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Transcription of the Glutamyl-tRNA Reductase (*hemA*) Gene in *Salmonella typhimurium* and *Escherichia coli*: Role of the *hemA* P1 Promoter and the *arcA* Gene Product

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In *Salmonella typhimurium* and *Escherichia coli*, the *hemA* gene encodes the enzyme glutamyl-tRNA reductase, which catalyzes the first committed step in the heme biosynthetic pathway. It has recently been reported that a *lac* operon fusion to the *hemA* promoter of *E. coli* is induced 20-fold after starvation for heme. Induction was dependent on the transcriptional regulator ArcA, with a second transcriptional regulator, FNR, playing a negative role specifically under anaerobic conditions (S. Darie and R. P. Gunsalus, J. Bacteriol. 176:5270–5276, 1994). We have investigated the generality of this effect by examining the response to heme starvation of a number of *lac* operon fusions to the *hemA* promoters of both *E. coli* and *S. typhimurium*. We confirmed that such fusions are induced during starvation of a *hemA* auxotroph, but the level of induction observed was maximally sixfold and for *S. typhimurium* fusions it was only two- to fourfold. Sequences required for high-level expression of *hemA* lie within 129 bp upstream of the major (P1) promoter transcriptional start site. Mutants defective in the P1 promoter had greatly reduced *hemA-lac* expression both in the presence and in the absence of ALA. Mutations in *arcA* had no effect on *hemA-lac* expression in *E. coli* during normal growth, although the increase in expression during starvation for ALA was half that seen in an *arcA*$^+$ strain. Overexpression of the *arcA* gene had no effect on *hemA-lac* expression. Primer extension analysis showed that RNA 5′ ends mapping to the *hemA* P1 and P2 promoters were not expressed at significantly higher levels in induced cultures. These results differ from those previously reported.

In *Salmonella typhimurium* and *Escherichia coli*, heme plays a role both in respiration and in defense against the toxic oxygen metabolite H_2O_2. Heme *b* (Fe protoporphyrin IX) and various modified hemes are cofactors for a number of cytochromes as well as two catalases (2, 7, 30). The heme biosynthetic pathway also branches to produce two other tetrapyrroles: siroheme, the cofactor for sulfite and nitrite reductases (26, 41), and cobalamin (vitamin B_{12}). *S. typhimurium* synthesizes cobalamin de novo, but only during growth under anaerobic or low-oxygen conditions (1, 24). Thus, the products of this branched pathway have a variety of functions related to oxygen, respiration, and electron transfer.

The biochemistry of heme synthesis is well established, and with the exception of the initial reactions leading to 5-aminolevulinic acid (ALA), the pathway is conserved among all organisms that make heme (9, 25). There are two routes to ALA: either by a C_5 route from glutamate or by a C_4 route from succinyl coenzyme A and glycine (5, 21). *S. typhimurium* and *E. coli* use the C_5 route (3, 14, 28, 32). The C_5 route enzyme glutamyl-tRNA reductase converts charged glutamyl-$tRNA^{Glu}$ to glutamate-1-semialdehyde (or its cyclic form). The *hemL*-encoded enzyme, glutamate-1-semialdehyde aminotransferase, converts that intermediate to ALA (21). Since only a small fraction of the cell's charged $tRNA^{Glu}$ is used to make heme, the reductase reaction is considered to be the first committed step in heme and tetrapyrrole biosynthesis.

Heterologous expression in *Saccharomyces cerevisiae* (40) and $tRNA^{Glu}$ substrate specificity studies (4) showed that *hemA* encodes glutamyl-tRNA reductase. Cells with null mutations in *hemA* exhibit a severe ALA auxotrophy, confirming its role in the pathway (15). Both a 45- and an 85-kDa glutamyl-tRNA reductase have been purified from *E. coli* cells; the former is the HemA protein, while the latter's origin and role are unknown (20). Genetic tests indicate that some flow through the pathway may occur independently of *hemA* (15), which is consistent with the existence of a second, minor enzyme.

Synthesis of heme is clearly a regulated process in enteric bacteria. First, the levels of heme in the membrane vary, depending on the mode of growth (e.g., see references 15, 18, and 34). Second, the amount of heme and, in particular, of glutamyl tRNA reductase activity can be increased dramatically by treatment with exogenous thiols, and this increase is blocked by chloramphenicol (23). Third, it is commonly observed that *E. coli* strains carrying multicopy plasmids encoding heme proteins (whether a catalase, cytochrome, or hemoglobin) are markedly red and overproduce heme by 10- to 20-fold (e.g., see reference 16). In many organisms other than the enteric bacteria, ALA production is known to be regulated at the levels of both gene expression and enzyme activity. It is likely but not proven that ALA synthesis determines the rate of heme synthesis in *E. coli*. Strains carrying cloned *hemA* genes of various species excrete ALA and have a fluorescent red phenotype due to tetrapyrrole overproduction (6, 8, 28), while cells overproducing the HemL and HemB enzymes do not have a fluorescent phenotype (8), which suggests that additional HemA enzyme may increase flux through the pathway but additional HemL or HemB does not.

We have previously constructed *lac* fusions to *hemA* and the other genes in the heme pathway, in an attempt to understand the regulation of *hem* gene expression in *S. typhimurium* (13,

