutants described above map in or near the eut operon; their Lac⁺ phenotype cotransduced with the drug resistance genes of eut::Mud and eut::Tn10 elements.

**Regulatory mutations affecting the P₁ region.** The majority of mutants with a Lac⁺ phenotype in the presence of only one inducer were found to contain a mutation located in the primary promoter region; the new mutation causes constitutive expression of the eutD::lac fusion. The operon expression in these mutants (Table 5, lines 3 to 5) is independent of the eutR function; the mutants remained Lac⁺ when a eutR205::Tn10 insertion was introduced (data not shown). Since operon expression in these mutants does not require EutR function or respond to the standard inducers, it seems likely that mutations of this type (eut-253, eut-254, and eut-259) render the P₁ promoter fully constitutive or (more likely) delete the eut promoter and fuse the operon to a foreign constitutive promoter of moderate strength.

Seven constitutive mutants, including the three described above and the eut-260 mutant described below, were mapped. All showed close transductional linkage to the promoter-proximal eutD18::MudJ insertion (98 to 100% cotransduction of Lac⁺ and Kan⁺). The constitutive mutants were crossed with a recipient strain containing a deletion which removes the left end of the operon and extends into the eutD gene (eut-230). In this cross, Eut⁺ transductants
TABLE 5. Effect of regulatory mutations on eutD::lac fusion expression

<table>
<thead>
<tr>
<th>Line</th>
<th>Strain</th>
<th>Relevant markers*</th>
<th>cAMP added to medium</th>
<th>β-Galactosidase activity (U) in cells grown in medium withβ:</th>
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<td></td>
<td></td>
<td>No inducer</td>
</tr>
<tr>
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<td>eutD::lac</td>
<td>-</td>
<td>1 1  2</td>
</tr>
<tr>
<td>2</td>
<td>TT10271</td>
<td>eutD::lac</td>
<td>+</td>
<td>1 2  5</td>
</tr>
<tr>
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<td>TT14498</td>
<td>eutD::lac eut-253</td>
<td>-</td>
<td>60 60 60</td>
</tr>
<tr>
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<td>-</td>
<td>110 120 120</td>
</tr>
<tr>
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<td>TT14503</td>
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<td>-</td>
<td>70 80 70</td>
</tr>
<tr>
<td>6</td>
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<td>-</td>
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</tr>
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<td>-</td>
<td>1 5  3</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>TT14493</td>
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<td>-</td>
<td>1 4  7</td>
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<tr>
<td>14</td>
<td>TT14493</td>
<td>eutD::lac eut-246</td>
<td>+</td>
<td>1 9  14</td>
</tr>
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</table>

* See Table 1 for complete genotypes.
β The carbon source was glycerol, and the nitrogen source was ammonia in NCE medium. EA, ethanolamine.

arose by recombination between the donor eutD18::MudJ insertion and the recipient deletion. When the seven regulatory mutants carrying the eutD18::MudJ insertion were used as donors in the same cross, all failed to generate Eut+' recombinants, indicating that the constitutive mutants are defective for the eutD structural gene. The results suggest that the constitutive regulatory mutations either generate a new promoter within the eutD gene or (more likely) are deletions or insertions which damage the eutD gene and fuse the eut operon to a foreign promoter.

Another regulatory mutant (eut-260) showed an increased basal level but, unlike the mutations described above, showed a further increase in expression to the normal induced level upon addition of the two inducers to the growth medium (Table 5, line 6). These results suggest that the constitutive mutation does not remove the main promoter but may have created an additional promoter or allowed the main promoter to act at a low level in the absence of induction.

Regulatory mutations that generate promoters within the operon. Unlike the mutations described above, which permit EutR-independent growth on lactose without any inducer, a few of the regulatory mutants showed a Lac+ phenotype if either ethanolamine or B12 was individually present; these mutants showed no growth on lactose unless at least one inducer was provided. The parent strain requires both ethanolamine and B12 to permit growth on lactose.