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TABLE 1. Bacterial strains

Strain	Genotype	Source and/or reference
E. coli		
MC4100	K-12 F$^-$ λ$^-$ *araD139* Δ(*lacIpoZYA argF*)*U169 flb5301 relA1 rpsL150 deoC1 ptsF25 rbsR*	J. S. Parkinson
BW12629	*lac-169 phoR68 arcA148*(*dye*)::Tn*5-132* (Tet^r)	B. Wanner
BW13711	DE3(*lac*)*X74*	B. Wanner
DH5α	F$^-$ λ$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*dlacZ*ΔM15)	P. Higgins
ECL547	MC4100 Δ*frd-101 sdh*$^+$ Φ(*sdh-lac*) (λp*lac*Mu9 Kan^r)	E. C. C. Lin (19)
ECL585	MC4100 Δ*frd-101 sdh*$^+$ Φ(*sdh-lac*) (λp*lac*Mu9 Kan^r) *zjj*::Tn*10 arcA1*	E. C. C. Lin (19)
RZ8480	*lacZ*Δ*145 narG234*::MudI1734 Δ*fnr* (*Bsm*I-*Mlu*I)::Ω-Sm	P. Kiley (27)
SASX41B	HfrPO2A *relA1 spoT1 metB1 hemA41*	B. Bachmann
TE2680	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lac*]	13
TE5811	MC4100 *trpDC701*::Kan	P1.TE2620 × MC4100
TE5814	MC4100 *hemA41*	P1.SASX41B × TE5811
TE5815	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op][a] (ST, −693 to +91)[b]	This study
TE5840	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	This study
TE5841	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −693 to +584)	This study
TE5861	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53)	This study
TE5862	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53, P1 T-7G)	This study
TE5863	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53, P1 Δ-10)	This study
TE5864	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −127 to +91)	This study
TE5874	DE3(*lac*)*X74 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53)	This study
TE5875	DE3(*lac*)*X74 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [pr][c] (ST, −364 to +53)	This study
TE5878	DE3(*lac*)*X74 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53, ATG → ACG)	This study
TE5879	DE3(*lac*)*X74 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [pr] (ST, −364 to +53, ATG → ACG)	This study
TE5939	MC4100 *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −128 to +289)	This study
TE5975	MC4100 Δ*frd-101 hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289) arcA148::Tn*5-132* (Tet^r)	This study
TE5976	MC4100 Δ*frd-101 hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289) zjj::Tn*10 arcA1*	This study
TE5977	MC4100 Δ*frd-101 hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	This study
TE5978	MC4100 Δ*frd-101 hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289) zjj::Tn*10 arcA*$^+$	This study
TE5989	MC4100 Δ*frd-101 zjj*::Tn*10* Δ(*creBCD-arcA*) (*Kpn*I-*Spe*I)::Kan	This study
TE5990	MC4100 Δ*frd-101 zjj*::Tn*10* Δ(*creBCD*) (*Kpn*I-*Kpn*I)::Kan	This study
TE5991	MC4100 Δ*frd-101 zjj*::Tn*10* Δ(*creBCD-arcA*) (*Kpn*I-*Spe*I)::Kan *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	P1.TE5989 × TE5977
TE5992	MC4100 Δ*frd-101 zjj*::Tn*10* Δ(*creBCD*) (*Kpn*I-*Kpn*I)::Kan *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	P1.TE5990 × TE5977
TE6033	MC4100 Δ*frd-101* Δ*fnr* (*Bsm*I-*Mlu*I)::Ω-Sm *hemA41 trpDC700*::*putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	P1.RZ8480 × TE5977
TE6038	TE5991/pTE552 (P_{tac} vector, Amp^r)	
TE6039	TE5991/pTE552 (P_{tac}-*arcA*$^+$, Amp^r)	
TE6057	MC4100 Δ*frd-101* Δ*fnr* (*Bsm*I-*Mlu*I)::Ω-Sm *hemA41 zjj*::Tn*10* Δ(*creBCD-arcA*) (*Kpn*I-*Spe*I)::Kan *trpDC700*::*putPA1303*::Kan^r-*hemA*-lac [op] (EC, −1418 to +289)	P1.TE5989 × TE6033
S. typhimurium		
TE719	*hemA60*	15
TE1295	*hemA60 env-53*	15
TE2962	*putPA1308*::[′*prsA-orf2-orf1-hemA702*::Kan-*dorf-kdsA′*]	15
TE2986	*hemA60 putPA1308*::[′*prsA-orf2-orf1-hemA702*::Kan-*dorf-kdsA′*]	P22.TE2962 × TE719
TE3000	*hemA60 putPA1303*::Kan^r-*hemA-lac* [op] (ST, −693 to +91)	This study
TE5869	*hemA60 putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	This study
TE5871	*hemA60 putPA1303*::Kan^r-*hemA-lac* [op] (ST, −693 to +584)	This study
TE5993	*hemA60 env-53 putPA1303*::Kan^r-*hemA-lac* [op] (EC, −1418 to +289)	This study
TE6015	*putA1306*::Tn*10d*-Tet *putPA1303*::Kan^r-*hemA-lac* [op] (ST, −364 to +53, P1 T-7G)	This study
TE6029	*putA1306*::Tn*10d*-Tet *putPA1303*::Kan^r-*hemA-lac* [op] (ST, −693 to +53, P1 wild type) *hemA60*	P22.TE6015 × TE719
TE6030	*putA1306*::Tn*10d*-Tet *putPA1308*::′*prsA-orf2-orf1-hemA-lac* [op] (ST, −2030 to +53, P1 T-7G) *hemA60*	P22.TE6015 × TE2986
TE6031	*putA1306*::Tn*10d*-Tet *putPA1308*::′*prsA-orf2-orf1-hemA-lac* [op] (ST, −2030 to +53, P1 wild type) *hemA60*	P22.TE6015 × TE2986

[a] [op] indicates an operon fusion.
[b] Species and coordinates in parentheses indicate the DNA segment used to make the fusion. EC, *E. coli*; ST, *S. typhimurium.*
[c] [pr] indicates a protein fusion.