For one type of mutant, operon expression was inducible to a level that exceeded that of the parent eutD::lac fusion strain. Three examples of this type are eut-247, eut-250, and eut-262 (Table 5). The induced enzyme level in these mutants is similar to that in strains which are able to increase eutR gene expression in the course of operon induction.

The strain used in isolating regulatory mutants (TT10271) carries a eutD18::MudJ insertion which exerts a strong polar effect on expression of the promoter-distal eutEABC genes as well as on the eutR gene. Because of this polarity effect, the parent strain cannot use ethanolamine as a nitrogen source. Some regulatory mutations (such as eut-247 and eut-250), identified by their effect on expression of the eutD::lac fusion, also enable the parent strain to use ethanolamine as a nitrogen source and thus appear to express the eutABC genes. The new promoters provided by these mutations must lie downstream of the polar parental eutD::lac fusion (whose expression was scored in isolating the mutants) and upstream of the eutABC genes, whose expression is required for growth on ethanolamine. The eut-247 mutation cotransduced with the eutD::MudJ element at a frequency of 85%, and the eut-250 mutation cotransduced at a frequency of 100%. This suggests that eut-247 lies in the distal portion of the eutD gene and that eut-250 is in the distal portion of the MudJ element.

The eut-262 mutation was generated by localized mutagenesis of the eutABC region. This mutation provides a Lac+ phenotype for the parental eutD::lac fusion and causes an increase in eutR gene expression (see below). However, unlike the mutations described above, it does not provide a Eut(N+2) phenotype. The mutation was mapped (as described in Materials and Methods) and was found to recombine with deletion DEL742, which includes the eutR gene. Thus, mutation eut-262 increases eutR gene expression but does not provide expression of all of the eutABC genes and does not affect the eutR gene itself. We suggest that mutation eut-262 generates a new promoter somewhere within the eutABC gene cluster (Fig. 1). The effect of both the eut-247 and eut-262 mutations is mediated by functional EutR protein because these strains become Lac- upon introduction of the eutR25::Tn10 insertion. However, the eut-247 and eut-262 mutations recombine with deletion DEL742 and thus do not affect eutR gene structure directly. Later we will describe the effect of the eut-247 and eut-262 mutations on eutR gene transcription.

Three additional mutations that show properties like those described above but were extremely unstable were isolated. Mutations eut-249 and eut-252 provide a Lac+ Eut(N+) phenotype; however, they revert to a Lac- Eut- phenotype with high frequency. Mutation eut-251 causes a Lac- phenotype but does not allow use of ethanolamine as a nitrogen source and therefore does not express eutABC genes. Like
**FIG. 2.** A duplication that can provide a new promoter for part of the eut operon. The duplicated segment is indicated by parentheses. Note that $P_x$ is a foreign promoter that expresses some of the eut genes by directing transcription across the duplication join point. One copy of the eut operon and one copy of the gene normally expressed by $P_x$ remain intact; the only novel sequence is at the duplication join point.

_eut-249_ and _eut-252_, it is unstable, reverting to Lac" with high frequency.

This instability phenomenon has been seen previously for mutants that restore function to promoterless genes. This has been described for the histidine operon (1). In one case, a silent _hisD_ gene is activated by fusion to the _argA_ promoter at the join point of a duplication (12, 27). It seems likely that the same explanation may account for the unstable eut regulatory mutations described above.

We infer that all three unstable mutations are duplications which fuse a foreign promoter to the eut operon, allowing expression of genes downstream of the join point (including the eutR gene). The proposed structure of these duplications is diagrammed in Fig. 2. In the two Eut(N") strains, the fusion join point must be placed so as to activate the _eutABC_ genes (i.e., between the _eutD::lac_ insertion and the _euta_ gene, as depicted in Fig. 2); in the Eut(N") mutant the fusion could be located anywhere within the _eutABC_ region.

**The internal regulatory mutations increase eutR gene transcription.** We have hypothesized that both the stable and unstable internal regulatory mutations described above exert their effect on the main promoter by increasing the level of _eutR_ gene expression. To test this, mutations _eut-247_ and _eut-262_ were each added singly to a strain with a _eutR::lac_ fusion (instead of the original _eutD::lac_ fusion). Both mutations caused a ninfold increase in expression of the _eutR::lac_ fusion (Table 6). The $\beta$-galactosidase activity measured in these strains is due to the native internal promoter plus the novel internal promoters (generated by the regulatory mutations); the primary promoter is not active in the absence of EutR function, and in any case, any residual transcripts are blocked by a _eutD::Tn10_ insertion.

**Elevated EutR allows partial induction by one effector and increased maximal induction.** All of the mutations that increase _eutR_ gene expression have two additional properties. They allow partial transcriptional activation when only one of the inducers is present, and they allow a higher than normal level of operon induction when both inducers are provided (compare line 1 with lines 7 to 12 in Table 5). Both of these effects were seen earlier in strains whose EutR level was increased by the presence of the F' 606 _eut^+_ plasmid (Table 3, compare lines 1 and 3).

**A regulatory mutation within the eutR gene.** One of the regulatory mutations (_eut-246_), whose effect is dependent on the _eutR_ activator function, differs in phenotype and map location from the mutations described above. Mutation _eut-246_ allows partial induction of the _eutD::lac_ fusion by a single effector but normal induction in the presence of both inducers (compare lines 13 and 14 with line 1 in Table 5). This suggests that the _eut-246_ mutation might alter regulation in a manner that does not involve an increase in _eutR_ gene expression (which would increase the maximum induced level).

The _eut-246_ mutation was mapped as described in Materials and Methods by using a series of deletions which enter the _eutR_ gene from the 3' end of the operon (Fig. 1). No recombinants were found with any of the deletions which enter the _eutR_ gene (DEL742, DEL758, and DEL760). Deletion DEL863 is a constructed EutR" deletion that extends from the _eut-38::MudA_ insertion site rightward to the nearby _cysA_ locus (23). Two of 80 recombinants selected for repair of DEL863 deletion did not inherit the donor _eut-246_ regulatory mutation, indicating that mutation _eut-246_ maps to the left of the end point of deletion DEL863. We infer that the _eut-246_ mutation must lie at the extreme downstream end of the _eutR_ gene or just outside of the distal end of this gene. It seems likely that this mutation owes its effects to alteration of the _eutR_ gene rather than an increase in _eutR_ gene expression.

**DISCUSSION**

**Conclusions.** Several conclusions are drawn from the results presented here.

(i) The _eut operon is controlled by a transcriptional activator (EutR) encoded within the operon_. A model for the mechanism of regulation is shown in Fig. 3. Null mutations in the _eutR_ gene prevent induction of transcription of the operon in response to the availability of ethanolamine plus B$_{12}$; the

**TABLE 6.** Effect of internal regulatory mutations on _eutR::lac_ expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant markers</th>
<th>$\beta$-Galactosidase activity (U) in cells grown in medium with*</th>
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</thead>
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<td>eutD::Tn10 eut-247 eutR156::lac</td>
<td>17</td>
</tr>
<tr>
<td>TT14845</td>
<td>eutD::Tn10 eut-262 eutR156::lac</td>
<td>19</td>
</tr>
</tbody>
</table>

* Cells were grown in NCE medium with glycerol as the carbon source and ammonia as the nitrogen source. EA, ethanolamine.
The fact that either ethanolamine alone or $B_{12}$ alone causes some induction in mutants with elevated eutR gene expression implies that ethanolamine and $B_{12}$ both participate in conversion of inactive EutR protein to the form capable of activating transcription. We suggest that the EutR protein may bind either effector singly and thereby stabilize distinct protein conformations that exist in equilibrium with the conformation that activates transcription. This active form may be stabilized by the simultaneous binding of both effectors (Fig. 3). In strains with a high level of EutR protein, the presence of a single effector could lead to an increased level of one of the intermediate conformations. Since these forms are in equilibrium with the active form, the high level of EutR protein could lead, by mass action, to a significant level of the active conformation when only a single effector is present.