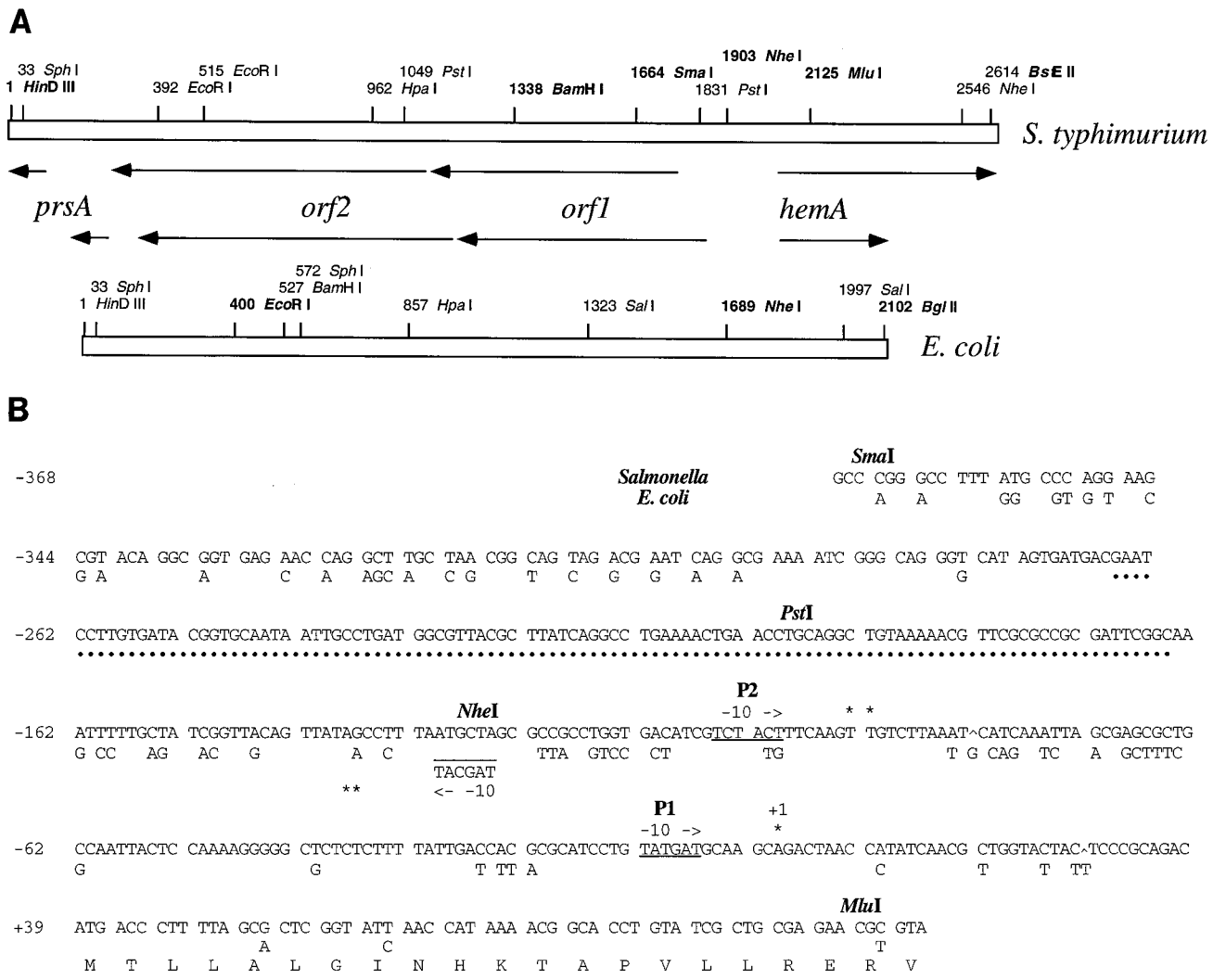

FIG. 1. (A) Restriction maps of the region between *prsA* and *hemA* in *S. typhimurium* (top) and *E. coli* (bottom). Each gene and open reading frame (*orf*) is indicated by an arrow. Only a part of the *hemA* and *prsA* genes is indicated. Restriction sites shown in boldface type flank the DNA segments used to construct various *lac* fusions discussed in the text. The two maps are aligned by using the N terminus of the *hemA* gene. The *S. typhimurium* genome has two insertions of ≈100 bp with respect to the *E. coli* sequence: one in the *hemA* promoter region and one in the *orf2-prsA* intergenic region (12, 33). (B) DNA sequence of the *hemA* promoter region in *S. typhimurium* and *E. coli*. The complete *S. typhimurium* sequence is given and differences in the *E. coli* sequence are shown below the *S. typhimurium* sequence, including a deletion of 102 bp indicated by black dots. Asterisks indicate the 5′ ends of transcripts as determined by primer extension (P1 and P2) or S1 nuclease protection (*orf1* promoter [39]). Potential −10 regions of σ^{70} promoters are underlined. Base pairs are numbered with respect to the 5′ end of the P1 transcript (+1). The numbering of fusion segments in the text uses the same scheme.

44–46). No evidence for regulation of transcription was found. In contrast, similar experiments have been reported recently in *E. coli*, with quite different results (10). In *E. coli*, a *hemA-lac* operon fusion was induced 20-fold during ALA starvation. Induction of *hemA* required *arcA* and was affected negatively by *fnr*, two genes encoding transcriptional regulators of respiratory proteins.

Here, we report experiments showing that *S. typhimurium hemA-lac* fusions, tested in *E. coli*, are induced by ALA starvation. We also explore the sequence requirements for induction. Verkamp and Chelm mapped two RNA 5′ ends in the *E. coli hemA* promoter region by using an S1 nuclease protection assay (39); these indicate the presence of a stronger promoter, P1, proximal to *hemA*, and a weaker distal promoter, P2. The 5′ ends of the P1 and P2 transcripts are indicated in Fig. 1. Assuming that these are σ^{70} promoters, their presumed −10 regions are also indicated. Mutations inactivating the major promoter serving the *hemA* operon, P1, eliminate the induction seen during starvation for ALA. However, direct analysis of RNA levels by primer extension could not confirm the model of transcriptional regulation. The role of the *arcA* gene in *hemA* expression was also investigated.

MATERIALS AND METHODS

Bacterial strains and construction. The *E. coli* and *S. typhimurium* strains used in this study are listed in Table 1. The wild-type *S. typhimurium* strain LT-2 does not carry the *lac* operon. Phage P1 *vir* was used for transduction between *E. coli* strains by standard methods (37). The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (36) was used for transductions in *S. typhimurium* by standard methods (11). Phage P22 transducing lysates were grown in *E. coli* as described previously (12). Fusions to *lac* of various DNA fragments from the *hemA* promoter region of *S. typhimurium* or *E. coli* were constructed as described below; these were transferred to the chromosome of an *E. coli recD* mutant by linear transformation exactly as described previously (13).

TABLE 2. Induction of *hemA-lac* expression during starvation for ALA in *E. coli*

Strain[a]	Origin of *hemA* promoter	Coordinates of DNA segment fused to *lac*[b]	β-Galactosidase activity of cultures grown to stationary phase (U)		Induction ratio	β-Galactosidase activity[c] of cultures grown to exponential phase with ALA	Induction ratio
			Without ALA	With ALA			
TE5815	*S. typhimurium*	−693 to +91 (codon 18)	870	370	2.4	350	2.5
TE5864	*S. typhimurium*	−127 to +91 (codon 18)	970	350	2.8	370	2.6
TE5841	*S. typhimurium*	−693 to +584 (codon 181)	4,740	1,280	3.7	1,140	4.2
TE5861	*S. typhimurium*	−364 to +53 (P1 wild type)	450	200	2.3	190	2.4
TE5862	*S. typhimurium*	−364 to +53 (P1 T-7G)	32	23	1.4	ND[d]	
TE5863	*S. typhimurium*	−364 to +53 (P1 Δ-10)	33	20	1.7	ND	
TE5840	*E. coli*	−1418 to +289 (codon 83)	3,010	800	3.8	530	5.7
TE5939	*E. coli*	−128 to +289 (codon 83)	3,010	740	4.1	530	5.7

[a] All strains are derivatives of *E. coli* MC4100 carrying the *hemA41* allele, which confers a requirement for ALA.
[b] Numbered relative to the A residue (+1) assigned as the 5′ end of the *hemA* transcript from the P1 promoter.
[c] Units of β-galactosidase according to reference 29.
[d] ND, not determined.

Each resulting strain carries a *lac* fusion as an insertion of a Kan^r-promoter-*lac* fragment in the *trp* operon (in *E. coli*) or in the *put* operon (in *S. typhimurium*).

Media and growth conditions. Strains were grown at 37°C in Luria-Bertani (LB) medium (prepared as described in reference 37). For starvation experiments, the medium contained, in addition, 50 mM KPO_4, pH 7.2, and 20 mM sodium pyruvate (10). Plates were prepared with NB agar (8 g of Difco nutrient broth, 5 g of NaCl, and 15 g of Difco Bacto Agar per liter). When present, ALA (Sigma) was used at 250 μM. Antibiotics were added to final concentrations as follows (per milliter): 50 μg of sodium ampicillin, 20 μg of chloramphenicol, 50 μg of kanamycin sulfate, and 20 μg of tetracycline hydrochloride. MacConkey lactose agar and TTC lactose agar were prepared as previously described (29). Difco EMB agar was prepared according to the manufacturer's instructions and used routinely to screen for the dye-sensitive phenotype of *arcA* mutants (35). Equivalent results were obtained with toluidine blue agar prepared according to Iuchi and Lin (19).

Construction of *lac* fusions, *hemA* promoter mutants, and *arcA* deletion mutants. The fusion system we used has been described previously (13). All constructs were made in the plasmids pRS550, pRS551, and pRS577 (38). The *S. typhimurium hemA-lac* fusions used a *Bam*HI site at −693, a *Sma*I site at −364 (with a *Bam*HI linker), and an *Nhe*I site at −127 (filled in and joined to *Bam*HI). Downstream fusion joints at +91 and +584 were made by digesting with *Mlu*I or *Bst*EII and with S1; these are joined to an *Eco*RI site. The downstream fusion joint at +53 was made by PCR. In each case, the specified base pair is immediately adjacent to the *Eco*RI site. The *E. coli hemA-lac* fusion was constructed by cloning an *Eco*RI-*Bgl*II fragment from pRSA34 into pRS551. The deletion to the *Nhe*I site was made by digesting with *Nhe*I and *Eco*RI, filling in with Klenow fragment, and religating.

Mutations in the P1 promoter −10 region were constructed by PCR using segment overlap extension, with minor modifications (17). In this method, one DNA fragment containing the desired mutation is synthesized in a first PCR and then itself is used as a primer in a second PCR. The method allows substitution of mutations incorporated into the primer sequence without the need for a nearby restriction site. All constructs were sequenced completely, or the authentic wild-type sequence was substituted using convenient restriction sites. The ATG → ACG mutant was constructed directly by PCR, with an extended version of the primer used to construct the fusion with a joint at +53.

The *arcA* deletion was constructed by digesting the plasmid pMW2 (42) with *Kpn*I and *Spe*I and then blunting and filling in with Klenow fragment, before inserting a Kan^r fragment derived from pUC4K that had been digested with *Bam*HI and filled in. The deletion of *creBCD* used as an *arcA*$^+$ control was constructed in the same way, except that pMW2 was digested with *Kpn*I alone. The Δ*creBCD-arcA*::Kan construct lacks sequences downstream from codon 46 of *creB* as well as all of *creC*, *creD*, and *arcA*. Both deletions were transferred to the chromosome by linear transformation of a *recD*::mini-Tet strain and then placed next to the *zjj*::Tn*10* insertion by transduction. The resulting strains, TE5989 and TE5990, were used as donors to transduce these deletions into other backgrounds by linkage to *zjj*::Tn*10*.

The wild-type *arcA* gene was isolated by PCR using chromosomal DNA from ECL547 as the template. The sequence of the product was determined completely and is identical to that submitted under GenBank accession no. U14003. The *arcA* expression plasmid was constructed from pTM30 (a gift of J. S. Parkinson), which contains *lacI*^{q} and the *tac* promoter (31). The plasmid was modified to remove an existing *Nde*I site and to substitute an *Nde*I site positioned to overlap the ATG initiation codon, resulting in plasmid pTE552. The *arcA* gene was then cloned as an *Nde*I-*Bam*HI fragment, yielding plasmid pTE553. This plasmid expresses ArcA protein that should have the native sequence.

Starvation protocol and β-galactosidase assays. Cultures were grown for β-galactosidase assays in LB medium containing pyruvate (described above). A small volume of culture medium without ALA was inoculated from a single colony and then split to two cultures, one with 250 μM ALA and one without ALA. The cultures were incubated for 15 h on a roller drum at 37°C, stored on ice, and then diluted 1:1,000 to inoculate a second set of identical cultures. These cultures were incubated for 15 h on a roller drum at 37°C. Two cycles of growth without ALA produced a maximum differential between the ALA-supplemented and unsupplemented cultures. Assays were performed by using a kinetic method on cells permeabilized by treatment with sodium dodecyl sulfate and chloroform (29). The results in each table are from a single experiment; each experiment was repeated several times with similar results.