Only one of the regulatory mutations described here (eut-246) maps in or very near the eutR gene. The phenotype of this mutant differs from that of mutations that are thought to cause increased expression of the eutR gene. The level of induction achieved in the presence of both inducers is the same level seen for the parental eutD::lac fusion strain. The results suggest that the eut-246 mutation may allow the EutR protein (shown in Fig. 3) to assume the active form when either one of the two inducers is present singly.

The model for regulation. The model presented here suggests that a very high concentration of activator protein might allow the operon to be fully induced by only one effector. The regulatory mutations which generate new internal promoters permit weak activation of transcription by a single effector. These effects should also be seen when eutR levels are high as a consequence of a normal induction. That is, once the operon is fully induced (by both effectors), one might expect that the induced state could be maintained by a single effector using the elevated levels of eutR function. This could be an important feature of the regulatory mechanism.

During anaerobic growth, the de novo $B_{12}$ biosynthetic pathway produces very little $B_{12}$ (about 60 molecules per cell) (3). Induction of the eut operon results in high levels of the $B_{12}$-dependent enzyme ethanolamine ammonia lyase. When $B_{12}$ is scarce, induction may relax the requirement for $B_{12}$ binding to the eut activator and serve to free $B_{12}$ from its inducer role so that it can function as a cofactor for ethanolamine ammonia lyase. We suggest that $B_{12}$ is needed for the initial induction of the operon but that the induced state may be maintained by ethanolamine alone, allowing all of the $B_{12}$ supply to be available for ethanolamine ammonia lyase once induction has been achieved.

ACKNOWLEDGMENTS

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REFERENCES


eutR defect can be corrected in trans by an F' eutR+ plasmid.

(ii) The EutR protein seems to play a purely regulatory role. Some suppressors of eutR mutations express all functions necessary for ethanolamine utilization with no functional eutR gene.

(iii) The quantity of EutR protein appears to limit operon expression. Insertion mutations that block transcription of the eutR gene from the primary promoter ($P_1$) leave the eutR gene expressed only by a weak constitutive internal promoter and reduce operon expression to 20% of the level seen when eutR gene expression is inducible. Mutations that, we argue, generate new internal promoters for a noninducible eutR gene increase operon expression.

(iv) Increased expression of the EutR protein allows operon induction by either ethanolamine alone or $B_{12}$ alone. Strains which overexpress eutR by virtue of a new internal promoter and strains which carry F' 606 eut+ and also express the eutR gene from the primary eut operon promoter show significant induction by a single effector.

General points. Fusions of the lac operon to the eutD gene, to the eutR gene, and to an untranslated region promoter distal to the eutR gene were used to report operon expression. All fusions show EutR-dependent induction when ethanolamine and $B_{12}$ are available. However, the fusions differ in their basal and fully induced levels of β-galactosidase activity. Several factors may account for this variation. The level of the primary promoter activity is very sensitive to the expression and inducibility of the eutR gene. Expression and inducibility of eutR varies in each fusion. The position of the fusions relative to the two promoters also influences lac expression. Fusions located downstream of the internal promoter show higher basal transcription. The downstream fusions also show a lower maximal induced expression level, possibly due to termination of some of the transcripts originating at the $P_1$ promoter before they reach the end of the operon. In previous experiments we have seen a generally decreasing gradient of induced eut::lac fusion expression as the fusion point is moved farther downstream in the operon (23). Finally, variation in the structure of individual fusion transcripts could cause differences in transcript stability or translation efficiency; these effects would be a characteristic of each fusion strain.

FIG. 3. The EutR regulatory protein responds to two effectors. The model proposes that each effector binds independently. Normally both effectors must be present to stabilize the active conformation and ensure sufficient activator for operon induction. In cells with a high level of regulatory protein, a single effector might generate a large enough pool of an intermediate conformation to lead, by simple equilibrium considerations, to a significant level of active regulator. EA, ethanolamine.