DNA sequencing. Sequencing of double-stranded plasmid DNA was done as previously described (12) by using Sequenase version 2.0 (U.S. Biochemical Corp.) according to the manufacturer's instructions. Plasmid DNA for sequencing was isolated by using Qiagen columns according to the manufacturer's instructions. Sequencing of amplified chromosomal DNA used PCR fragments synthesized with one biotinylated primer for the desired template strand. Single-stranded DNA was isolated by NaOH treatment of double-stranded DNA captured on streptavidin-coated magnetic beads (Dynal).

Primer extension analysis. Primer extension analysis of RNA was performed as previously described (43). RNA was isolated by a hot-phenol method from cells grown as described in Results.

RESULTS

Increased expression of *hemA-lac* fusions during starvation for ALA in *E. coli* cells. When an *E. coli hemA* mutant, auxotrophic for ALA, was grown in LB medium containing 20 mM pyruvate but no ALA, expression of a *hemA-lac* operon fusion carried on a λ prophage was reported to be 20-fold higher than in cells grown in the same medium supplemented with 250 μM ALA (10). By contrast, we have never observed more than a twofold increase using a similar protocol with *S. typhimurium* strains grown in LB medium. In order to determine the reason for this difference, we carried out experiments with *E. coli* using *lac* operon fusions to both the *E. coli* and *S. typhimurium* *hemA* promoters. The fusions were placed in single copy in the chromosome of a parental *E. coli* MC4100 strain carrying the *hemA41* allele (strain TE5814; Table 1). Cultures were grown in LB medium containing 50 mM KPO_4, pH 7.2, and 20 mM sodium pyruvate (10). The pyruvate in this medium allows the *hemA* mutant to grow without added ALA, although the strain grows more slowly than when ALA is provided, and the yield is also reduced. Using the starvation protocol described in

TABLE 3. An ATG → ACG mutation in the *hemA* initiation codon reduces expression of β-galactosidase from a *hemA-lac* protein fusion

Strain[a]	Fusion[b]	β-Galactosidase activity (U)	Fraction of wild-type activity
TE5874	*hemA-lac* [op]	130	
TE5878	*hemA-lac* [op] (ATG → ACG)	89	0.68
TE5875	*hemA-lac* [pr]	9.1	
TE5879	*hemA-lac* [pr] (ATG → ACG)	0.21	0.023

[a] All strains are *E. coli* DE3(*lac*)*X74*.
[b] [op] indicates an operon fusion, and [pr] indicates a protein fusion.

Materials and Methods, we measured the levels of β-galactosidase in a series of strains grown in the presence or in the absence of ALA.

The results are shown in Table 2 and can be summarized as follows. (i) Expression of several different *lac* fusions to the *S. typhimurium hemA* promoter (in *E. coli)* was increased 2.5- to 4-fold during starvation for ALA. (ii) Expression of the *E. coli hemA-lac* fusion was increased as much as 6-fold during starvation for ALA. (iii) Expression from the *E. coli* fusion in unstarved cultures was lower during exponential phase than in stationary phase, increasing the induction ratio compared with that for the *S. typhimurium* fusions. (iv) A *lac* fusion to codon 181 of the *S. typhimurium hemA* gene had a higher induction ratio than a fusion to codon 18 (compare strains TE5841 and TE5815). (v) Expression from both *E. coli* and *S. typhimurium hemA-lac* fusions was unchanged by deletion of DNA upstream of the *Nhe*I site. This site lies at −127 (*S. typhimurium*) or −128 (*E. coli*) with respect to the 5′ end of the transcript from P1, the major *hemA* promoter (39) (Fig. 1). The induction ratio for the *E. coli* fusions was not as high in our experiments as those reported previously (10).

We constructed two mutants with alterations in the −10 region of the stronger *S. typhimurium hemA* promoter, P1. The most conserved position, T at −7, has been changed to G (T-7G), and in another mutant, 9 bp have been deleted, including all of the −10 sequence. When fused to *lac*, these mutant P1 promoters directed less than 10% of the level of β-galactosidase seen with a functional P1 promoter (Table 2, compare strains TE5861, TE5862, and TE5863). In the experiment presented, a small induction by ALA starvation is apparent in the P1 mutants, but this was not observed consistently. Assuming that induction occurs by an increase in transcription from one of the *hemA* promoters, these results would suggest that induction occurs by an increase in expression from the P1 promoter rather than by activation of P2 or a silent promoter. However, this assumption may not be justified (see below). As mentioned above, all promoter elements apparently lie downstream of the *Nhe*I site.

Mutation affecting the *hemA* initiation codon. The 45-kDa glutamyl-tRNA reductase encoded by *hemA* has not been highly purified, and particularly, its N-terminal amino acid sequence has not been determined (20, 40). We tested whether the N-terminal sequence shown in Fig. 1 is correctly assigned, by constructing a mutant in which the initiation codon ATG was changed to ACG. This mutation is predicted to drastically reduce expression of a *hemA-lacZ* protein fusion only if this ATG codon is the *hemA* initiation codon. The ATG → ACG mutation reduced expression of a *hemA-lac* operon fusion slightly, probably because of polarity, but had a severe effect on a *hemA-lacZ* protein fusion, reducing expression to about 2% of the level seen with the wild type (Table 3). This result confirms the assignment of the initiation codon shown in Fig. 1B.

Increased expression of *hemA-lac* fusions during starvation for ALA in *S. typhimurium* cells. We also measured the levels of β-galactosidase from *hemA-lac* operon fusions in *S. typhimurium* (Table 4). Expression from the *E. coli hemA* promoter (tested in *S. typhimurium*) was increased about fourfold during starvation for ALA, compared with the sixfold increase seen with *E. coli* cells. The *S. typhimurium hemA* promoter fusions were induced two- to threefold. Similar to the previous experiments, the *E. coli hemA* promoter directed a lower level of *lac* expression during exponential growth than in stationary phase for cultures grown with ALA. We tested two other wild-type *S. typhimurium* strains; both had induction ratios for the *E. coli hemA-lac* fusion similar to those shown in Table 4. We also used an *S. typhimurium* strain which is permeable to heme (22) to determine whether heme is as effective as ALA in reducing the expression of *hemA-lac*. No substantial difference was seen between the induction behavior of strains grown with heme and that of strains grown with ALA (Table 5). These results suggest that heme limitation rather than the low level of heme biosynthetic intermediates is responsible for induction.

Effect of *arcA* function on *hemA-lac* expression and induction by ALA starvation. We first examined the effect of two *arcA* alleles on expression of a *hemA-lac* fusion: *arcA1* (19) and an *arcA*::Tn*5* insertion that lies in the middle of the gene (8). Neither mutation significantly affected expression of the *hemA-lac* fusion in our *E. coli hemA*$^+$ background (8). Other phenotypes characteristic of *arcA* mutants were as expected: the mutant strains were sensitive to toluidine blue and, when tested with an *sdh-lac* reporter fusion, failed to show repression of *sdh-lac* during anaerobic growth. We also confirmed the identity of the *arcA1* allele by DNA sequencing of the relevant part of the *arcA* gene amplified from TE5976.

For a more rigorous test, we used the *arcA*$^+$ *sdh-lac* fusion strain used by Iuchi and Lin, an *E. coli* MC4100 derivative in which *arcA* function is known to be robust (19). Otherwise isogenic *arcA*$^+$ and *arcA* mutant strains were constructed by P1

TABLE 4. Induction of *hemA-lac* expression during starvation for ALA in *S. typhimurium*

Strain[a]	Origin of *hemA* promoter	Coordinates of DNA segment fused to *lac*[b]	β-Galactosidase activity of cultures grown to stationary phase (U)		Induction ratio	β-Galactosidase activity of cultures grown to exponential phase with ALA (U)	Induction ratio
			Without ALA	With ALA			
TE3000	*S. typhimurium*	−693 to +91	1,250	520	2.4	440	2.8
TE5871	*S. typhimurium*	−693 to +584	3,350	1,940	1.7	1,720	1.9
TE5869	*E. coli*	−1418 to +289	2,650	1,310	2.0	660	4.0

[a] All strains are derivatives of TE719 (*hemA60*), an ALA auxotroph.
[b] Numbered relative to the A residue (+1) at the 5′ end of the *hemA* transcript from the P1 promoter.

TABLE 5. Comparison of starvation for ALA or heme in *S. typhimurium*

Strain	β-Galactosidase activity of cultures grown to stationary phase (U)			Induction ratio	β-Galactosidase activity of cultures grown to exponential phase (U)		Induction ratio
	Untreated (no addition)	With ALA	With heme		With ALA	With heme	
TE5869	2,330	1,390	ND[a]	1.7	520	ND	4.5
TE5993[b]	2,600	1,520		1.7	550		4.7
TE5993	2,600		1,340	1.9		670	3.9

[a] ND, not determined.
[b] Strain TE5993 is a derivative of TE1295 (*hemA60 env-53*), an ALA auxotroph which is permeable to heme. Both TE5869 and TE5993 carry the same fusion of *lac* to the *E. coli hemA* promoter (−1418 to +289).

transduction, and the effect of *arcA* on *hemA-lac* expression was determined (Table 6, strains TE5975 to TE5978). The results show that (i) the *arcA1* and *arcA*::Tn*5* mutations actually increased the expression of *hemA-lac* in a strain grown in the presence of ALA by 30 to 40% and that (ii) induction during starvation for ALA was reduced but not eliminated in the *arcA* mutants. These results differ substantially from those reported previously (10).

One possible explanation for the difference is that their *arcA* mutant strain was a deletion mutant. To address this possibility, we constructed a complete deletion of the *arcA* gene as described in Materials and Methods. It was convenient to construct this deletion in a way that included the *creBCD* genes, which are adjacent to *arcA*. Thus, the *arcA*$^+$ control strain carries a deletion of *creBCD* only. We found that the effect of the *creBCD-arcA* deletion was the same as that seen with the *arcA1* and *arcA*::Tn*5* alleles (Table 6; strains TE5991 and TE5992).

Overexpression of *arcA*. A plasmid that expresses the *E. coli arcA* gene under the control of the P_{tac} promoter and *lac* repressor was constructed (see Materials and Methods for details). This plasmid directs some expression of ArcA protein even without the inducer, IPTG (isopropyl-β-D-thiogalactopyranoside), as seen by complementation of the dye-sensitive phenotype conferred by the chromosomal *arcA* deletion present in strain TE6039. Growth of strain TE6039 carrying this plasmid showed inhibition of growth at an IPTG concentration as low as 5 μM. Strains with a *lac* fusion to the *E. coli hemA* promoter and a deletion of the chromosomal *arcA* gene, and carrying either the vector or the P_{tac}-*arcA* construct, were induced with 5 to 200 μM IPTG and assayed for β-galactosidase. Expression of *hemA-lac* was not increased by overexpression of *arcA* in this manner (8). We found that 1 μM IPTG still allowed good growth of TE6039 carrying the *arcA* expression plasmid. Cultures grown with 1 μM IPTG were tested for the effect of *arcA* overexpression in an ALA starvation experiment. The results were comparable to those seen with *arcA* mutant and wild-type alleles carried in single copy (Table 6). Thus, expression of *arcA* to a level just below that which inhibits growth did not result in a significant increase in *hemA-lac* expression, nor did it result in an increased induction ratio during starvation for ALA.

Effect of an *fnr* mutation. Since *fnr* mutations were previously shown to allow increased expression of *hemA-lac* fusions during anaerobic growth, we examined the effect of an *fnr* deletion (27) on expression of an *E. coli hemA-lac* fusion after starvation for ALA by the standard protocol (aerobic growth). We also looked at a double mutant defective in both *fnr* and *arcA*. The *fnr* mutation did apparently increase the induction ratio slightly, but this was due to a decrease in expression in the presence of ALA rather than to enhancement of expression during starvation. Again, the *arcA* mutation resulted in a modest decrease in the induction ratio during starvation for ALA. Therefore, the difference between our experiments and the previous study is not due to repression by FNR in our strains.

Primer extension mapping of *hemA* transcripts. Primer extension analysis was used to confirm the effect of the *S. typhimurium* P1 promoter mutants described above. A signal was obtained from both the P1 and P2 transcripts in cells containing a plasmid with a wild-type *hemA* promoter region, but only the P2 signal was seen in RNA from cells containing plasmids with P1 promoter mutations (Fig. 2A). The P2 signal was shifted to a shorter length in the P1 deletion mutant, as predicted. These results confirm that the mutations specifically damage the P1 promoter.

TABLE 6. Effect of *arcA* and *fnr* on expression of *hemA-lac* in *E. coli*

Strain[a]	Genotype[b]	β-Galactosidase activity of cultures grown (U)[c]		Induction ratio
		Without ALA	With ALA	
TE5975	*hemA-lac* [op] *arcA140*::Tn*5-132*	2,150	920	2.3
TE5977	*hemA-lac* [op] *arcA*$^+$	2,920	755	3.9
TE5976	*hemA-lac* [op] *arcA1 zjj*::Tn*10*	2,550	905	2.8
TE5978	*hemA-lac* [op] *arcA*$^+$ *zjj*::Tn*10*	2,760	745	3.7
TE5991	*hemA-lac* [op] Δ(*creBCD-arcA*)::Kan *zjj*::Tn*10*	2,820	890	3.2
TE5992	*hemA-lac* [op] Δ(*creBCD*)::Kan *zjj*::Tn*10*	3,150	740	4.3
TE6038[d]	TE5991/pTE552 (P_{tac} vector)	3,360	1,170	2.9
TE6039[d]	TE5991/pTE553 (P_{tac}-*arcA*$^+$)	3,420	970	3.5
TE6057	*hemA-lac* [op] Δ*fnr*::Ω-Sm Δ(*creBCD-arcA*)::Kan *zjj*::Tn*10*	3,020	900	3.4
TE6033	*hemA-lac* [op] Δ*fnr*::Ω-Sm	2,870	610	4.7

[a] All strains are *E. coli* MC4100 Δ*frd-101 hemA41*, carrying a fusion of *lac* to the *E. coli hemA* promoter (see strain TE5840 in Table 2).
[b] [op] indicates an operon fusion.
[c] Cultures were grown to stationary phase as described for the experiment reported in Table 2.
[d] Cultures were grown in medium containing 1 μM IPTG.

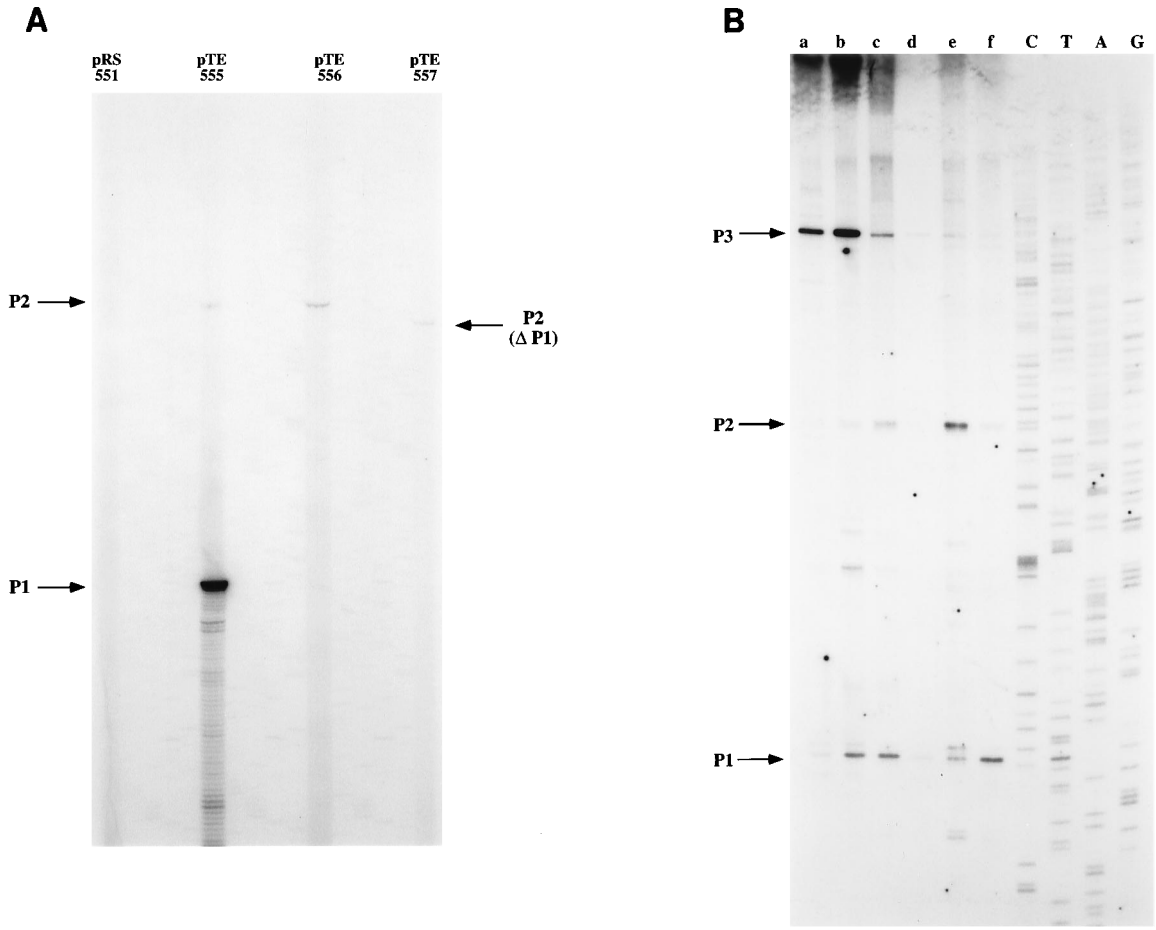

FIG. 2. Primer extension mapping of the 5′ ends of RNA transcripts in the *hemA* promoter region. (A) Primer extension products obtained by using a ^{32}P-labeled *lac*-specific primer and template RNA isolated from *E. coli* DH5α carrying the following plasmids: a control *lac* fusion plasmid with no promoter insert (pRS551); *S. typhimurium hemA-lac* fusion (−364 to +53) with the wild-type P1 promoter (pTE555); pTE555 with the P1 T-7G mutation (pTE556) or the P1Δ mutation (pTE557). The intervening lanes, which are not identified, contained DNA sequencing reaction products obtained with the same labeled *lac* primer and the cognate plasmid DNA template. These products, used as size markers, are faint but visible in the original autoradiogram. (B) Primer extension products obtained by using a ^{32}P-labeled *hemA*-specific primer and template RNA isolated from an *E. coli hemA41* mutant strain (TE5814). RNA was isolated from cultures grown in buffered LB medium containing pyruvate, without ALA (lanes a to c) or with ALA (lanes d to f). Primer extension reaction mixtures contained 1.5 μg (a and d), 5 μg (b and e), or 15 μg (c and f) of RNA. Lanes C, T, A, and G contained DNA sequencing reaction products obtained by using the same labeled *hemA* primer with pTE541, a plasmid DNA template carrying the *E. coli hemA* gene. The primer sequence corresponds to bp +96 to +76 (Fig. 1B).

RNA purified from an *E. coli hemA* auxotroph was also examined by primer extension to test whether starvation for ALA increases the abundance of P1-derived transcripts. The results are shown in Fig. 2B. We note three points: (i) the signal from P1 was not substantially increased in RNA prepared from cells starved for ALA (in other experiments, a slight increase was noted); (ii) two strong signals were apparently induced by ALA starvation, one band (designated P3) indicating an RNA 5′ end that would map approximately 350 nucleotides upstream of P1, just downstream of the *Sma*I site (Fig. 1B), and a smeared band indicating a group of 5′ ends that would lie even farther upstream; and (iii) the signal strength was not strictly proportional to RNA concentration (compare the P3 signals in Fig. 2B, lanes b and c, and compare the P2 signals in lanes d to f). The loss of signal strength at higher RNA concentrations probably indicates the presence of competitor RNA which can hybridize to the template RNAs.

The strongly inducible P3 signal is apparently an artifact arising by primer extension on RNA from a gene other than *hemA*. This conclusion is based on (i) the absence of the P3 and upstream signals in experiments with other primers derived from the *hemA* promoter sequence; (ii) a striking conflict with the deletion analysis using *lac* fusions, which showed that all sequences required for *hemA* promoter activity lie downstream of the *Nhe*I site, as do all sequences needed for induction of *hemA-lac* expression during ALA starvation; and (iii) the requirement of P1 for high-level expression of *hemA* under all conditions examined (see below). We considered the possibility that the P3 signal results from extension on traces of DNA contaminating the RNA preparation. Appearance of the P3 signal was sensitive to treatment of the template RNA with RNase but not to treatment with DNase, confirming that the P3 signal results from extension to an RNA 5′ end (8).

Genetic tests were designed to detect the activity of the putative P3 promoter in *S. typhimurium* using strains and methods described previously (see Fig. 4D of reference 13). We constructed strains carrying *lac* fusions with DNA extending as far upstream as the *Hin*dIII site (Fig. 1A) that differ only in the presence of the P1 promoter mutation (T-7G) described above (Table 7; strains TE6030 and TE6031). These strains were compared with one carrying the parental fusion with DNA only to the *Bam*HI site (−693) (Table 7, strain TE6029). Substitu-

TABLE 7. Effect of additional upstream DNA and a P1 promoter mutation on expression of *hemA-lac* in *S. typhimurium*

Strain[a]	Coordinates of DNA segment fused to *lac*	β-Galactosidase activity of cultures grown (U)[b]		Induction ratio
		Without ALA	With ALA	
TE6029	−693 to +53, P1 wild type	1,210	410	3.0
TE6030	−2030 to +53, P1 T-7G	32	42	0.8
TE6031	−2030 to +53, P1 wild type	780	420	1.9

[a] All strains are *S. typhimurium hemA60 putA1306*::Tn*10d*-Tet, carrying the indicated fusion of *lac* to the *S. typhimurium hemA* promoter. For complete genotypes, see Table 1.

[b] Cultures were grown to stationary phase as described for the experiment reported in Table 2.

tion of additional upstream DNA caused no additional induction; furthermore, the P1 mutation severely reduced expression both in the presence and in the absence of ALA. These results are not consistent with the presence of an inducible promoter lying near −350.

DISCUSSION

The results of this study partially confirm the report of Darie and Gunsalus regarding induction of *hemA-lac* fusions in *E. coli* cultures starved for ALA (10). One significant difference is a substantially lower level of induction (maximally 6-fold, compared with 20-fold), even for a *lac* fusion to the *E. coli hemA* promoter. We believe that several factors acting incrementally account for the larger induction ratios we obtained in this study compared with those obtained in our previous work with *S. typhimurium*: (i) *lac* fusions to the *E. coli hemA* promoter are more highly induced than fusions to the *S. typhimurium hemA* promoter; (ii) induction in *E. coli* cells is greater than that in *S. typhimurium* cells; and (iii) the buffered LB medium contains pyruvate, which allows slow exponential growth of *hemA* mutants in the absence of ALA and is essential to obtain the results shown here.

A more important discrepancy is the lack of a clear effect of *arcA* mutations in our experiments. This is not due to residual *arcA* function in the mutants we used nor to poor *arcA* function in our wild-type strain, nor is it due to repression by FNR. We also saw no effect of expression of ArcA at higher levels than normally found in cells, even to levels that inhibit growth. Recent work in R. Gunsalus's laboratory is in agreement with these findings (15a).

We confirmed the primary role of the P1 promoter in *hemA* transcription by examining the effects of mutations in the −10 region on expression of *hemA-lac* fusions and also showed that all sequences important for expression of *hemA* under the conditions examined lie within 128 bp upstream of the P1 RNA 5′ end. We also confirmed the identity of the ATG initiation codon of the *hemA* gene.

Primer extension analysis showed no evidence of a large increase in the abundance of RNA from the *hemA* promoters during starvation for ALA, which suggests that lower levels of induction in our experiments are not due simply to a technical problem with the particular fusion system we used. It also suggests that induction of *hemA-lac* fusions is mediated at a step after transcription initiation.

Remarkably, a signal for an RNA that is highly inducible by starvation for ALA but that apparently originates from elsewhere in the genome was found. We found that this signal was independent of *arcA* function (8). The P3 RNA can base pair to one (but not other) primer used for primer extension experiments (Fig. 2B); the sequence of this primer lies within the N-terminal segment of *hemA*. One speculative explanation is that this region is conserved in the 85-kDa secondary glutamyl-tRNA reductase (20), whose gene has not been identified. If this is so, we would predict that the secondary enzyme is induced during starvation for ALA in *E. coli* (but not in *S. typhimurium* [8]).

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